UNIVERSITÉ DU QUÉBEC À MONTRÉAL

MÉCANISMES MOLÉCULAIRES ET CELLULAIRES DE L'APOPTOSE INDUITE PAR L'ACROLÉINE: IMPLICATION SUR LA SANTÉ HUMAINE

THÈSE PRÉSENTÉE À LA FACULTÉ DES SCIENCES
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LISTE DES ABRÉVIATIONS

AFC	Amino trifluorocoumarin
AIF	Facteur inducteur d'apoptose
ALMA	Acide mercapturique
ARE	Élément de réponse aux antioxydants
AIF	Facteur inducteur d'apoptose
AKT	Protéine kinase B
AMC	Amino méthylcoumarin
AMPc	Adénosine monophosphate cyclique
ANT	Translocateur d'adénine
AP-1	Protéine activatrice-1
APAF-1	Facteur activateur de protéase apoptotique
ASK1	Kinase régulatrice du signal apoptotique
ATM	Protéine mutée d'ataxia-telangiectasia
ATP	Adénosine triphosphate
ATR	Protéine similaire à Rad3
BH	Domaine homologue à Bcl-2
BIR	Répétition de IAP du baculovirus
BRCA1	Antigène 1 du cancer de sein
L-BSO	L-buthionine-(S,R)-sulphoximine
BSA	Albumine du sérum bovin
BSAO	Amine oxydase de sérum bovin
CAD	ADNase caspase-dépendante
CARD	Domaine recruteur de caspase
CHAPS	Acide 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonique
Chk1	Kinase de vérification 1

Chk2	Kinase de vérification 2
CHO	Ovaire de hamster chinois
CK	Créatinine kinase
CD	Cyclophiline D
CrmA	Cytokine modifiant de la réponse
Cyt-c	Cytochrome c
DD	Domaine de mort
DED	Domaine effecteur de mort
DIABLO/Smac	Protéine liante de l'IAP avec un dérivé de la mitochondrie activateur de caspase
DISC	Le complexe inducteur de signal de mort
DNA-PK	Kinase DNA-dépendante
EGF	Facteur de croissance épidermale
EGFR	Récepteur du facteur de croissance épidermale
ERK	Kinase régulée par des stimuli extracellulaires
EOR	Espèce réactive de l'oxygène
FADD	Protéine associée au Fas avec un domaine de mort
Fas	Protéine associée au fibroblaste
FasR	Récepteur Fas
FasL	Ligand Fas
FBS	Sérum foetal bovin
FCCP	P-trifluoromethoxy-phenyl-hydrazone
FDP-lysine	N^{ε}-(3-formyl-3,4-deshydropipéridino) lysine
bFGF	Facteur basique de croissance de fibroblaste
FLICE	Caspase-8, une protéine associée au ligand Fas
FLIP	Protéine inhibitrice de FLICE
FSH	Hormone stimulante des follicules
GADD45	Protéine induite par les dommages à l'ADN et arrêtant la croissance

GAPDH	Glycéraldehyde-3-phosphate déshydrogénase
γ-GCS	γ-glutamyl-cystéine-synthétase
GPx	Glutathion-péroxydase
GSH	Glutathion réduit
GSSG	Glutathion disulfure
GST	Glutathion-S-transférase
HDL	Lipoprotéine de haute densité
HK	Hexokinase
4-HNE	4-hydroxynonénal
HOCL	Acide hypochloreux
HPMA	Acide 3-hydroxypropylmercapturique
HSPs	Protéines de choc thermique
IAP	Inhibiteur de protéine apoptotique
ICAD	Inhibiteur de CAD
IGF	Facteur de croissance ressemblant à l'insuline
IK	Inhibiteur du facteur NF-κB
IKK	Kinase du κβ inductible
IL-1	Interleukine-1
JNK	Kinase du terminus-N de c-jun
LDL	Lipoprotéine de faible densité
LPO	Péroxydation lipidique
MAPKs	Protéines kinases activées par les mitogènes
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MDM2	Murine double minute
MEK	MAPKK de ERK
α-MEM	Milieu essentiel minimum alpha
MOPS	Acide 3-(N-morpholino)-propane sulfonique

NAC	N-acétylcystéine
NADH	Nicotinamide adénine dinucléotide
NF-κB	Facteur nucléaire-κB
NO	Oxyde nitrique
Nrf2	Facteur de transcription nucléaire dérivé des érythroides
$O_2^{\cdot-}$	Superoxyde
$OH^{\cdot}$	Radical hydroxy
ONOO-	Péroxynitrite
α-OH-PdG	Isomères 6α et 6β de 3*H*-6-hydroxy-3-(β-D-2'désoxyribofuranosyl)- 5,6,7,8-tétrahydropyrido[3,2-a]purine-9-one)
γ-OH-PdG	Isomères 8α et 8β de 3*H*-8-hydroxy-3-(β-D-2'désoxyribofuranosyl)-5,6,7,8-tétrahydropyrimido[3,2-a]purine-9-one ou 1,N(2)-gamma-hydroxypropano déoxyguanosine
OTC	2-oxo-4-thiazolidine carboxylate
PARP	Poly-ADP-ribose-polymérase
PBS	Tampon phosphate salin
PdG	1,N^2-(1,3-propano)-2'-désoxyguanosine
PIKK	Kinase similaire à la phosphoinositide-3-kinase
PI3-K	Phosphatidylinositol-3-kinase
PKB	Protéine kinase B
PMSF	Fluorure de phénylméthylsulfonyl
PS	Phosphatidylsérine
PTP	Pore de transition de perméabilité
PVDF	Difluorure de polyvinylidene
RAIDD	Protéine associée au RIP par un DD
RE	Réticulum endoplasmique

RIP	Récepteur interagissant de protéine
SDS	Sodium dodesylsulfate
SDS-PAGE	Électrophorèse sur gel de polyacrylamide en présence du SDS
SEM	Erreur standard de la moyenne
SMAC	Activateur mitochondrial des caspases
TNF-α	Facteur de nécrose tumorale-α
TNFR	Récepteur du facteur de nécrose tumorale
TRADD	Protéine associée au TNF-R1 avec un domaine de mort
VDAC	Canal ionique voltage-dépendant
VLDL	Lipoprotéine à très faible densité
Wt	Wortmannine

RÉSUMÉ

L'acroléine est un aldéhyde α,β-insaturé extrêmement réactif et elle est un produit de la péroxydation lipidique. Elle est un polluant environnemental qui a été impliqué comme un facteur de risque de développer plusieurs maladies respiratoires (bronchite, asthme) et neurodégénératives (Alzheimer) ainsi que l'athérosclérose. L'acroléine qui se trouve à des concentrations élevées au niveau de l'hippocampe des patients atteints de l'Alzheimer est proposée comme marqueur biochimique pour le pronostic de la maladie. L'acroléine est utilisée comme herbicide et elle est générée par la fumée de cigarette, la cuisson d'huile, le métabolisme de certains agents anticancéreux, lors de la péroxydation des lipides et après exposition aux rayons UV. L'acroléine est libérée lors des feux de forêt, ce qui représente un grand risque pour les pompiers et les secouristes. Il a été démontré que l'acroléine est un fort irritant des muqueuses et du système respiratoire. L'acroléine est produite lors de la désamination oxydative par la spermine oxydase. Les produits d'oxydation des polyamines sont impliqués dans l'inhibition de la prolifération cellulaire, l'apoptose, et l'inhibition de la synthèse de l'ADN et des protéines. Ainsi, à cause de l'impact important de l'acroléine sur la santé humaine, nous avons entrepris cette étude afin de déterminer, et ce pour la première fois, les mécanismes moléculaires impliqués dans la mort cellulaire induite par cet aldéhyde.

Le premier objectif visait à définir l'implication de la voie mitochondriale dans l'apoptose induite par l'acroléine. Cette étude est réalisée chez les cellules ovariennes de hamster chinois (CHO). L'acroléine induit l'apoptose en entraînant la baisse du potentiel membranaire mitochondrial, la libération du cytochrome-C, l'activation de la caspase-9 initiatrice et la caspase-7 exécutrice. D'ailleurs, l'acroléine a inhibé l'activité enzymatique de la caspase-3 exécutrice tout en clivant la forme proenzyme de la protéine. L'activation des caspases-7 et -9 a été confirmée par le clivage de leurs proenzymes. L'apoptose induite par l'acroléine a été inhibée par un inhibiteur de la caspase-9 mais pas avec un inhibiteur de la caspase-3. L'induction de l'apoptose par l'acroléine a été confirmée morphologiquement par la condensation nucléaire de la chromatine et par le clivage de l'inhibiteur de la DNase activée par les caspases (ICAD). Le clivage de l'ICAD libère l'endonucléase CAD qui emmène la fragmentation de l'ADN. Ces résultats démontrent que l'acroléine induit l'apoptose par la voie mitochondriale.

Le deuxième objectif visait à établir l'implication de la voie des récepteurs de mort dans l'induction de l'apoptose par l'acroléine. L'exposition des cellules ovariennes de hamster chinois (CHO) à l'acroléine entraînait la translocation de l'adapteur de domaine de mort de Fas (FADD) à la membrane plasmique et l'activation de la caspase-8 initiatrice. Kp7-6, un antagoniste du récepteur Fas, bloquait les évènements apoptotiques en aval de la caspase-8, comme l'activation de la caspase-7 exécutrice et la condensation de la chromatine nucléaire. L'acroléine induit une transduction croisée entre la voie de signalisation des récepteurs de mort et

celle de la mitochondrie en clivant la protéine Bid en sa forme tronquée t-bid qui se transloque à la membrane mitochondriale pour stimuler cette voie. L'inhibition spécifique du récepteur Fas ou de la caspase-8 inhibait partiellement l'activation de la caspase-9 par l'acroléine. Ces résultats démontrent que l'acroléine active la voie du récepteur Fas en amont de la voie mitochondriale. La caspase-9 demeurait active malgré l'inhibition du récepteur Fas et de la caspase-8 suggérant que l'acroléine peut induire la voie mitochondriale indépendamment de la voie des récepteurs de mort.

Le troisième objectif était d'examiner l'implication des protéines kinases activées par des mitogènes (MAPK) dans l'apoptose induite par l'acroléine. Quelques études ont démontré que l'acroléine active les MAPKs et d'autres ont rapporté que l'acroléine induit l'apoptose. Cependant, aucune étude n'a examiné le lien entre l'activation des MAPK et l'apoptose induite par l'acroléine. Nous avons démontré pour la première fois que l'apoptose induite par l'acroléine est dépendante des MAPK. Une heure d'exposition à l'acroléine induisait une forte phosphorylation de la kinase régulatrice extracellulaire (ERK), de la p38 et du substrat de la kinase N-terminale de c-jun (JNK), c-jun, chez les cellules CHO. L'inhibition de la condensation de la chromatine induite par l'acroléine à l'aide de l'inhibiteur de ERK, le U126, et l'inhibiteur de la p38, le SB203580, démontre clairement l'implication de ces deux voies dans l'apoptose induite par l'acroléine. En plus, le U0126 et le SB203580 inhibaient l'activation des caspases-7 et -9 et le clivage de l'ICAD induites par l'acroléine. D'autre part, les voies de JNK et de la protéine kinase B (AKT) semblent être impliquées dans la survie contre l'agression par l'acroléine, puisque des inhibiteurs pharmacologiques de ces deux voies, le SP600125, le LY294002 et le Wortmannin changeaient la mort par apoptose en nécrose. Enfin, l'acroléine a entraîné la phosphorylation de p53, une protéine responsable de la transcription de plusieurs gènes impliqués dans l'apoptose dont ceux de Bax et le ligand Fas.

Le quatrième objectif visait à déterminer l'implication du N-acétylcystéine (NAC), un précurseur du glutathion, dans la protection contre la toxicité engendrée par l'acroléine. L'exposition des cellules CHO à une concentration non-cytotoxique d'acroléine (4 *f*mol/cell), baissait le glutathion intracellulaire de 45% de son taux initial. Le NAC augmentait le niveau du glutathion intracellulaire et offrait une protection contre la cytotoxicité et l'apoptose induite par l'acroléine. Le NAC affectait l'apoptose induite par l'acroléine en inhibant la voie mitochondriale. Le NAC inhibait la translocation de bad à la mitochondrie et la baisse du bcl-2 mitochondriale induites par l'acroléine. D'autre part, le NAC inhibait la baisse du potentiel membranaire, le clivage de la procaspase-9, l'activation des caspases-9, -7 et -8 ainsi que le clivage de PARP. L'inhibition par NAC de l'apoptose induite par l'acroléine était confirmée morphologiquement par la baisse de la condensation de la chromatine nucléaire. Ces résultats suggèrent que NAC peut être utilisé comme un antidote pour traiter les gens exposés à l'acroléine.

En conclusion, ces résultats ont permis une meilleure compréhension de la toxicité de l'acroléine, un des produits majeurs de l'oxydation des polyamines

impliqués dans la régulation de la prolifération cellulaire et la croissance des tumeurs. En plus, cette étude a permis de proposer une explication possible de l'activité pharmacologique et/ou de la toxicité du cyclophosphamide, un agent anticancéreux, qui est métabolisé en acroléine et en un mélange de phosphoramides. Ceci ouvre la voie au développement de l'enzymothérapie par l'amine oxydase du sérum de bovin (BSAO) pour lutter contre les cancers. Le milieu cancéreux contient des concentrations élevées en polyamines qui sont des molécules nécessaires à la division et la prolifération cellulaire. Ces polyamines seront oxydées par la BSAO et libéreront les produits d'oxydation dont l'acroléine et le péroxyde d'hydrogène qui sont impliqués dans l'inhibition de la prolifération cellulaire, l'apoptose, et l'inhibition de la synthèse de l'ADN et des protéines. Ce faisant, la taille de la tumeur régresse et la santé du patient s'améliora. D'autre part, les résultats de ce projet permettent d'envisager la possibilité d'utiliser les composés qui augmentent le niveau de glutathion (GSH) dont le NAC dans des cas de toxicité à l'acroléine, molécule qui prend de l'ampleur dans la recherche sur la maladie d'Alzheimer.

Mots clés : acroléine, apoptose, mitochondrie, voie des récepteurs, MAPK, NAC.

CHAPITRE I
INTRODUCTION

1.1. L'ACROLÉINE

1.1.1. Généralités

Le 26 avril 1914, M. Thomas Edison écrit une lettre à M. Henry Ford et je cite : *"The injurious agent in cigarettes comes principally from the burning paper wrapper. The substance thereby formed, is called "Acrolein". It has a violent action on the nerve centers, producing degeneration of the cells of the brain, which is quite rapid among boys. Unlike most narcotics this degeneration is permanent and uncontrollable. I employ no person who smokes cigarettes".* Cette célèbre lettre de l'inventeur du télégraphe montre bien la nocivité de cette substance et le danger du tabagisme. De nos jours, l'acroléine soulève de plus en plus d'intérêt dans la recherche surtout au niveau des maladies respiratoires et neurodégénératives.

L'acroléine (2-propenal) est la plus électrophile des aldéhydes α,β-insaturées. Elle est extrêmement volatile, piquante, lacrymogène et inflammable. L'humain est exposé à l'acroléine de multiples façons et elle est un polluant omniprésent dans l'environnement (Takeuchi *et al.*, 2001; Li *et al.*, 1999). Cet aldéhyde est dix fois plus toxique que le formaldéhyde qui est largement étudié en toxicologie de l'environnement (Sun *et al.*, 2006).

L'acroléine est utilisée comme herbicide aquatique depuis plus de 40 ans (Bowmer *et al.*, 1976), car elle est cytotoxique pour les tissus des plantes et se décompose rapidement dans l'eau (Kissel *et al.*, 1978). Son utilisation s'étend à la synthèse des polymères de l'acrylate, de l'acide acrylique, de l'immunosuppresseur FR901483 (Maeng et Funk, 2001) et de la cytotoxine lepadiformine (Greshock et Funk, 2001).

L'acroléine se trouve dans tous les types de fumée, notamment la fumée de cigarette (25 à 468 µg par unité) (Esterbauer *et al.*, 1991; Carmines et Gaworski, 2005; Fujioka et Shibamoto, 2006), l'émission des moteurs et les feux de forêts ce qui représente un risque pour les pompiers et les secouristes (Caux *et al.*, 2002). L'acroléine est aussi libérée dans la vapeur de la cuisson d'huile dont on a rapporté des cas sévères de toxicité (Beauchamp *et al.*, 1985). En plus, elle peut être produite par l'oxydation des lipides et former de l'acrylamide, un puissant cancérigène, suite à la cuisson de la patate par de l'huile végétale (Ehling *et al.*, 2005). Durant la combustion de matériaux organiques, par exemple le diesel et le plastique, l'acroléine est libérée en grandes concentrations (Ghilarducci *et al.*, 1995; Uchida *et al.*, 1998). "In vivo", l'acroléine est un des métabolites des agents anticancéreux cyclophosphamide et ifosfamide et elle semble responsable de l'effet thérapeutique (Schwerdt *et al.*, 2006; Kehrer et Biswal, 2000) et de l'hémorragie cystique causée par une exposition chronique à ces agents (Batista et al., 2006). De plus, elle est formée à partir de l'oxydation de la thréonine par la myéloperoxidase du neutrophile aux sites d'inflammation (Shao *et al.*, 2005; Vasilyev *et al.*, 2005; Anderson *et al.*, 1997). L'acroléine est produite par l'oxydation des polyamines (Hoet et Nemery, 2000; Agostinelli *et al.*, 1996), par le métabolisme de l'allylamine qui est une toxine cardiovasculaire (He *et al.*, 1998) et par l'oxydation des lipides insaturés par l'ozone (Medina-Navarro *et al*, 1999). Elle est aussi formée par des réactions photochimiques dans l'air pollué. Selon une étude, le tabagisme est une source significative d'acroléine à l'intérieur des maisons mal aérées de l'Ile du Prince-Edouard du Canada (Gilbert *et al.*, 2005). On estime à 5% la concentration de l'acroléine dans l'air pollué par rapport au total des aldéhydes, notons que le formaldéhyde représente le taux le plus élevé.

L'acroléine est un irritant sensoriel puissant. Une courte exposition à 0.67 ppm endommage les muqueuses olfactives et respiratoires (Cassee *et al.*, 1996). D'autre part, une courte exposition à 6 ppm peut emmener une baisse de 50% dans le taux de respiration chez le rat et une inhibition accrue des mouvements des cils

(Bergers *et al.*, 1996; Cassee *et al.*, 1996). Une exposition prolongée à 10 ppm d'acroléine peut entraîner la mort d'une personne et des cas d'irritation sévère des poumons et de la trachée ont été rapportés avec une exposition allant de 0.17 ppm à 0.43 ppm (Agency for Toxic Substances, 1989). Des analyses récentes montraient des concentrations au-delà de 3 ppm dans 10% des feux à la ville de Boston aux États-Unis (Treitman *et al.*, 1980). Notons qu'on retrouve une concentration de 0.04 à 0.08 ppm d'acroléine dans l'air ambiant (Costa et Amdur, 1996). On estime la concentration d'acroléine inhalée en fumant ou en respirant dans un environnement de fumeurs à 80 μM dans le fluide du tractus respiratoire (Eiserich *et al.*, 1995). Sa production annuelle aux États-Unis est estimée à 60 millions de livres sans compter la partie utilisée pour la synthèse de l'acide acrylique (Huang *et al.*, 2002).

1.1.2. Propriétés physiques

L'acroléine est un liquide de couleur blanche ou jaune. Elle a une odeur très désagréable qui peut être sentie à aussi peu que 0.2 ppm. La formule chimique est C_3H_4O et la masse molaire est de 56.06 g/mol. La pression de vapeur de l'acroléine est de 220 mm Hg à 20^0C et son coefficient de partition Log K_{ow} est -0.01 (Agency for Toxic Substances, 1989). Pour l'acroléine, 1 ppm représente une concentration de 2.29 mg/m^3.

1.1.3. Propriétés biochimiques

1.1.3.1. La réaction d'addition de Michael

L'acroléine est capable de réagir selon la réaction d'addition de Michael (Kaminskas *et al.*, 2005; Maeda et Kraus, 1997) qui est une façon classique de former des liaisons carbone-carbone (Figure 1.1). Il s'agit simplement de la conjugaison d'un énolate nucléophile à un composé carbonyle α,β-insaturé tel que l'acroléine. L'acroléine peut réagir avec les acides nucléiques, les acides aminés et les thiols par la réaction d'addition de Michael, et de ce fait créer des liaisons protéine-acroléine-

protéine (Burcham et Pyke, 2006), ADN-acroléine-ADN (Sanchez *et al.*, 2005; Kozekov *et al.*, 2003) ou protéine-acroléine-ADN (Minko et al., 2005).

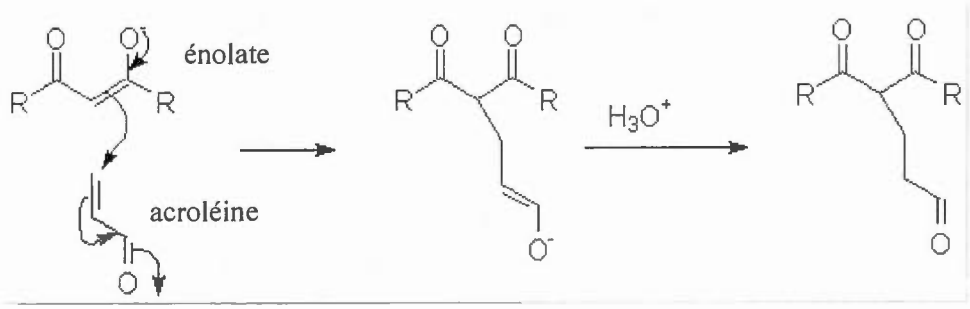


Figure 1.1 : La réaction d'addition de Michael entre un énolate et l'acroléine (Maeda et Kraus, 1997).

1.1.3.2. Interactions au niveau cellulaire : mécanismes de toxicité

1.1.3.2.1. Modifications des acides nucléiques par l'acroléine

L'acroléine réagit avec les bases azotées de l'ADN (Pawlowicz *et al.*, 2006; Kitamura *et al.*, 2006; Takeuchi *et al.*, 2001; Kehrer 2000). L'acroléine réagit avec le désoxyguanosine (dG) de l'ADN pour former des exocycles stéréoisométriques dans les tissus humains (Chung *et al.*, 1999; De Los Santos *et al.*, 2001) entraînant une substitution de la guanine en thymine ou en adénine lors de la réplication de l'ADN. Il a été démontré que la forme 1 (voir Figure 1.2) est dominante après une réaction entre l'acroléine et le 2'-désoxyguanosine de l'ADN tandis que la forme 3 est la structure la plus stable (Khullar *et al.*, 1999; Kanuri *et al.*, 2002). Ces exocycles préviennent la formation des ponts hydrogènes Watson-Crick et ainsi inhibent la synthèse de l'ADN jusqu'à 70% et emmènent des erreurs durant la réplication chez Escherichia coli (Yang *et al.*, 2001). Ces nouvelles liaisons peuvent contribuer à la mutagenèse spontanée et jouent un rôle dans le vieillissement et le cancer (Yang *et al.*, 2002; Chung *et al.*, 1999). L'addition de l'acroléine à la guanine se fait par la réaction d'addition de Michael (Nechev *et al.*, 2002; Pan and Chung, 2002). Une récente étude démontrait par simulation d'énergie libre (ΔG) que la N-alkylation de la

guanine était plus favorisée que la O^6-alkylation (Balu *et al.*, 2002). L'acroléine stimule la carcinogénèse de l'appareil urinaire chez le rat (Cohen *et al.*, 1992). Elle est une inhibitrice potentielle des enzymes de réparation de l'ADN, par exemple la poly-ADP-ribose-polymérase (PARP) (Grafstrom *et al.*, 1994; Dypbukt *et al.*, 1993). Cela explique sa génotoxicité et son activité mutagénique chez la drosophile (Sierra *et al.*, 1991) et les bactéries (Parent *et al.*, 1996; Van Beerendonk *et al.*, 1998).

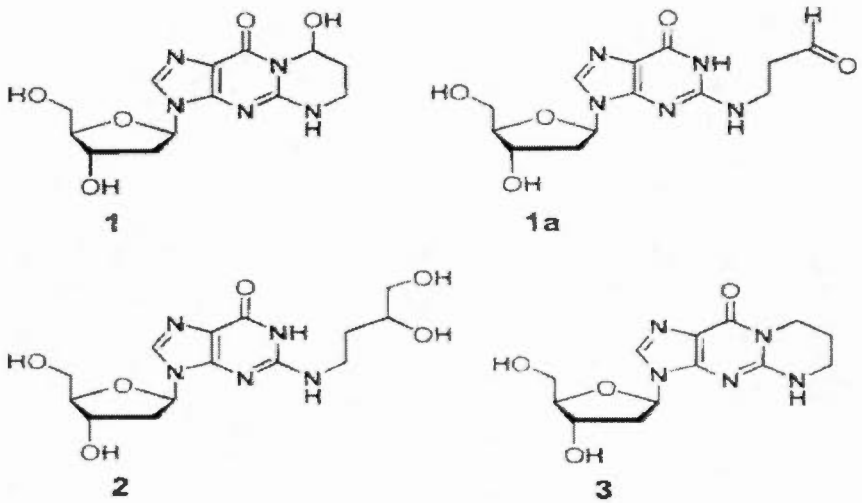


Figure 1.2: Des cycles formés par la réaction entre la désoxyguanosine de l'ADN et l'acroléine (Yang *et al.*, 2001, 2002; Khullar *et al.*, 1999). Il a été démontré que la forme 1 est dominante tandis que la forme 3 est la structure la plus stable (Khullar *et al.*, 1999; Kanuri *et al.*, 2002).

1.1.3.2.2. Modifications des acides aminés par l'acroléine

L'acroléine réagit avec le groupe sulfhydryl de la cystéine (Starkenmann *et al.*, 2005), l'imidazole de l'histidine et l'amine de la lysine des protéines (Kitamura *et al.*, 2006; Takeuchi *et al.*, 2001; Kehrer, 2000). D'autre part, l'acroléine modifie les résidus histidine de l'anhydrase carbonique (Tu *et al.*, 1989) pour former deux nouveaux composés, le N^{α}-acétyl-N^{α}-formyléthylhistidine (Figure 1.3B) et le N^{α}-acétyl-$N\tau$-formylethylhistidine (Figure 1.3C) (Uchida *et al.*, 1998). Bien qu'il était

proposé que la réaction de l'acroléine avec les groupements amine forme les propanals β-substitués (R---NH---CH_2---CH_2---CHO) et la base de Schiff's (R---NH---CH_2---CH_2----CH-----N---R), une structure acroléine-lysine ou N^{ε}-(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine) a été identifiée comme le produit majeur de la réaction (Esterbauer *et al.*, 1991) (Figure 1.3A). Une récente étude a démontré le potentiel électrophilique de la FDP-lysine (Furuhata *et al.*, 2002). Ainsi la structure N-α-acetyl-FDP-lysine, générée par la réaction de la N-α-acétyl-lysine avec l'acroléine, a été covalement liée à la glycéraldehyde-3-phosphate déshydrogénase (GAPDH). Notons que la GAPDH est responsable de la production du nicotinamide adenine dinucléotide (NADH). La structure FDP-lysine réagissait aussi avec le glutathion pour former un nouveau conjugué protéique (Figure 1.4) (Furuhata *et al.*, 2002).

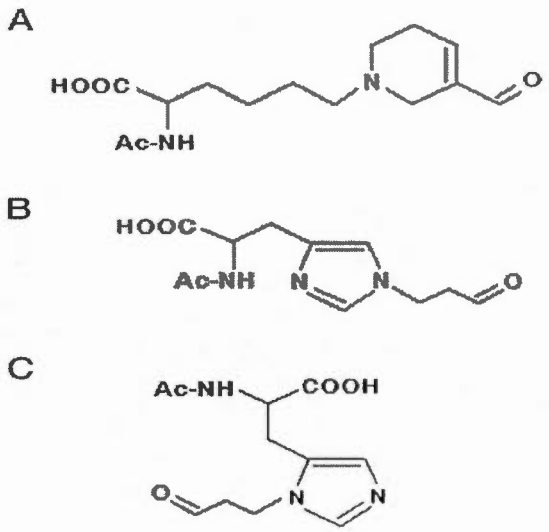


Figure 1.3: L'acroléine réagit avec le groupe sulfhydryl de la cystéine (Starkenmann *et al.*, 2005), l'imidazole de l'histidine et l'amine de la lysine des protéines (Kitamura *et al.*, 2006; Takeuchi *et al.*, 2001; Kehrer, 2000). Cette figure illustre les structures de l'acroléine-lysine (*A*) et l'acroléine-histidine (*B* et *C*). *A*, N^{α}-acetyl-N^{ε}-(3-formyl-3,4-dehydropiperidino) lysine ou N^{α}-acétyl-FDP-lysine. *B*, N^{α}-acetyl-N^{α}-formylethylhistidine. *C*, N^{α}-acétyl-$N\tau$-formylethylhistidine. (Uchida *et al.*, 1998).

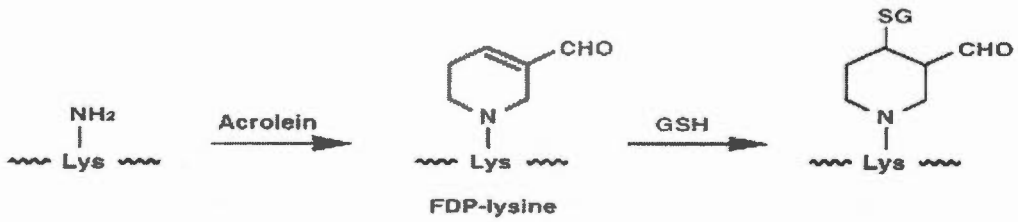


Figure 1.4: Une illustration d'une glutathiolation d'une protéine par l'acroléine (Furuhata *et al.*, 2002). L'acroléine réagit avec le groupe amine de la lysine des protéines pour former la FDP-lysine, le produit majeur de réaction (Kitamura *et al.*, 2006; Takeuchi *et al.*, 2001; Kehrer, 2000). La structure FDP-lysine réagit ensuite avec le glutathion pour former un nouveau conjugué protéique (Furuhata *et al.*, 2002).

1.1.3.2.3. Interactions avec les thiols et les facteurs de transcription

L'acroléine est la plus réactive des aldéhydes α,β-insaturées. Elle se lie rapidement aux molécules nucléophiliques comme le glutathion qui est un thiol essentiel pour la défense antioxydante. On peut voir dans la Figure 1.5 la réaction d'un thiol (glutathion) avec l'acroléine par la réaction d'addition de Michael.

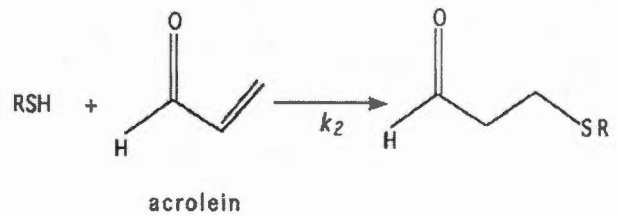


Figure 1.5: Réaction d'un thiol (exemple : glutathion) avec l'acroléine (Tacka *et al.*, 2002). L'acroléine, une molécule électrophilique, se lie rapidement aux molécules nucléophiliques comme le glutathion qui est un thiol essentiel pour la défense antioxydante. L'acroléine réagit avec le groupe sulfhydryl de la cystéine selon le mécanisme d'addition de Michael (Starkenmann *et al.*, 2005).

De plus, l'acroléine inactive l'enzyme glutathione réductase qui régénère la forme réduite du glutathion (GSH), lorsque le GSH est oxydé en disulfure de glutathion (GSSG) (Nunoshiba et Yamamoto, 1999). Ces réactions vont diminuer les concentrations intracellulaires des thiols, créant un déséquilibre dans la balance d'oxydo-réduction (Finkelstein *et al.*, 2001; Nunoshiba et Yamamoto 1999).

La balance d'oxydo-réduction des thiols est critique pour plusieurs fonctions cellulaires (Arrigo, 1999) et des changements induits par l'acroléine semblent affecter plusieurs voies de signalisation. Notons qu'il y a un grand nombre de gènes et de facteurs de transcription sensibles aux changements oxydo-réducteurs dont le facteur nucléaire-κβ (NF-κβ) et la protéine activatrice-1 (AP1) (Allen et Tresini, 2000; Arrigo, 1999). La régulation des facteurs de transcription a suscité un grand intérêt ces dernières années. Ces protéines se lient au promoteur des gènes cibles déclenchant la transcription. La régulation se fait par activation de ces facteurs dans le cytosol pour une redistribution dans le noyau, ou en affectant leur liaison au promoteur. Ce processus requiert souvent de la cystéine au domaine de liaison à l'ADN. En se liant à un acide aminé nucléophile comme la cystéine d'un facteur de transcription, l'acroléine peut directement interférer avec cette transcription, ou indirectement en diminuant le glutathion intracellulaire. Plusieurs gènes et facteurs de transcription impliqués dans le cycle cellulaire et l'apoptose ont été identifiés. "In vitro", l'acroléine inhibe quelques-uns de ces facteurs notamment le facteur nucléaire kappa beta (NF-κB) et la protéine activatrice-1 (AP-1) (Horton *et al.*, 1999; Li *et al.*, 1999). Ceci se produit par liaison à une cystéine sur la sous-unité p50 et/ou la sous-unité p65 dans le cas du facteur NF-κB (Kumar *et al.*, 1992; Toledano *et al.*, 1993). Plus récemment, on a démontré que l'acroléine pourrait réagir avec la kinase inductible κβ (IKK) et supprimer sa fonction et de ce fait inhiber l'activité du NF-κB. Normalement, l'IKK enlève l'inhibiteur kinase (IK) du NF-κB par phosphorylation et ainsi active le facteur de transcription NF-κB (Valacchi *et al.*, 2005)

L'acroléine inactive la disulfure-isomérase qui participe à la maturation des protéines nouvellement synthétisées en permettant une bonne formation des ponts disulfures au niveau du réticulum endoplasmique (Carbone *et al.*, 2005; Liu et Sok, 2004). En plus, l'acroléine inhibe la thioredoxine et la thioredoxine-réductase qui jouent un rôle dans la prolifération cellulaire et la régulation de plusieurs facteurs de transcription qui sont sensibles aux conditions d'oxydoréduction (Yang *et al.*, 2004).

Une exposition des cellules A549 provenant d'un carcinome de poumon à une concentration non-létale d'acroléine (150 *f*mol/cellule pour une heure) diminue le glutathion intracellulaire de 80%. De plus, l'acroléine augmente la transcription de l'enzyme de synthèse de GSH, la γ-glutamylcystéine synthétase (γ-GCS). En ce faisant, la γ-GCS aide à rétablir le niveau de glutathion réduit. Notons que cette enzyme appartient à la famille des éléments de réponse aux antioxydants (ARE). Cette activation de la transcription de la γ-GCS est médiée par l'activation du facteur de transcription nucléaire dérivé des érythroides (Nrf2) qui se lie aux gènes de la phase II et induit leur transcription (Tirumalai *et al.*, 2002). Uchida (1999) a rapporté que l'acroléine est capable d'induire l'expression de la glutathion-S-transférase (GST) qui est une enzyme impliquée dans la détoxification de nombreux xénobiotiques.

1.1.3.2.4. Physiopathologies associées à l'acroléine

L'acroléine augmente l'expression des gènes de mucines MUC5AC et MUC5B qui emmènent une hypersécrétion de mucus (Borchers *et al.*, 1998, 1999). Cette hypersécrétion est responsable de plusieurs maladies respiratoires notamment la maladie chronique pulmonaire obstructive (Hogg 2001), l'asthme, la fibrose cystique (Witschi *et al.*, 1997; Samet *et al.*, 1994), la bronchite chronique et l'emphysème (Saetta, 1999).

En parallèle, l'acroléine peut altérer la fonction des neutrophiles humains en inhibant leur apoptose et en accentuant la libération de l'interleukine-8

(chimioattracteurs des neutrophiles). Cette altération favorise le processus d'inflammation des voies respiratoires (Akgul *et al.*, 2001; Finkelstein *et al.*, 2001) puisque l'apoptose est l'étape clé dans la résolution de l'inflammation (Haslett 1999; Fadok *et al.*, 1998). En plus, l'acroléine inhibe la NADPH-oxydase des neutrophiles qui est essentielle dans la défense cellulaire contre les pathogènes (Nguyen *et al.*, 2001). Plusieurs études ont démontré des effets multiples de l'acroléine sur la fonction des macrophages alvéolaires, notamment une inhibition de la phagocytose (Low *et al.*, 1977). De plus, l'acroléine inhibe la production de superoxyde dans les macrophages alvéolaires de rat et dans les neutrophiles humains (Nguyen *et al.*, 2001; Witz *et al.*, 1987). L'acroléine induit un taux élevé de mort cellulaire, une production altérée de cytokines ainsi qu'une inhibition de la synthèse des macromolécules et de l'activité ATPase dans les macrophages (Li *et al.*, 1997, 1999), ce qui est probablement la cause de l'immunosuppression pulmonaire (Li et Holain, 1998). De nouvelles observations démontrent qu'à un taux de respiration normal de 2 à 4 L/min, un enfant peut théoriquement recevoir une dose immunosuppressive d'acroléine (10-30 μg) pour une heure d'exposition, une dose qui correspond à une exposition typique à la fumée secondaire de cigarette dans un restaurant. Cette dose supprimerait la réponse des lymphocytes T au niveau des poumons des fumeurs (Lambert *et al.*, 2005) .

Des liaisons acroléine-protéines ont été trouvées chez des personnes souffrant de néphropathie diabétique (Suzuki and Miyata, 1999), de la maladie d'Alzheimer (Seidler *et al.*, 2006; Williams *et al.*, 2006; Kawaguchi-Niida *et al.*, 2006; Seidler et Squire, 2005; Arlt *et al.*, 2002; Pocernich *et al.*, 2001; Calingasan *et al.*, 1999) et de l'ischémie cérébrale (Adibhatla *et al.*, 2003), qui sont des maladies associées avec une élévation de la peroxydation lipidique. On a suggéré l'utilisation de l'acroléine comme marqueur biochimique en clinique pour le prognostic de la maladie d'Alzheimer (Calingasan *et al.*, 1999), des lésions diabétiques glomérulaires (Suzuki *et al.*, 1999), de l'athérosclérose (Biswal *et al.*, 2002; Uchida *et al.*, 1998), du stress oxydatif chez les nouveaux-nés (Tsukahara *et al.*, 2004), du diabète du type I (Hata *et*

al., 2006), de l'insuffisance rénale chronique (Igarashi *et al.*, 2006), et pour le diagnostic des accidents cérébraux (Tomitori *et al.*, 2005). Le suivi peut se faire en testant la concentration du conjugué acroléine-lysine dans l'urine du patient (Yan *et al.*, 2006). Au niveau de l'hippocampe des patients atteints de la maladie d'Alzheimer, l'acroléine atteint 2 nM (Lovell *et al.*, 2000, 2001). Récemment, on a observé que l'acroléine était augmentée au niveau de la moelle épinière suivant un traumatisme (Liu-Snyder *et al.*, 2006; Luo et Shi, 2004). De récents résultats suggèrent que l'acroléine joue un rôle critique dans l'athérogénèse chez les humains et ceci, en altérant le déplacement et le nettoyage du cholestérol à partir de la paroi artérielle et en rendant moins stable la plaque d'athérome déjà fragile (Vindis *et al.*, 2006). Le problème du nettoyage du cholestérol est dû au fait que l'acroléine interfère avec le transport de la lipoprotéine de haute densité (HDL) en modifiant la protéine apoA-I à la surface du HDL (Shao *et al.*, 2005). En plus, l'acroléine est impliquée dans le développement de l'angiopathie dont souffrent les fumeurs, et le sang de ces gens ainsi que celui des diabétiques contient des concentrations élevées en acroléine (Misonou *et al.*, 2006).

La peau est aussi une des cibles de l'acroléine, puisque la molécule est formée de façon endogène suite aux irradiations des lipides par les rayons ultraviolets du soleil (Niyati-Shirkhodaee et Shibamoto, 1992). Dans la peau, l'acroléine peut causer l'hypersensibilité non-immunologique (Verrier *et al.*, 1999; Coverly *et al.*, 1998), l'inflammation, le développement de cancer, le vieillissement, la cytotoxicité et la génotoxicité (Grafstrom *et al.*, 1994; U.S. Department of Health and Human Sciences, 1993). L'acroléine est capable d'activer le récepteur du facteur de croissance épidermale (EGFR) chez les kératinocytes humains, emmenant l'activation des protéines kinases activées par les mitogènes (MAPKs) et ainsi induire la mort cellulaire par apoptose (Takeuchi *et al.*, 2001). Mentionnons ici que l'activation des MAPKs peut prendre différentes voies de signalisation et ainsi induire l'apoptose dans le cas des kératinocytes humains (Takeuchi *et al.*, 2001) et l'inhibition de l'apoptose chez les neutrophiles humains (Finkelstein *et al.*, 2001).

Enfin, l'acroléine semble changer le phénotype bénin des tumeurs de colon à celui de malin et ceci probablement en inhibant la synthèse du p53 (Zarkovic *et al.*, 2006).

En général, on remarque que l'acroléine induit la mort cellulaire par nécrose (Rudra et Krokan, 1999) et cause une inhibition de la prolifération cellulaire (Agostinelli *et al.*, 1994, 1996; Biswal *et al.*, 2002). Toutefois, l'acroléine peut induire l'apoptose ou l'inhiber dans le même type cellulaire et ceci dépendamment de la concentration et le type cellulaire utilisé. Par exemple, une faible concentration en acroléine (<10 μM) induit l'apoptose chez les neutrophiles tandis qu'une plus grande concentration (>10 μM) l'inhibe (Finkelsetin *et al.*, 2005).

1.1.3.2.5. Métabolisme de l'acroléine

Jusqu'à récemment, il n'y avait pas eu d'étude détaillée sur le métabolisme de l'acroléine chez aucune espèce. On rapportait que l'acroléine était métabolisée en glycidaldéhyde, glyceraldéhyde et l'acide acrylique dans les microsomes de foie de rat (Patel *et al.*, 1980). Sharp *et al.* proposait la voie métabolique ci-dessous (Figure 1.6) pour l'acroléine chez la poule. Le métabolisme et la distribution du 2,3-C^{14} de l'acroléine étaient étudiés chez 10 poules qui ont reçu oralement des doses de 1.09 mg/kg de masse corporelle par jour pour 5 jours. La radioactivité était mesurée dans les œufs, les excréments et l'air expiré.

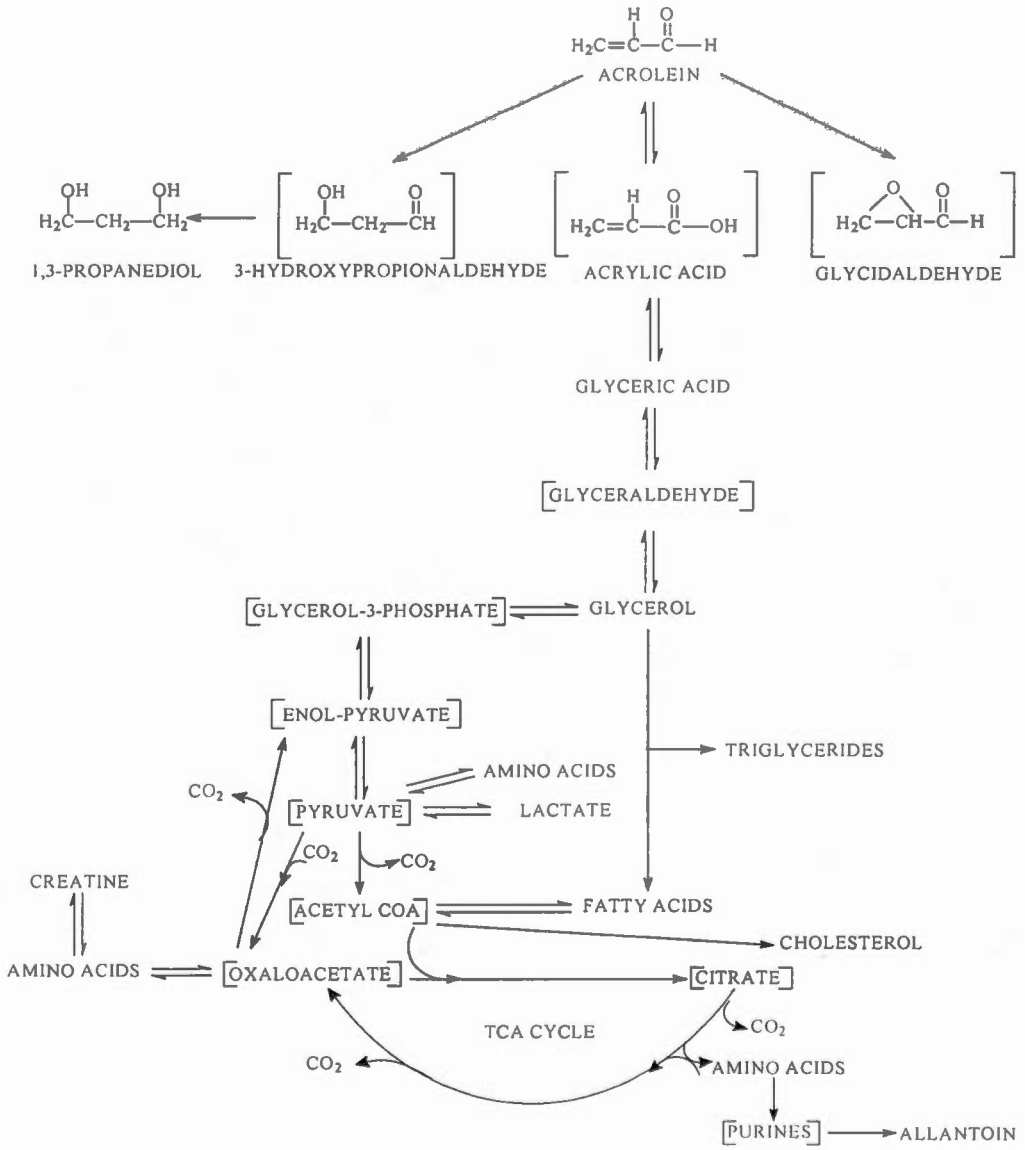


Figure 1.6: Voie du métabolisme de l'acroléine proposée chez la poule, en étudiant la radioactivité du 2,3-^{14}C de l'acroléine. Les composés entre parenthèses sont des intermédiaires qui ne sont pas isolés (Sharp *et al.*, 2001). Le métabolisme et la distribution du 2,3-C^{14} de l'acroléine étaient étudiés chez 10 poules qui ont reçu oralement des doses de 1.09 mg/kg de masse corporelle par jour pour 5 jours. La radioactivité était mesurée dans les œufs, les excréments et l'air expiré.

1.1.4. Protection cellulaire contre les effets néfastes de l'acroléine

La glutathion-S-transférase (GST) lie l'acroléine au glutathion (GSH) qui est impliqué dans la défense cellulaire et ainsi éviter à la cellule les effets indésirables de l'acroléine (Meacher et Menzel, 1999; Berhane *et al.*, 1994; Eder *et al.*, 1982). D'autre part, l'acroléine induit la GST et la GSH-péroxydase *in vivo* suite à une agression vasculaire chez le rat pour contrer la baisse accrue de la GSH-péroxydase et de la GST au début de l'agression (Yousefipour *et al.*, 2005). L'aldéhyde-déshydrogénase-1 est une enzyme importante dans la défense cellulaire contre les aldéhydes toxiques dont l'acroléine chez l'humain (Lindahl et Peterson, 1991; Burcham et Fontaine, 2001). Cependant, l'acroléine entraîne une inhibition de l'enzyme aldéhyde déshydrogénase-1 chez l'humain (Ren *et al.*, 1998; Bunting et Townsend, 1998), ce qui bloquera une des voies d'élimination de l'acroléine.

L'enzyme O^6-alkylguanine-ADN-transférase enlève la modification apportée à la position O^6 de la guanine après une alkylation par l'acroléine (Cai *et al.*, 2000; Balu *et al.*, 2002). De plus, récemment, on a découvert que la cellule possède un mécanisme de réparation pour contourner les dommages infligés à la guanine. En fait, la protéine Rev1 ajoute un résidu C en face du G alkylé par l'acroléine et la polymérase ζ continue la réplication et ainsi la mutation est réparée (Wolfle *et al.*, 2005; Washington *et al.*, 2004).

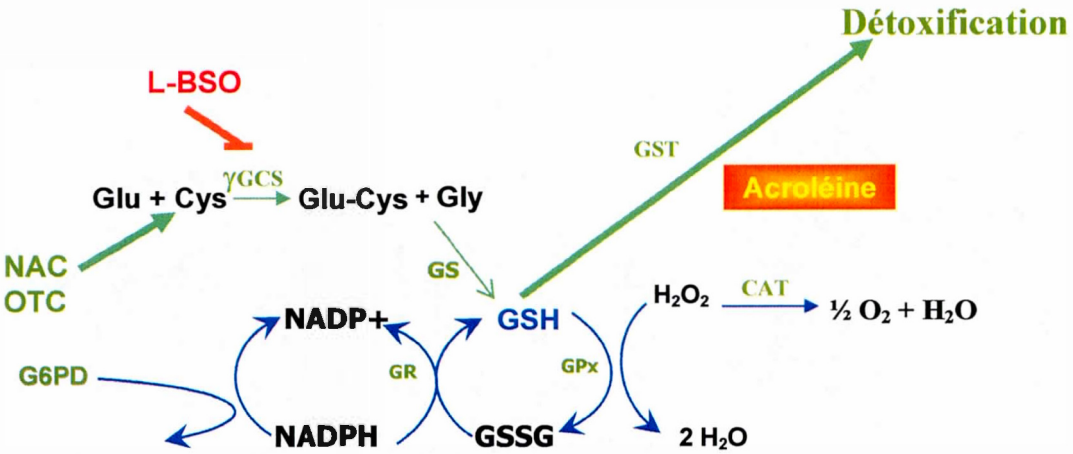
L'hydralazine, qui est un antihypertensif, est un piégeur efficace de l'acroléine (Burcham et Pyke, 2006; Liu-Snyder *et al.*, 2006; Kaminskas *et al.*, 2004; Burcham *et al.*, 2000). En plus, la carnosine, un puissant antioxydant, est aussi un piégeur de l'acroléine (Aldini *et al.*, 2005). Une nouvelle étude vient de démontrer que la vitamine C ou l'ascorbate peut protéger des lipoprotéines telles que les lipoprotéines de très faible densité (VLDL) et les lipoprotéines à faible densité (LDL) contre l'oxydation par l'acroléine. Les VLDL oxydées seront phagocytées par les macrophages, une étape clef dans l'initiation de l'athérogénèse (Arai *et al.*, 2005).

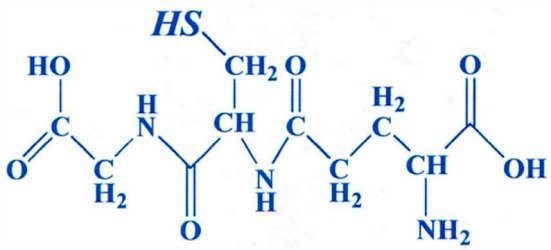
1.1.4.1. Le glutathion (GSH)

L'acroléine a été identifiée comme un initiateur et un produit de la peroxydation lipidique (Biswal *et al.*, 2002; Uchida, 1999). De plus, elle induit la production de l'oxyde nitrique (NO), du superoxyde ($O_2^{\cdot-}$), du peroxynitrite ($ONOO^-$) et du peroxyde d'hydrogène (H_2O_2) (Misonou *et al.*, 2006). Comme ce processus d'oxydation des lipides existe naturellement chez toutes les cellules et qu'il est augmenté dans plusieurs pathophysiologies, un potentiel de toxicité existe après une exposition continue à un agent oxydatif tel que l'acroléine.

Le glutathion (GSH), le plus important antioxydant intracellulaire, peut détoxifier l'acroléine, une réaction catalysée par la GST (Figure 1.7). Trois produits de détoxification sont formés de la réaction entre l'acroléine et le GSH, soient les deux produits majeurs, l'acide 3-hydroxypropylmercapturique (HPMA) et l'acide 2-carboxyethylmercapturique (CEMA), et l'acide mercapturique (ALMA) (Athersuch *et al.*, 2006). De plus, le cycle du glutathion est le mécanisme central pour éliminer le peroxyde d'hydrogène (H_2O_2) qui est aussi produit dans la cellule en présence d'acroléine (Luo *et al.*, 2005).

D'autres substrats métabolisés par le glutathion incluent les larges molécules d'hydroperoxydede lipides, formés par l'attaque de radicaux libres sur les lipides membranaires, ainsi que des produits de la réaction catalysée par la lipooxygénase (Heffner et Repine, 1989). L'enzyme clé du cycle d'oxydoréduction responsable de la réduction du H_2O_2 est la glutathione péroxydase (GPx) (Hashida *et al.*, 2002) (Figure 1.7). Cette réaction requiert spécifiquement le glutathion réduit (GSH) qui sert de donneur d'électrons. Le glutathion disulfure (GSSG) formé au cours de la réaction est réduit de nouveau en GSH par la glutathion réductase (GR) (Halliwell et Gutteridge, 1999), qui utilise le NADPH généré par le cycle des pentose phosphates (CPP) qui sert de donneur d'électron (Rahman *et al.*, 1999). Des cellules non stressées maintiennent un rapport intracellulaire GSH:GSSG élevé pour assurer une disponibilité élevé de GSH (Deneke et Fanburg, 1989).





Glutathion

GSH : glutathion
L-BSO : L-buthionine sulfoximine
NAC : N-acétylcystéine
OTC : 2-oxo-4-thiazolidine carboxylate
GPx : glutathion peroxydase
GS: glutathion synthétase
GST: glutathion S-transférase
GR : glutathion réductase
γ-GCS : γ-glutamylcystéine synthétase
CAT : catalase
G6PD: glucose 6-phosphate déshydrogénase

Figure 1.7: Mécanismes de détoxication cellulaire de l'acroléine impliquant le glutathion (adapté de Anderson, 1997; Rahman *et al.*, 1999; Logan *et al.*, 2005). Le GSH détoxifie l'acroléine à l'aide de la GST et le peroxyded'hydrogène (H_2O_2) à l'aide de la GPx. Le glutathion disulfure (GSSG) formé au cours de la réaction est réduit de nouveau en GSH par la glutathion réductase (GR) (Halliwell et Gutteridge, 1999), qui utilise le NADPH généré par le cycle des pentose phosphates (CPP) qui sert de donneur d'électron (Rahman *et al.*, 1999).

Le GSH est essentiel pour la défense antioxydante, il réagit directement avec les radicaux intermédiaires des réactions non-enzymatiques. L'élimination du superoxyde $O_2^{\cdot-}$ par GSH entraîne la formation des radicaux thiols (GS·) et de H_2O_2 par l'intermédiaire de plusieurs étapes, qu'on appelle une réaction de propagation radicalaire.

En plus de son rôle comme cofacteur du GPx et de la GST, le GSH est impliqué dans d'autres processus métaboliques, tel le maintien de l'acide ascorbique sous sa forme réduite, la communication entre les cellules et la prévention de l'oxydation des groupements thiols des protéines et des liaisons croisées entre les protéines (Halliwell et Gutteridge, 1999). Le GSH participe à la synthèse des précurseurs de la synthèse protéique et de l'ADN et au transport d'acides aminés (Oiry *et al.*, 1999). Le GSH peut chélater les ions de cuivre et ainsi diminuer leur disponibilité pour générer des radicaux libres. Par ailleurs, le GSH joue un rôle dans le repliement protéique et dans la dégradation des protéines avec liaison disulfure.

Le GSH protège les cellules épithéliales pulmonaires de l'oxydation et de l'inflammation (Rahman *et al.*, 1999, 2000). Une récente étude a démontré que le GSH peut aussi atténuer le développement de l'athérosclérose (Rosenblat *et al.*, 2002). Enfin, on le retrouve dans le cytosol à des concentrations de l'ordre du millimolaire chez les plantes, les animaux et les bactéries aérobiques (Anderson, 1997).

1.1.4.1.1. Biosynthèse du GSH

Le GSH est synthétisé en deux étapes. Premièrement, l'enzyme γ-glutamylcystéine synthétase (E1) catalyse la formation du premier lien peptidique, ensuite le produit est converti en GSH par la glutathion synthétase (E2) (Équation 1) (Anderson, 1997; Rahman *et al*, 1999):

Équation 1:

$$\text{L-glutamate} + \text{L-cystéine} + \text{ATP} \xrightarrow{\text{E1}} \text{L-}\gamma\text{-glutamyl-L-cystéine} + \text{ADP} + P_I$$

$$\text{L-}\gamma\text{-glutamyl-L-cystéine} + \text{glycine} + \text{ATP} \xrightarrow{\text{E2}} \text{GSH} + \text{ADP} + P_I$$

Les cellules peuvent produire la cystéine nécessaire à partir de la méthionine ou bien elles peuvent la prendre dans les fluides avoisinants. Souvent, les cellules utilisent la forme disulfure (cystine) et la réduisent en cystéine à l'intérieur de la cellule. Pour sa part, la γ-glutamylcystéine synthétase est rétro-inhibée par le GSH et ne semble pas être saturée à des niveaux cellulaires normaux de cystéine (Halliwell et Gutteridge, 1999). Une élévation en cystéine peut promouvoir la synthèse du GSH dans certaines circonstances.

1.1.4.2. Modulation du glutathion intracellulaire par des composés synthétiques

L'acroléine et les EOR, lorsque générées en excès et avec un mécanisme de défense insuffisant, peuvent engendrer des dommages à l'ADN, aux lipides membranaires, aux protéines et aux carbohydrates (Gillissen et Nowal, 1998). Chez l'humain, plus particulièrement dans les poumons, plusieurs maladies sont associées aux métabolites oxygénés qui ont un rôle prédominant dans la pathogénicité. Par conséquent, une approche thérapeutique consisterait en une augmentation de produits antioxydants dans l'organisme, tel le N-acétyl-L-cystéine (NAC), le 2-oxo-4-thiazolidine carboxylate (OTC) et le chélateur desferroxamine (Gillison et Nowak, 1998).

1.1.4.2.1. Le N-acétyl-L-cystéine (NAC)

Le NAC qui est un dérivé thiol, augmente le niveau de GSH intracellulaire chez les cellules animales. Le NAC est désacétylé intracellulairement afin de céder

une cystéine (Cotgreave, 1997). Cette dernière peut ensuite être rapidement utilisée pour la synthèse du GSH (Issels *et al.*, 1988). À cause de son groupement SH, le NAC élimine également le peroxyded'hydrogène (H_2O_2), les radicaux libres hydroxyles ($OH^{\bullet}$) et l'acide hypochloreux (HOCl). De plus, le NAC inhibe la propriété immunosuppressive de l'acroléine (Lambert *et al.*, 2005) mais réduit la production cellulaire des médiateurs pro-inflammatoires tels que le facteur de nécrose tumorale (TNF-α) et l'interleukine-1 (IL-1) (Gillissen et Nowak, 1998). Le NAC inhibe l'activation des facteurs de transcription "redox-sensitive", comme le AP-1 (Lo *et al.*, 1995) et le NFκB (Schreck *et al.*, 1991), et bloque l'activation des MAPKs (Chen *et al.*, 1995; Sekharam *et al.*, 1998). Par ailleurs, le NAC est utilisé cliniquement pour traiter les surdoses d'acétaminophène, un médicament détoxifié par le GSH (Perry et Shannon, 1998). Le NAC est aussi un agent chimioprotecteur réduisant les effets secondaires de nombreux composés cytotoxiques. Il stimule le système immunitaire, prévient le cancer, détoxifie les cellules des métaux lourds, traite la bronchite et la toux des fumeurs, prévient les maladies cardiaques et ralentit la progression du SIDA (De la Fuente *et al*, 2000; Droge, 1999; Faintush *et al*, 1999). Le NAC diminue le potentiel mutagénique de l'ADN oxydé (Malins *et al.*, 2002), et peut augmenter l'expression de l'ARNm du p53 (Liu *et al.*, 1998). Enfin, le NAC peut inhiber complètement l'apoptose induite par la fumée de cigarette au niveau des cellules épithéliales des alvéoles (Hoshino *et al.*, 2001).

1.1.4.2.2. Le 2-oxo-4-thiazolidine carboxylate (OTC)

L'OTC est converti intracellulairement en S-carboxy-L-cystéine à l'aide de l'enzyme 5-oxo-L-prolinase et ensuite décarboxylé spontanément pour libérer la L-cystéine (Dizdar *et al.*, 2000) nécessaire pour la synthèse du glutathion (Rosenblat *et al*, 2002). L'OTC augmente le taux de survie des patients atteints de SIDA (Oiry *et al.*, 1999), protège les lymphocytes du sang (après exposition au peroxyde d'hydrogène (Corsi *et al.*, 1998) et protège les poumons de l'hyperoxie chez les rats

(Levy *et al.*, 1998). Néanmoins, les effets bénéfiques de ces molécules (NAC et OTC) sont restreints "in vivo" par les grandes concentrations nécessaires pour avoir des effets biologiques, par leur métabolisme extracellulaire rapide et par la faible concentration plasmique (Oiry *et al.*, 1999).

1.1.4.2.3. Le L-buthionine sulfoximine (BSO)

Le L-BSO est un inhibiteur spécifique de la γ-glutamylcystéine synthétase (GCS) (Griffith et Meister, 1979). Il est utilisé afin de démontrer l'implication d'un stress oxydatif lors de l'étude de composés toxiques (Sakurai *et al.*, 1998). Le BSO augmente la susceptibilité à la mort cellulaire induit par plusieurs stress, tels que le stress oxydatif (Przybytkowski et Averill-Bates, 1996), le choc thermique (Lord-Fontaine et Averill-Bates, 1999; Mitchell et Russo, 1983) et des agents anticancéreux (Anderson *et al.*, 1997). Ce dernier inhibe la synthèse du GSH plutôt que la réserve intracellulaire en GSH. Le 10 à 20% de GSH restant dans la cellule suite à un traitement avec le L-BSO se retrouve à l'intérieur des mitochondries (Anderson, 1997). Ces dernières, ne pouvant pas synthétiser le GSH, l'importent à partir du cytosol. De ce fait, les cellules ayant subit un traitement avec le L-BSO s'avèrent être plus sensibles face à une attaque de radicaux libres (Anderson *et al*, 1999). Enfin, la diminution du GSH réduit l'activité enzymatique de la gluthation péroxidase (Flohe L, 1982; Lord-Fontaine et Averill, 1999; Nomura *et al.*, 1999).

La cellule se dote d'un mécanisme de défense très bien régulé impliquant des protéases afin de s'autodétruire en cas de dérèglement en faveur des dommages. Un dérèglement peut être causé par des substances toxiques tels que l'acroléine ou l'acétaminophène et / ou la diminution des défenses cellulaires. D'ailleurs, il est connu que les dommages cellulaires qui nuisent à la fonction normale de la cellule sont à l'origine de plusieurs cancers. Ainsi, si les dommages infligés à la cellule par les ERO sont plus importants que la défense cellulaire, la cellule s'autodétruit par apoptose ou meurt par nécrose.

1.2. MÉCANISMES DE MORT CELLULAIRE

1.2.1. Nécrose

La mort cellulaire nécrotique est un processus passif résultant d'une séquence d'événements cellulaires et tissulaires, qui survient à la suite de perturbations profondes de l'environnement cellulaire. Elle se produit dans des conditions pathologiques telles que l'hypoxie sévère, la privation de glucose, le traumatisme, la variation de l'osmolarité, de la température de l'environnement, et du pH, ainsi qu'à l'exposition à des toxines et des agents infectieux. La nécrose conduit à une perte cellulaire toujours secondaire à des conditions pathologiques (Choi *et al.*, 1999) et elle se caractérise de façon constante par une réaction inflammatoire.

Les anomalies morphologiques observées dans la nécrose résultent des perturbations des fonctions cellulaires. Au niveau de la cellule, deux phénomènes principaux conduisent irréversiblement à la mort des cellules nécrotiques affectées : la perte de l'intégrité membranaire et l'altération des fonctions mitochondriales. Un des premiers événements est la rupture de la membrane plasmique et donc la perte de sa capacité à réguler les gradients osmotiques et ioniques. Cela engendre une entrée massive d'eau accompagnée d'électrolytes, provoquant ainsi un gonflement de la cellule et une augmentation de la perméabilité aux molécules extracellulaires. Ainsi les morphologies cellulaires sont rapidement modifiées et vont s'accompagner d'une augmentation du volume cellulaire, d'un gonflement des organites et du noyau.

Dans le cytoplasme, les organites commencent à dégénérer et de profondes perturbations de l'activité mitochondriale sont observées. La mitochondrie et le réticulum endoplasmique se dilatent, des vacuoles intracytoplasmiques se forment et les polysomes se détachent du réticulum endoplasmique. Les organites intracellulaires sont dispersés dans l'espace extracellulaire. La structure nucléaire est également perturbée, le noyau est gonflé et la chromatine digérée par des protéases et des endonucléases notamment des sérines protéases et ainsi l'ADN nucléaire va être

dégradé de manière ' aléatoire ' (Bicknell et Cohen, 1995 ; Dong *et al.*, 1997) générant des fragments dépourvus d'extrémité 3' sortante.

La masse de cellules nécrotiques peut se limiter à une nécrose localisée, ou évoluer vers une nécrose diffuse qui résulte de l'action d'enzymes lysosomiales dérivées de la cellule elle-même (autolyse) ou de celles voisines (hétérolyse). La mort cellulaire par nécrose déclenche souvent une forte réaction inflammatoire locale. Les cellules nécrotiques lysées libèrent des substances dont les leucotriènes qui sont générés par la peroxydation des lipides membranaires, et des composants de la mitochondrie activant le complément (Giclas *et al.*, 1979) qui ne sont pas encore identifiés (Kagiyama *et al.*, 1989). Ces substances induisent une réaction inflammatoire typique de la nécrose (Kroemer *et al*, 1998) et les débris cellulaires sont alors phagocytés par les cellules immunitaires de l'inflammation.

1.2.2. Apoptose

1.2.2.1. Introduction

La mort cellulaire programmée fait partie intégrante de la physiologie normale d'un organisme. Ainsi au cours des nombreuses mitoses et différenciations cellulaires qui permettront de créer un organisme à partir d'un œuf, il est nécessaire d'éliminer continuellement les cellules superflues ou potentiellement dangereuses. Ce phénomène d'élimination sélective des cellules est médié par un processus appelé **APOPTOSE.** Le nom apoptose fait référence à la chute programmée des feuilles à l'automne; ***apo*** pour éloignement et ***ptose*** pour chute. La notion d'apoptose a été introduite en 1972 par Kerr et coll. pour désigner une forme de mort cellulaire totalement différente de la nécrose, tant d'un point de vue morphologique que biochimique. La nécrose était la seule forme de mort cellulaire connue à cette époque. L'apoptose est une réponse hautement conservée des eucaryotes unicellulaires jusqu'aux mammifères (Maarouf *et al.*, 1997; Ameisen *et al.*, 1995). L'apoptose survient naturellement au cours de l'embryogenèse, du renouvellement tissulaire et

lors du vieillissement. Cependant, elle peut également se produire dans diverses conditions pathologiques. De plus, un dysfonctionnement des mécanismes régulateurs de l'apoptose va donner lieu à de graves pathologies. Ainsi, un défaut d'apoptose va entraîner des syndromes prolifératifs associés à un processus de tumorisation, tout particulièrement au niveau des tissus à renouvellement rapide comme le système immunitaire (lymphome, leucémie). A l'inverse, une activation anormale va donner lieu à un phénomène de dégénérescence, comme cela est observé au cours de nombreuses maladies neurodégénératives telles que la maladie d'Alzheimer, la maladie de Parkinson, ou la sclérose latérale amyotrophique (Thompson, 1995).

L'apoptose est considérée comme une mort cellulaire "ordonnée ", procédant par différentes phases (Duvall et Wyllie, 1986; Kerr *et al.*, 1972). L'un des points morphologiques caractéristiques de l'apoptose est l'importante condensation à la fois du noyau et du cytoplasme ce qui induit une diminution significative du volume cellulaire. Ensuite, l'ADN est clivée en fragments réguliers d'environ 180 paires de base (Wyllie *et al.*, 1984). La membrane plasmique va bourgeonner et conduire à la formation de corps apoptotiques renfermant une partie du cytoplasme de la cellule. Afin de faciliter la reconnaissance des corps apoptotiques par les phagocytes, la cellule va signaler son état apoptotique à son environnement notamment grâce au changement de localisation des molécules de phosphatidylsérine qui passent d'une orientation cytoplasmique vers une orientation extracellulaire. L'un des points majeurs de l'apoptose est que l'intégrité de la membrane plasmique n'est jamais altérée au cours du processus, ce qui permet d'éviter tout déversement du contenu cellulaire et ainsi prévenir tout dommage infligé aux tissus avoisinant. Toutefois, l'on peut noter que l'inflammation n'est pas nécessairement totalement absente durant l'apoptose, en raison du relargage de l'interleukine-1 et -18 dans l'environnement, mais il s'agit d'une inflammation régulée.

1.2.2.2. Voies de signalisation de l'apoptose

De nombreux signaux très différents, physiologiques ou pathologiques, intracellulaires ou extracellulaires, peuvent déclencher l'apoptose. Les dommages cellulaires, tels que les agents cytotoxiques, les radiations ionisantes et les chocs thermiques, induisent des signaux apoptotiques. L'apoptose peut être induite, par exemple, par la carence en facteurs de croissance et par certains récepteurs nucléaires (récepteurs aux glucocorticoïdes) activés par leur ligand. Trois voies principales peuvent être décrites dans l'induction de l'apoptose: l'activation des récepteurs membranaires de "mort", comme le récepteur associé au fibroblaste (FasR) ou le récepteur du facteur de nécrose tumorale (TNF-R), l'activation de la voie mitochondriale et celle de la voie du réticulum endoplasmique. Les signaux vont être intégrés par la cellule et de nombreuses voies de signalisation vont être ainsi activées aboutissant à l'activation des caspases

1.2.2.2.1. La voie des récepteurs

De nombreux stimuli tels que la radiation et des agents chimiothérapeutiques sont capables d'induire l'apoptose. Toutefois il existe une famille de récepteurs spécialisés dans l'induction de la mort cellulaire programmée : les récepteurs de mort. Une fois stimulés par leurs ligands, ces récepteurs induisent l'activation des caspases (Longthorne et Williams, 1997). Cette voie d'activation est impliquée dans l'élimination des cellules potentiellement dangereuses pour l'organisme et notamment les lymphocytes autoréactifs. Les récepteurs de mort appartiennent à la famille du récepteur du facteur de nécrose tumorale (TNF-R) (Nagata, 1997). Les TNF-R peuvent promouvoir, selon le contexte cellulaire, soit la survie, soit la mort. Par exemple, les récepteurs CD27 (Camerini *et al.*, 1991) et CD30 (Durkop *et al.*, 1992) sont impliqués dans la survie cellulaire. Parmi les membres de la famille impliquée dans la mort cellulaire, il convient de citer Fas (Oehm *et al.*, 1992). L'interaction du récepteur Fas à son ligand (FasR/FasL), au même titre que celle du

granzymeB/perforine joue un rôle prépondérant dans la cytotoxicité des lymphocytes T (LTc) du système immunitaire. Ces deux mécanismes coopèrent pour permettre l'élimination de la cellule cible par un processus d'apoptose (Cohen, 1991; Golstein *et al.*, 1991). Au cours de la réponse immunitaire, le récepteur Fas va également être impliqué dans l'élimination des cellules infectées par des virus ou des parasites, ou des cellules tumorales. Bien que l'expression et le rôle de la protéine Fas dans le système nerveux ne soient pas clairement définis, elle a été impliquée au cours de pathologies neurodégénératives telles que l'ischémie ou la maladie d'Alzheimer.

Ce paragraphe décrira surtout le mécanisme d'activation du récepteur Fas. Une fois stimulé par son ligand spécifique (FasL), le récepteur Fas se trimérise et recrute une protéine adaptatrice, la protéine associée au Fas avec un domaine de mort (FADD) (Chinnaiyan *et al.*, 1995) (Figure 1.8). Le FADD présente la particularité de posséder, en plus de son domaine de mort cellulaire (DD), un domaine effecteur de mort cellulaire (DED). Le DED est nécessaire et suffisant pour induire l'apoptose. Dans certaines conditions, le TNF-R1 recrute également FADD. Ce couplage est cependant indirect et se fait par l'intermédiaire d'une protéine comportant un DD, la protéine associée au TNF-R1 avec un domaine de mort (TRADD) (Hsu *et al.*, 1995) (Figure 1.8). En définitive le FADD représente le point de convergence des voies de signalisation induites par Fas ou TNF-R1. Par la suite, le FADD peut recruter la procaspase-8 (Boldin *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996) ou la procaspase-10 (Vincenz et Dixit, 1997) par l'intermédiaire de ces DEDs et ainsi initier la cascade apoptotique (Figure 1.9) (Hirata *et al.*, 1998). La procaspase-8 sera clivée en caspase-8, qui activera ensuite les caspases effectrices -3, -6 et -7. Ces caspases effectrices cliveront leurs substrats correspondants dont l'inhibiteur de l'ADNase caspase-dépendant (ICAD) et entraîneront la mort par apoptose (Los *et al.*, 1999). Cette signalisation est vraie aussi pour la procaspase-10 (Los *et al.*, 1999).

Le TRADD peut aussi interagir avec le DD de "receptor interacting protein" (RIP) (Hsu *et al.*, 1996a) (Figure 1.8), une serine/thréonine kinase qui peut induire,

selon les circonstances, soit l'activation de NF-κB soit l'apoptose (Hsu *et al.*, 1996a). Le RIP interagit avec la protéine associée au RIP par un DD (RAIDD) (Duan et Dixit, 1997) qui lui-même lie directement la procaspase-2 mais pas avec les caspases 1, 3, 4, 6, 7 ou 9 (Duan et Dixit, 1997) (Figure 1.8). La caspase-2 activera ensuite la caspase-3 effectrice et conduira à l'apoptose (Tyagi *et al.*, 2006).

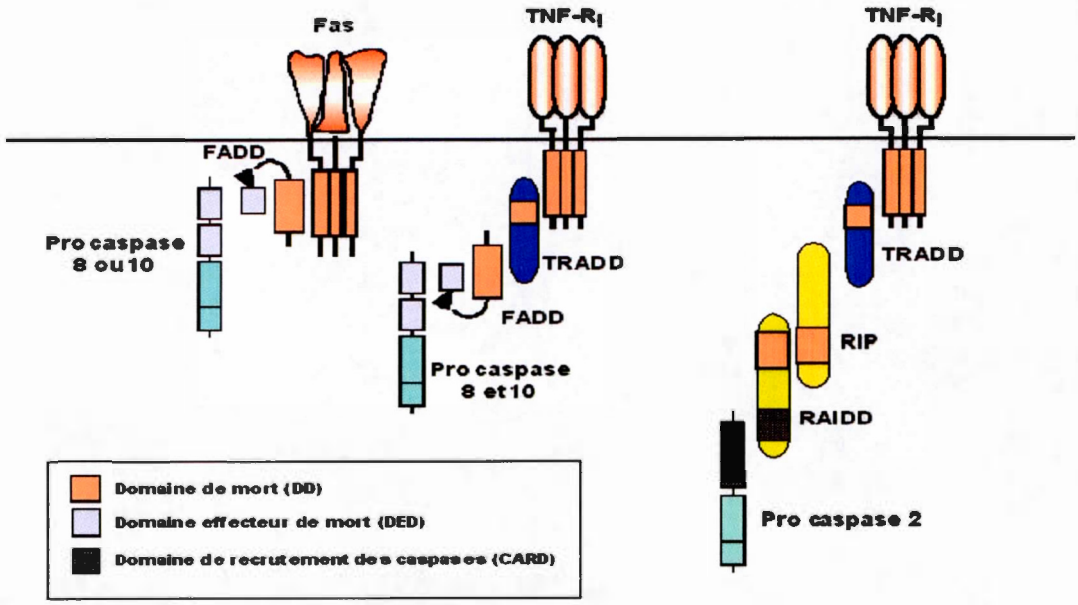


Figure 1.8: Activation des caspases par les récepteurs de mort. Les récepteurs de mort appartiennent à la famille du récepteur du facteur de nécrose tumorale (TNF-R) et ils comprennent plusieurs récepteurs dont le FasR et le TNF-R1 (Rathmell *et al.*, 1999).

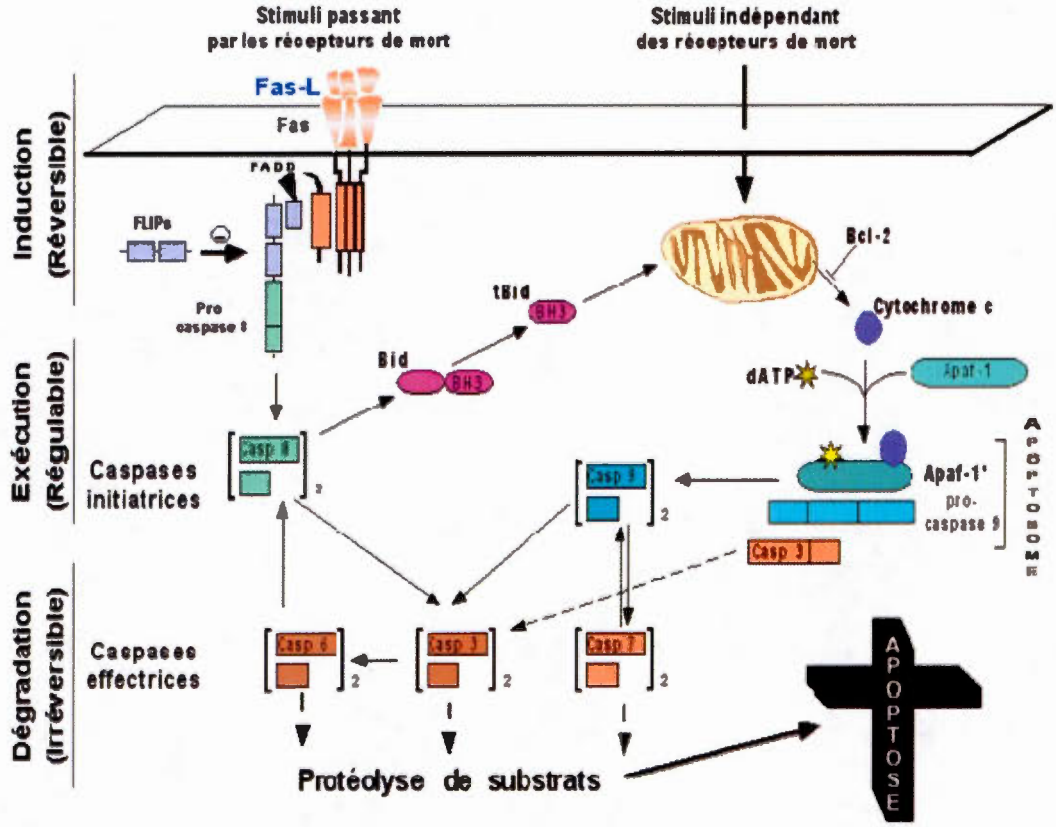


Figure 1.9 : Les deux principales voies conduisant à l'apoptose : la voie des récepteurs de mort et la voie mitochondriale. L'activation des caspases initiatrices -8 et -9 entraîne l'activation des caspases effectrices -3, -6 et -7 qui cliveront leurs substrats correspondants pour emmener la mort par apoptose. Notons que la caspase-8 peut activer la voie mitochondriale par l'intermédiaire de la protéine proapoptotique Bid. Adapté de Los *et al.*, 1999

1.2.2.2.2. La voie mitochondriale

1.2.2.2.2.1 Mitochondrie, potentiel membranaire et libération du cytochrome-c

La mitochondrie joue un rôle clé dans la régulation de l'apoptose (Brenner *et al.*, 1998; Kroemer, 1997). Cette voie est activée par de multiples stimuli notamment le choc thermique, la radiation, l'hypoxie, la privation en sérum et en facteurs de croissance, et par des agents chimiothérapeutiques comme l'étoposide. Ces facteurs causent un débalancement entre les facteurs anti-apoptotiques et les facteurs pro-apoptiques en faveur de ces derniers (Wilson, 1999). En effet, la phase effectrice de l'apoptose comporte la diminution du potentiel membranaire ($\Delta\Psi m$) qui permet l'ouverture des pores de transition de perméabilité (PTP) spécialisés (Vander Heiden *et al.*, 1997) et la libération de molécules apoptogènes de l'espace intermembranaire vers le cytoplasme (Figure 1.9, 1.10, 1.11) (Kohler *et al.*, 2002; Kluck *et al.*, 1997; Yang *et al.*, 1997). Ces molécules sont le cytochrome-c (Cyt-c), le facteur inducteur d'apoptose (AIF) (Figure 1.10), l'activateur mitochondrial des caspases (Smac) (Figure 1.11) et les protéines de choc thermique, HSP10 et HSP60. Dans un complexe appelé "apoptosome", le cyt-c se lie au facteur activateur de protéase d'apoptose (Apaf) afin d'activer la procaspase-9. Deux molécules de procaspase-9 clivées se lient ensemble pour former la caspase-9 active (Figure 1.9). La caspase-9 activera ensuite les caspases effectrices -3, -6 et -7. Ces caspases effectrices cliveront leurs substrats correspondants dont l'ICAD et entraîneront la mort par apoptose (Los *et al.*, 1999). Cette phase de libération des facteurs pro-apoptotiques de la mitochondrie est sous le contrôle des membres de la famille Bcl-2 (Figure 1.10). Ainsi, Bcl-2 et Bcl-X_L sont capables de bloquer la sortie du cyt-c dans le cytoplasme (Kluck *et al.*, 1997; Vander Heiden *et al.*, 1997), alors que les protéines pro-apoptotiques telles que Bax et Bak peuvent l'induire (Jurgensmeier *et al.*, 1998).

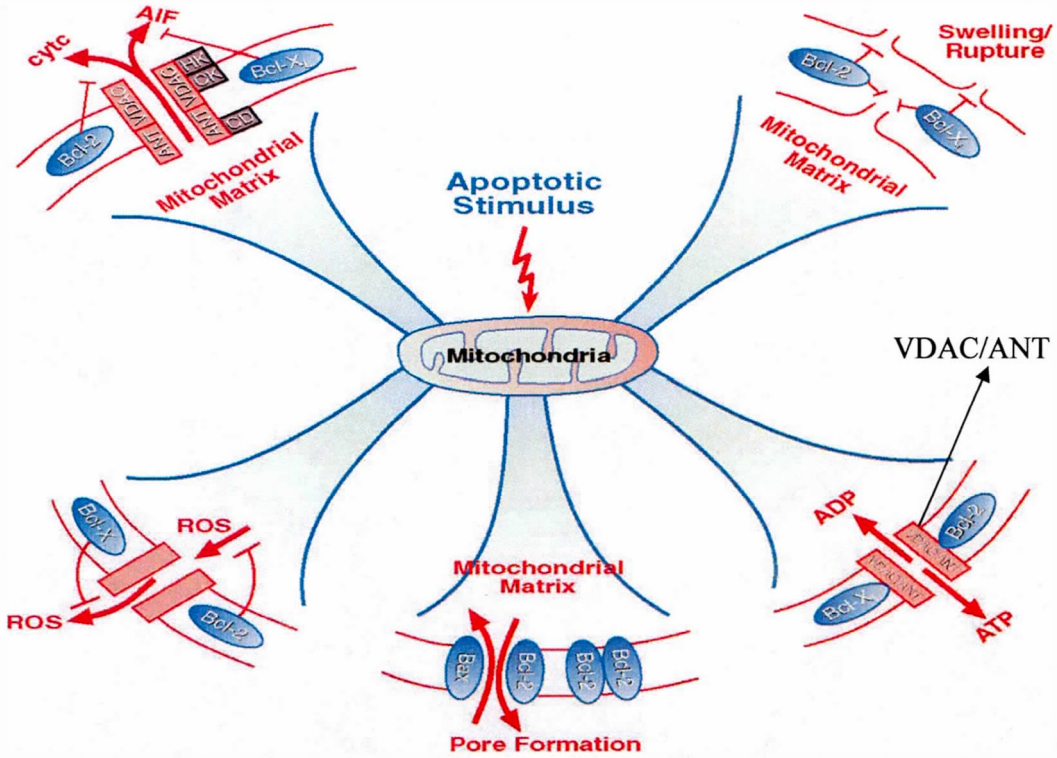
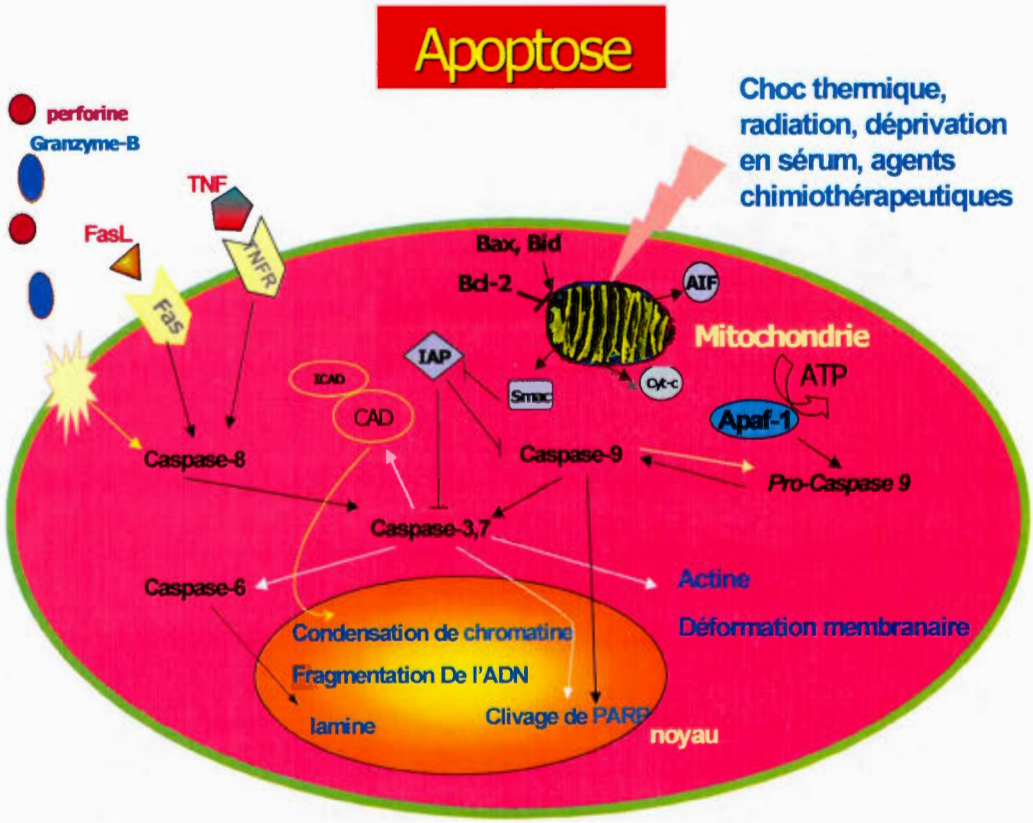


Figure 1.10: La régulation de l'apoptose au niveau de la mitochondrie. Cette organite joue un rôle important durant l'apoptose. Les membres anti-apoptotiques de la famille Bcl2, tels que Bcl-2 et Bcl-X_L, inhibent la mort cellulaire au niveau mitochondrial en maintenant la fonction physiologique de l'organite normale et en prévenant la libération du cytochrome-c et du facteur inducteur d'apoptose (AIF). Mais, les protéines pro-apoptotiques telles que Bax stimulent l'apoptose. Un des canaux libérant le cyt-c et l'AIF est formé de plusieurs protéines dont le canal ionique voltage-dépendant (VDAC), le translocateur d'adénine (ANT), la cyclophiline D (CD), la créatinine kinase (CK) et l'hexokinase (HK) (Bortner et Cidlowski, 2002).



AIF (Facteur inducteur d'apoptose)
APAF1 (Facteur activateur de protéase d'apoptose)
SMAC (Activateur mitochondriale des caspases)
ICAD (Inhibiteur de l'ADNase caspase-dependant)
PARP (poly ADP-ribose polymérase)
IAP (Inhibiteur de protéine apoptotique)

Figure 1.11: Induction de l'apoptose par la voie des récepteurs de mort et la voie mitochondriale. L'activation des caspases initiatrices -8 et -9 entraîne l'activation des caspases effectrices -3, -6 et -7 qui cliveront leurs substrats correspondants pour emmener la mort par apoptose. Adapté de Los *et al.*, 1999; Salvesen et Duckett, 2002; Stennicke *et al.*, 2002; Pharmingen; Sigma.

Il est admis que la libération du cytochrome c ainsi que la perte du $\Delta\Psi m$ induite par Bax/Bak peuvent être régulées par le PTP. Le pore PTP est un canal oligo-protéique constitué au niveau de la membrane externe de la mitochondrie par la porine VDAC, sur la membrane interne par l'ANT et d'une protéine matricielle la cyclophiline D (CD) (Figure 1.10) (Kroemer *et al.*, 1998). De plus, il a été montré que Bax pouvait interagir avec VDAC (Narita *et al.*, 1998; Shimizu *et al.*, 1999) et ANT (Marzo *et al.*, 1998; Shimizu *et al.*, 1999). Il semble que Bax/Bak puissent induire soit un changement de conformation du canal VDAC afin de former un pore permettant le passage des différentes molécules apoptogènes, soit interagir directement avec VDAC et participer ainsi à agrandir le pore.

Il semble que la mitochondrie joue le rôle d'intégrateur des différents signaux et qu'une fois le seuil atteint, la totalité du cyt-c est libérée en une seule étape. Toutefois toutes les mitochondries d'une même cellule ne vont pas être parfaitement synchronisées. Tout récemment, l'invalidation du gène codant pour le cytochrome c a confirmé l'importance cruciale de cette protéine dans l'apoptose (Li *et al.*, 2000). Les cellules Cyt $c^{-/-}$ provenant d'un embryon de souris ne peuvent pas induire l'activation de la caspase-3 en réponse à différents stimuli pro-apoptotiques. Enfin, les cellules Cyt $c^{-/-}$ sont résistantes à l'apoptose induite par les U.V. ou l'étoposide et très peu sensibles aux effets pro-apoptotiques de la privation en facteur de croissance ou de la staurosporine (Li *et al.*, 2000). De plus, Apaf-1 reste sous forme monomérique dans des conditions où l'apoptosome devrait se former. Ceci implique qu'aucune autre protéine cellulaire ne puisse remplacer le Cyt c pour l'oligomérisation d'Apaf-1 et pour l'activation de la caspase-3 induite par un stress cellulaire ou par un agent ciblant la mitochondrie.

Le facteur AIF est aussi une des molécules apoptogènes libérées de la mitochondrie. L'AIF transloque au noyau, condense la chromatine et fragmente l'ADN, indépendamment des cascades impliquant les caspases. (Susin *et al.*, 1999).

Durant l'apoptose, la protéine SMAC qui est un inhibiteur des IAPs est libérée de la mitochondrie, afin de potentialiser l'apoptose. Enfin, la relâche de Smac est inhibée à l'aide du Bcl-2/Bcl-X_L (MacFarlane *et al.*, 2002).

L'activation des caspases induite par le cytochrome c cytosolique associé à Apaf-1, ou l'apoptose induite par les récepteurs de mort, ne sont pas des mécanismes totalement indépendants (Figure 1.9). En effet, un nouveau membre pro-apoptotique de la famille Bcl2, la protéine Bid (Wang *et al.*, 1996), permet de faire le lien entre les récepteurs de mort et la libération du cytochrome c de la mitochondrie. Le Bid est directement clivé par la caspase 8 et le fragment C-terminal produit transloque à la mitochondrie et permet la libération du cytochrome c (Figure 1.9) (Li *et al.*, 1998; Luo *et al.*, 1998).

1.2.2.2.2.2 La famille de Bcl-2

Les protéines de la famille Bcl-2 jouent un rôle majeur dans la régulation des caspases et plus généralement de l'apoptose. Si l'on se réfère à leurs fonctions biologiques, on peut classer les membres de la famille Bcl-2 en deux sous-familles, les membres anti-apoptotiques tels que Bcl-2, mais aussi Bcl-X_L (Boise et Thompson, 1997), Bcl-w (Gibson *et al.*, 1996), BAR (Zhang *et al.*, 2000) et les membres pro-apoptotiques comme Bax (Oltvai *et al.*, 1993), Bak (Chittenden *et al.*, 1995;), Bcl XS (Boise et Thompson, 1997), Bad, Bid (Yang *et al.*, 1996) et Bim (O'Connor *et al.*, 1998). Cette famille se caractérise par la présence de domaines homologues à Bcl-2 1à 4 (BH 1 à 4) (Kelekar et Thompson, 1998). La distribution de ces domaines permet de regrouper cette famille en trois classes, la classe contenant BH1 à BH4, la classe contenant BH1 à BH3 et la classe contenant seulement BH3 (Figure 1.12).

Il semblerait que la balance entre la vie et la mort cellulaire soit influencée par le type et la proportion de dimères anti- ou pro-apoptotiques formés (Oltvai et Korsmeyer, 1994; Sedlak *et al.*, 1995). Ainsi par exemple, lorsque Bax est préférentiellement exprimé, des homodimères Bax-Bax se formeront et conduiront à

la mort cellulaire. En revanche, si c'est Bcl-2 qui est majoritairement exprimée, alors il y aura survie cellulaire. Cependant un article récent semble indiquer que, pour des raisons structurales, des homodimères Bcl-2 ne pourraient pas se former et que Bcl-2 jouerait son rôle anti-apoptotique sous forme monomérique (Conus *et al.*, 2000).

Les membres ne comportant qu'un domaine BH3, comme Bid, ne peuvent pas former des homodimères et ne possèdent pas d'activité intrinsèque. En fait, ils exerceraient leur rôle pro-apoptotique en formant des dimères avec des membres anti-apoptotiques réduisant ainsi la capacité de ceux-ci à exercer leurs effets protecteurs et dans le même temps favoriseraient la constitution de dimères pro-apoptotiques (Kelekar *et al.*, 1997; Sattler *et al.*, 1997).

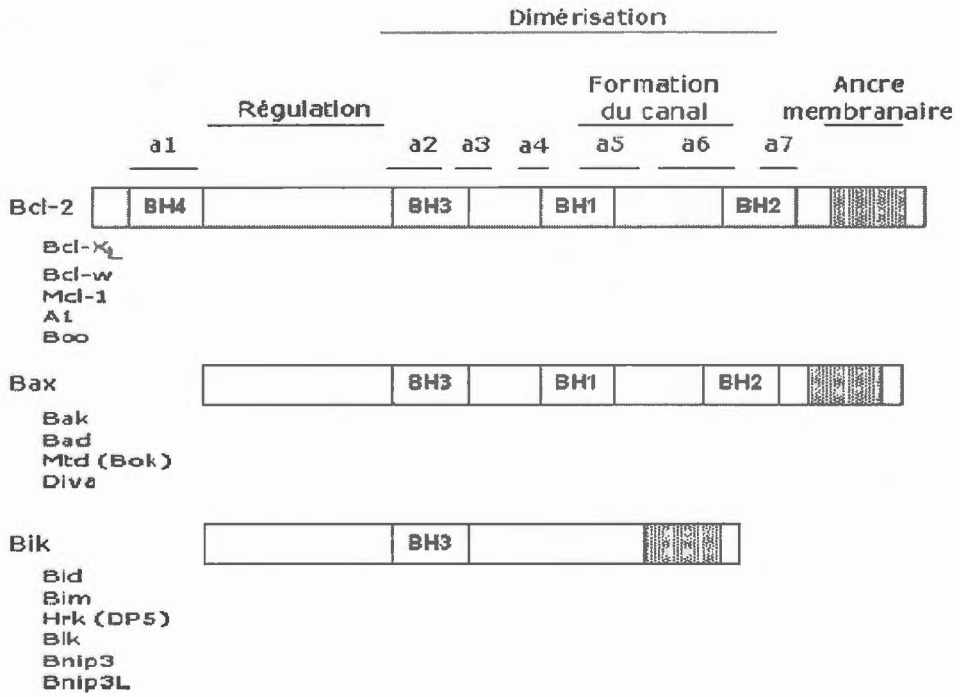


Figure 1.12: Représentation schématique des membres de la famille Bcl-2. Les domaines BH1 à BH4 sont des motifs très conservés. a1-a7 représentent les hélices alpha identifiées dans Bcl-X_L. a5 et a6 forment une structure hydrophobe entourée par 5 hélices amphipatiques. Le domaine entre a1 et a2 contient une boucle de régulation. Il est à noter que tous les membres de la famille Bcl-2 ne possèdent pas le domaine d'ancrage membranaire. Adapté de Tsujimoto *et al.*, 2000.

1.2.2.2.3. La voie du réticulum endoplasmique

La voie du réticulum endoplasmique est la troisième voie d'induction d'apoptose nouvellement identifiée. Cette voie est induite suite à l'accumulation excessive de protéines nouvellement synthétisées et qui sont mal repliées (Kadowaki *et al.*, 2004). Cette voie active principalement la caspase-12 initiatrice qui activera à son tour la caspase-9 (Kadowaki *et al.*, 2004). La caspase-7 et la calpaine qui est activée par le calcium sont aussi impliquées dans l'activation de la caspase-12 et l'exécution de l'apoptose par cette voie (Szegezdi *et al.*, 2003) (Figure 1.13).

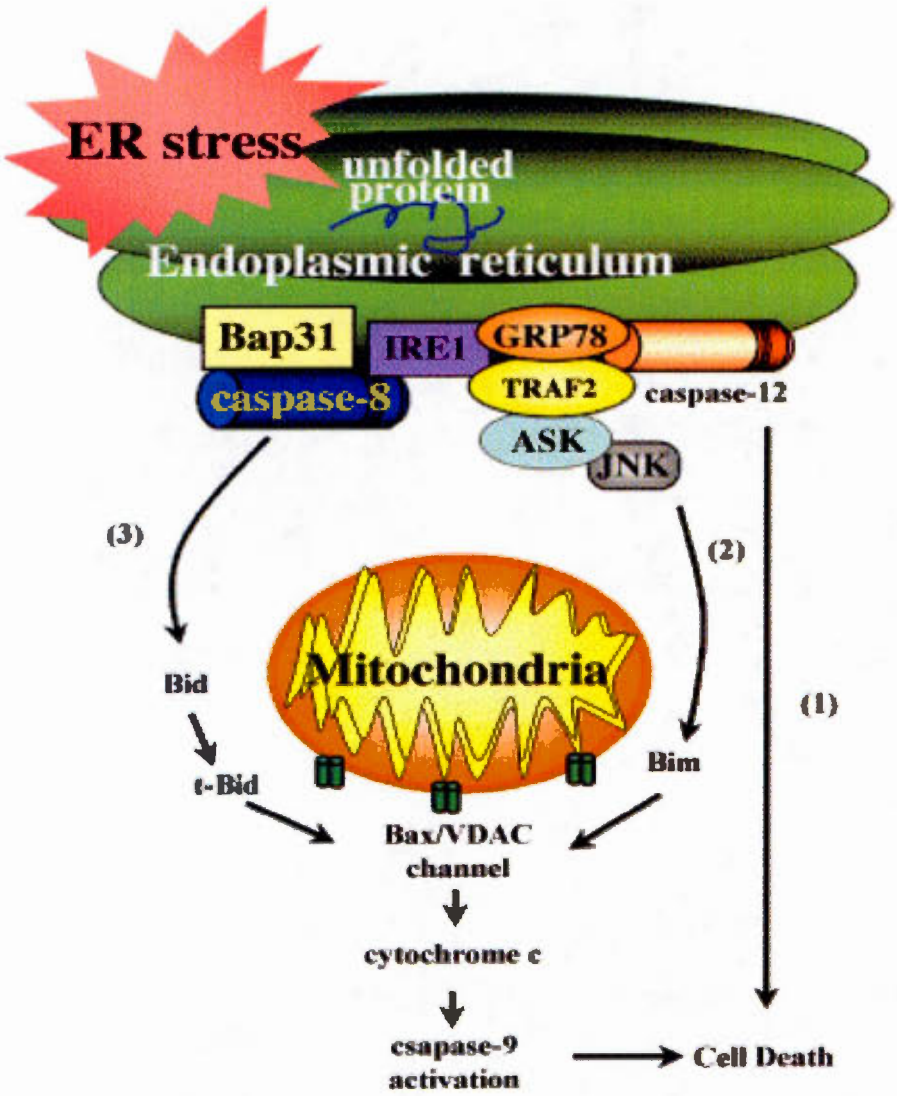


Figure 1.13: Signalisation apoptotique de la voie du réticulum endoplasmique (RE) en réponse à un stress. Trois voies majeurs d'induction de l'apoptose par la voie du RE ont été rapportées : (1) la voie caspase-12-dépendante; (2) la voie de la kinase régulatrice du signal apoptotique et de la kinase c-jun (ASK/JNK), qui induisent la libération du cyt-c de la mitochondrie et l'activation de la caspase-9; (3) la voie Bap31/caspase-8 qui induit aussi la libération du cyt-c de la mitochondrie et l'activation de la caspase-9 (Momoi, 2004).

1.2.2.3. Les caspases

1.2.2.3.1. Nomenclature

Une nouvelle nomenclature proposée par Alnemri *et al.* (1996) regroupe désormais les protéases apoptogènes sous le nom de CASPASE. Le *C* représente la cystéine du site actif (QACxG) et ASPASE définit la spécificité stricte de clivage des substrats de cette famille de protéases après un acide aspartique (Figure 1.14a). À ce jour 14 caspases ont été identifiées, mais il ne fait aucun doute que cette liste n'est pas exhaustive. Notons qu'on a classé les caspases sous trois groupes : il y a les caspases initiatrices (2, 8, 9, 10, 12), les caspases effectrices (3, 6, 7) et les caspases impliqués dans l'inflammation (1, 4, 5, 11, 13) (Figure 1.14b). Enfin on a la caspase-14 qui est impliquée dans la différenciation des kératinocytes (Kohler *et al.*, 2002).

1.2.2.3.2. Structure des caspases

Toutes les caspases ont une structure très conservée comprenant un prodomaine N-terminal de taille variable, un domaine qui deviendra après clivage la grande sous-unité (17-21 kDa) qui porte le site actif et un domaine qui deviendra après clivage la petite sous-unité (10-14 kDa) (Figure 1.14a). Certains membres de la famille des caspases possèdent un domaine de liaison entre la grande et la petite sous-unité. Les prodomaines sont variables, à la fois dans leur taille et dans leur séquence. Ainsi les caspases -3, -6 et -7 ont un petit prodomaine alors que les caspases -1, -2, -4, -5, -8, -9, -10, -11, -12 et -13 possèdent un grand prodomaine. Les caspases à petits prodomaines sont souvent regroupées sous le nom de caspases effectrices. Ces caspases sont activées par des caspases dites initiatrices. Les prodomaines semblent jouer un rôle dans les interactions protéines-protéines. Ainsi les prodomaines des caspases -8 et -10 contiennent des DEDs qui sont des structures permettant la liaison de la caspase aux molécules adaptatrices FADD (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995) ou TRADD (Hsu *et al.*, 1995). Certaines autres caspases (caspases -1, -2, -4 et -9) possèdent un domaine de recrutement des caspases (ou "caspase recruitment

domain": CARD) (Hofmann *et al.*, 1997). Ces domaines CARDs jouent un rôle dans l'interaction entre caspases ainsi qu'avec une grande variété de molécules adaptatrices ou régulatrices.

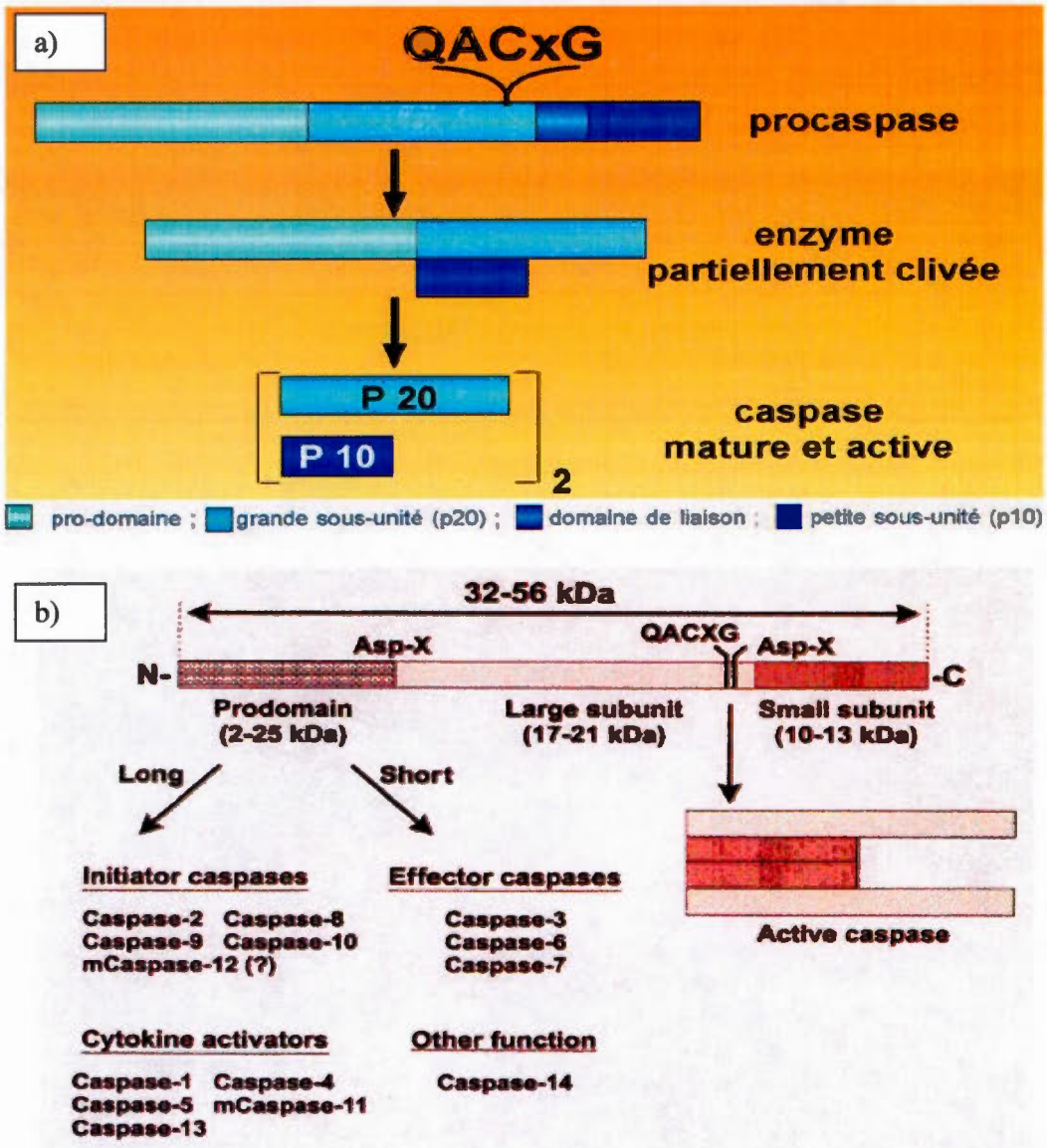


Figure 1.14: Structure et activation des caspases. Les caspases contiennent quatre domaines principaux qui sont clivés en deux étapes afin de donner une caspase active sous forme de dimères. a) Adapté de Rathmell *et al.*, 1999 et b) Adapté de Kohler *et al.*, 2002.

1.2.2.3.3. Activation des caspases

La conversion de la caspase à l'état de zymogène en une enzyme mature nécessite au moins deux clivages au niveau de lien Asp-X. Ces clivages successifs ont lieu de manière séquentielle : tout d'abord coupure entre la grande et la petite sous-unité (donc il y a libération de la petite sous-unité du reste de la molécule) suivie par la libération du prodomaine (Figure 1.14a). Les caspases vont pouvoir s'auto-activer et/ou être activées par d'autres caspases. Ce dernier concept introduit la notion de cascade d'activation. Ainsi une fois les caspases initiatrices activées, elles vont pouvoir cliver d'autres caspases encore à l'état de zymogène, notamment les caspases effectrices -3, -6 et -7. Ce type d'activation en cascade permet probablement la régulation et l'amplification du signal apoptotique.

1.2.2.3.4. Les substrats des caspases

Le rôle des caspases est principalement exécutif. Elles vont activer des molécules qui participeront à la destruction cellulaire. Les caspases sont des enzymes extrêmement sélectives. Les substrats clivés par les caspases comprennent des protéines du cytosquelette (α-fodrine, actine, gelsoline) (Villa *et al.*, 1998), nucléaires (lamines), ainsi que des protéines impliquées dans la réparation de l'ADN comme la poly ADP-ribose polymérase (PARP) (Salvesen et Dixit, 1997; Germain *et al.*, 1999) et la suppression tumorale comme la rétinoblastome (Rb) (Chang et Yang, 2000). De plus, des protéines impliquées dans la transduction du signal, dans l'expression de gènes, dans la régulation du cycle cellulaire (p27Kip1), dans les maladies génétiques ou des protéines de régulation de l'apoptose (ICAD) sont aussi des substrats des caspases. Notons que le complexe ICAD/CAD, une fois clivé, libère la protéase CAD qui entre dans le noyau pour condenser la chromatine et fragmenter l'ADN (Chang et Yang, 2000).

1.2.2.3.5. Régulation des caspases

Etant donné les effets dévastateurs que pourrait avoir une activation inappropriée des caspases, il n'est pas surprenant que cette étape soit étroitement modulée. En fait, non seulement l'activation, mais aussi l'activité et la production des caspases, sont régulées à plusieurs niveaux. La caspase-3, par exemple, est fortement exprimée dans de nombreuses cellules lymphoïdes et myéloïdes matures alors qu'elle n'est que faiblement présente dans l'épithélium mammaire et dans les neurones normaux (Krajewska *et al.*, 1997).

La p35, qui est une des protéines de baculovirus, possède un large spectre d'inhibition, en inhibant à la fois les caspases initiatrices et effectrices (Figure 1.15) (Bortner et Cidlowski, 2002).

La cytokine modifiant la réponse (CrmA) est issue d'un gène précoce du virus cowpox. Sa surexpression permet d'inhiber l'apoptose induite par la privation en facteurs de croissance et par le CD95 (ou récepteur Fas) ou le TNF (Figure 1.15) (Tewari et Dixit, 1995; Gagliardini *et al.*, 1994). La CrmA inhibe l'activité protéolytique des caspases -2 et -8 (Bortner et Cidlowski, 2002). Ces deux protéines virales (CrmA et p35) sont des inhibiteurs compétitifs. Une fois clivées par une caspase, elles vont se lier à ces enzymes et ainsi empêcher la dégradation de nouveaux substrats (Komiyama *et al.*, 1994; Bump *et al.*, 1995).

La stimulation des récepteurs de mort va conduire au recrutement de molécules adaptatrices puis au clivage de la forme zymogène de caspases initiatrices (caspases -8 et -10) et à l'activation des caspases effectrices. Cette étape de recrutement peut être régulée par une protéine inhibitrice de la caspase-8 (FLIP) (Rasper *et al.*, 1998; Irmler *et al.*, 1997).

Les baculovirus possèdent une autre protéine capable d'inhiber l'apoptose: l'inhibiteur de protéine apoptotique (IAP). Au moins 5 homologues ont été identifiés chez les mammifères. Il s'agit de NAIP, de XIAP et de la survivine (Liston *et al.*, 1996), de cIAP-1, et de cIAP-2 (Rothe *et al.*, 1995). L'effet protecteur des IAPs est dû

à leur capacité d'inhiber l'activation et donc l'activité de certaines caspases. Ainsi, XIAP, cIAP-1 et cIAP-2 inhibent les caspases 3, 7 et 9 mais pas les caspases 1, 6 et 8 (Deveraux *et al.*, 1997-1999; Roy *et al.*, 1997; Sun *et al.*, 2000; Takahashi *et al.*, 1998). La NAIP, pour sa part, est incapable d'inhiber les caspases 1, 3, 6, 7 ou 8 (Roy *et al.*, 1997). Les IAPs, à l'exception de la survivine, sont caractérisées par 3 domaines de répétition de IAP de la baculovirus (BIR) dans leur partie N-terminale et un domaine d'interaction protéine-protéine contenant un atome de zinc dans leur partie C-terminale. La survivine ne contient que les domaines BIRs et la plupart des carcinomes humains la surexpriment ce qui a comme conséquence la survie et la croissance des cellules tumorales (Choi *et al.*, 2003). De plus, il semble que cIAP-1 et cIAP-2 puissent se lier au facteur associé au TNF-R 1 et 2 (TRAF-1 et 2) grâce à leur motif BIR (Rothe *et al.*, 1995; Roy *et al.*, 1997). Ceci implique que les cIAPs, ainsi que les vIAPs (Vucic *et al.*, 1998), puissent exercer des effets inhibiteurs sur l'activation des caspases en aval des récepteurs de mort (Wang C.Y. *et al.*, 1998). Par ailleurs cIAP-1, cIAP-2 et XIAP ont été décrites comme des inducteurs de l'activation de NF-κB (Chu *et al.*, 1997). Ceci pourrait notamment contribuer à l'effet protecteur de NF-κB sur l'apoptose induite par le TNF-α (You *et al.*, 1997; Stehlik *et al.*, 1998; Wang C.Y. *et al.*, 1998).

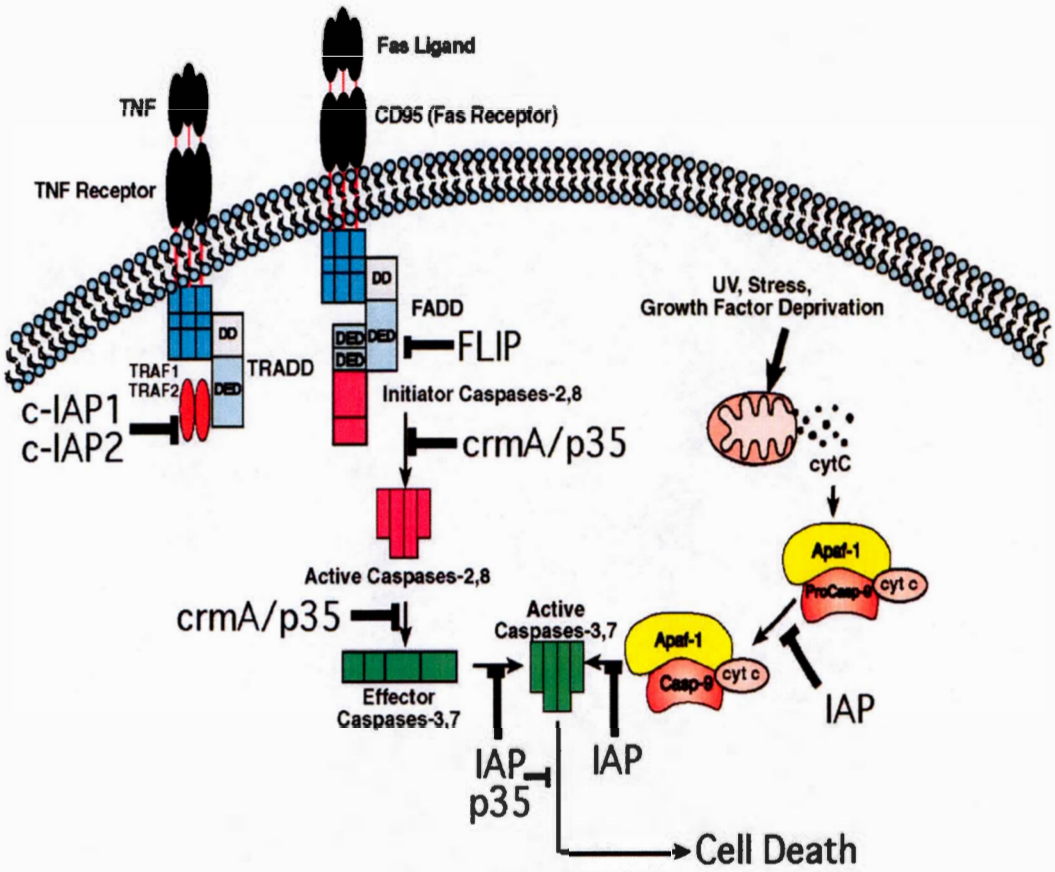


Figure 1.15: Inhibition de l'apoptose au niveau des caspases. Les IAP inhibent la caspase-9 (initiatrice) et les caspases -3 et -7 (effectrices). De plus, les cIAP-1 et cIAP-2 se lient au facteur associé au TNF-R 1 et 2 (TRAF-1 et 2) grâce à leur motif BIR (Rothe *et al.*, 1995; Roy *et al.*, 1997). Ceci implique que les cIAPs puissent exercer des effets inhibiteurs sur l'activation des caspases en aval des récepteurs de mort (Wang C.Y. *et al.*, 1998). CrmA est un inhibiteur des caspases initiatrices (caspase- 2 et -8), avec toutefois une capacité limitée d'inhiber les caspases effectrices. Un inhibiteur spécifique de la caspase-8 a été identifié et nommé "FLICE-inhibitory proteins" (FLIP) qui inhibe l'apoptose en se complexant avec le complexe inducteur de signal de mort (DISC) qui regroupe le récepteur de mort, le FADD et la procaspase-8 (aussi nommé FLICE). Enfin, le p35 isolé du baculovirus possède un large spectre d'inhibition, en inhibant à la fois les caspases initiatrices et effectrices (Bortner et Cidlowski, 2002).

1.3. LES VOIES DE SIGNALISATION DES MAPK, ASK1, AKT ET P53

1.3.1. Les voies de signalisation d'apoptose médiées par MAPK et ASK1

Il existe deux voies distinctes de signalisation de l'apoptose à partir du domaine intracellulaire du récepteur Fas : la première voie est celle qu'on a développée à la section de la voie des récepteurs (section 2-2-2.1) et qui implique la cascade des protéines adaptatrices qui interagissent via des domaines d'homologie, et qui finalement communiquent le message aux caspases (Figures 1.8 et 1.9); la deuxième implique, quant à elle, une cascade de protéines kinases. La cascade des protéines kinases activées par les mitogènes (MAPK) est multifonctionnelle et a été conservée durant l'évolution (Takeda *et al.*, 2003). Il y a trois différentes cascades qui convergent vers la kinase régulée par des stimuli extracellulaires (ERK), la kinase du terminus-N de c-jun (JNK) ou la p38 MAP kinase (Figure 1.16). Ces MAPK sont régulées par différents stimuli (Pearson *et al.*, 2001; Kyriakis et Avruch, 2001).

Souvent, la voie ERK est connue comme étant anti-apoptotique et de survie et elle est activée par des facteurs de croissance (Lee *et al.*, 2006; Park *et al.*, 2003) tandis que les voies de JNK et de la p38 MAP kinases sont connues comme étant pro-apoptotiques (Sarker *et al.*, 2003; Assefa *et al.*, 2000). Les JNK et p38 sont activées par le facteur de nécrose tumorale (TNF-α), le peroxyded'hydrogène, les rayons UV, les rayons X, le choc thermique et la privation de sérum et de facteurs de croissance qui entraînent l'activation de la protéine kinase régulatrice du signal apoptotique (ASK1) (Figure 1.16) (Takeda *et al.*, 2003; Raingeaud *et al.*, 1995; Xia *et al.*, 1995; Kyriakis et Avruch, 1996; Verheij *et al.*, 1996). Les JNK et p38 MAPK induisent la translocation de Bax vers la mitochondrie et la libération du cytochrome-c (Tsuruta *et al*,. 2004; Park *et al.*, 2003) activant de ce fait la voie mitochondriale, tandis que ERK inhibe l'activité de la caspase-8 (Park *et al.*, 2003), inhibant ainsi l'apoptose. En plus, la p38 peut phosphoryler le facteur pro-apoptotique p53 aux serines -15, -37 et -46, et

ainsi induire l'apoptose (Bulavin *et al.*, 1999; Kim *et al.*, 2002; Sanchez-Prieto *et al.*, 2000).

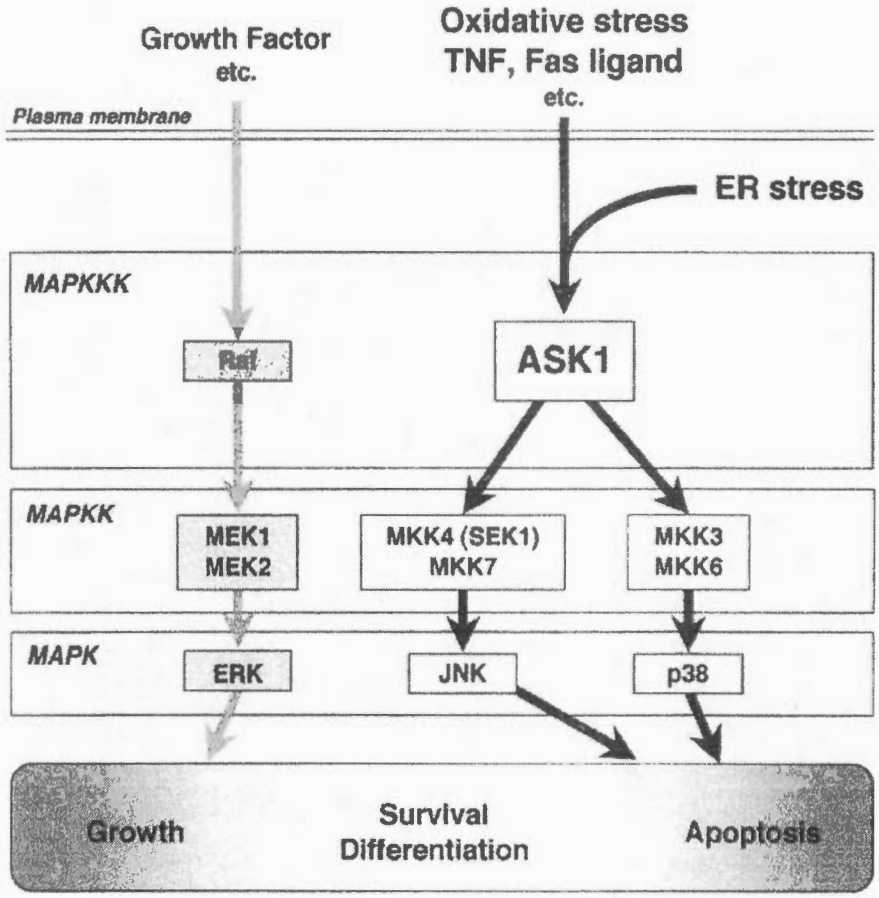


Figure 1.16: Signalisation des différentes MAPKs, ERK, JNK et p38. Suite à une stimulation par un facteur de croissance, la MAPK kinase kinase (MAPKKk) Raf active la MAPKK, MEK 1 et 2, qui active ERK pour induire la survie cellulaire. Cependant, une stimulation par un ligand Fas, un ligand TNF, un stress oxydatif ou un stress du RE activera la MAPKKK ASK1 qui activera à son tour des MAPKK qui activeront la JNK et la p38. Cette cascade emmène l'apoptose de la cellule (Takeda *et al.*, 2003).

Notons que la phosphorylation à la sérine 46 est spécifique à la p38 MAPK (Bulavin *et al.*, 1999). Le facteur de croissance épidermale (EGF) pourrait protéger les cellules de l'apoptose en activant la voie des ERK1/2 et en s'opposant à la voie de la p38 MAPK (Kanasaki *et al.*, 2000).

Des effets contradictoires ont été rapportés pour ces différentes MAPK. La p38 MAPK peut intervenir dans les mécanismes de survie cellulaire. En effet, l'hormone stimulante des follicules (FSH) induit la survie et l'arrondissement des cellules de granulosa de rat (Maizels *et al.*, 1998). Dans ce cas, la FSH induit la phosphorylation de la p38 MAPK, via la protéine kinase A (PKA). La phosphorylation de la p38 MAPK induit la phosphorylation de la protéine HSP-27 qui induit la réorganisation des filaments d'actine et leur stabilité (Huot *et al.*, 1997). Une récente étude a aussi démontré que la protéine ERK peut être impliquée dans la mort cellulaire des kératinocytes humains malins exposées aux chélateurs de fer (Lee *et al.*, 2006). La voie JNK peut aussi être impliquée dans la survie cellulaire des macrophages (Himes *et al.*, 2006) contrairement à son caractère apoptogène discuté plus haut.

Une importante MAP kinase kinase kinase (MAPKKK) du stress apoptotique, la kinase régulatrice du signal apoptotique (ASK1) a été identifiée. Elle active les deux voies de signalisation JNK et p38 (Ichijo *et al.*, 1997) (Figures 1.16, 1.17). La surexpression de ASK1 dans des cellules épithéliales incubées avec très peu de sérum induisait la mort cellulaire par apoptose. Tandis que la surexpression du mutant ASK1, ASK1-K709R, réduisait significativement l'apoptose induite par le récepteur TNF-α. Ceci suggère que ASK1 joue un rôle majeur dans l'induction de l'apoptose par les cytokines ou par différents stress (Ichijo *et al.*, 1997; Tobiume *et al.*, 1997).

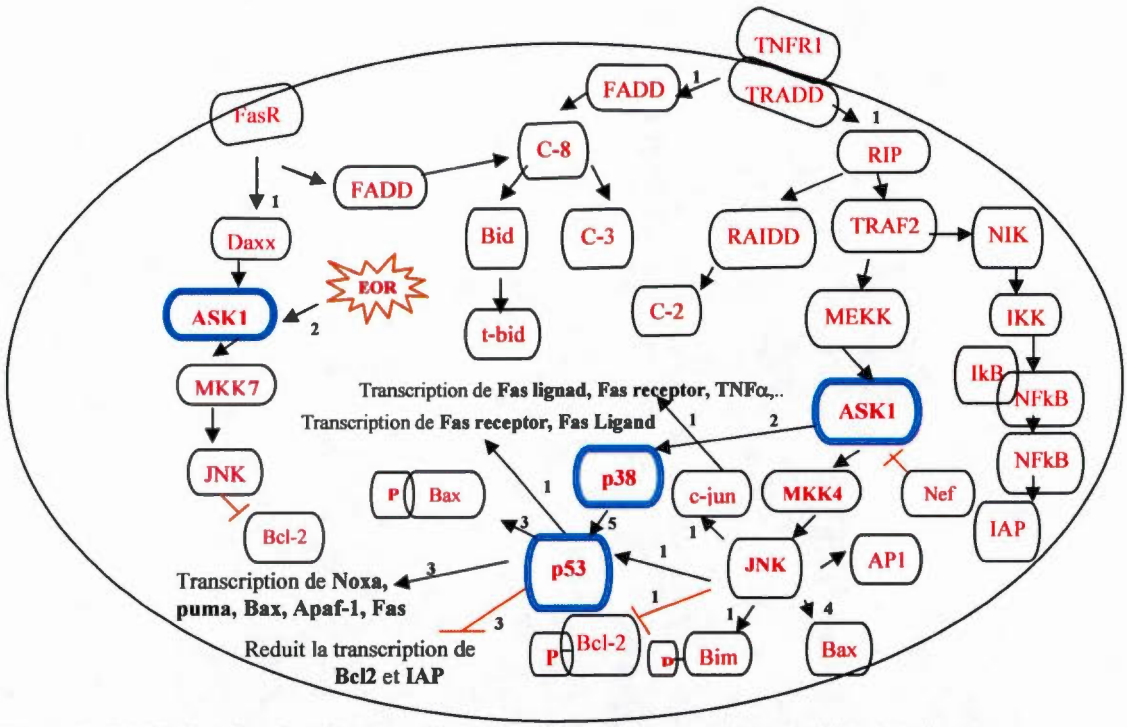


Figure 1.17: Voies de signalisation d'apoptose impliquant p53 et ASK1. (1. Curtin et Cotter, 2003; 2. Takeda *et al.*, 2003: 3. Bras *et al.*, 2005; 4. Tsuruta *et al.*, 2004; 5. Bulavin *et al.*, 1999). Les deux voies de signalisation des récepteurs de mort passant par FasR et TNFR convergent à l'activation de ASK1. En plus, les espèces réactives de l'oxygène (EOR) peuvent entraîner l'activation de ASK1. Ensuite, ASK1 active les deux voies JNK et p38 qui sont impliquées dans la transcription des gènes des protéines apoptotiques dont Bax, Bim, le ligand Fas et le récepteur Fas. D'autre part, p53 qui est induite suite aux dommages infligés à l'ADN par des composés toxiques tel que l'acroléine joue un rôle important dans la transcription du Bax, Bim, ligand Fas et du récepteur Fas.

1.3.2. La voie de survie AKT

L'AKT est une protéine pro-oncogène et qui est activée par la phosphoinositide-3 kinase (PI3K) et ceci par phosphorylation. L'activation de la protéine AKT est impliquée dans la croissance et la survie cellulaire et l'inhibition de l'apoptose (Asselin *et al.*, 2001; Downward, 1998). L'AKT inhibe l'apoptose en phosphorylant plusieurs protéines cibles dont la protéine pro-apoptotique Bad. Ce faisant, la protéine Bad sera reconnue par la protéine 14-3-3 qui induit sa dégradation et ainsi enlève l'inhibition de Bad sur la protéine anti-apoptotique Bcl-X_L (Sarker *et al.*, 2003; Franke *et al.*, 1997; Bellacosa *et al.*, 1998). Ainsi, l'inhibition de AKT induit la mort par apoptose chez les cellules cancéreuses.

1.3.3. Le facteur de transcription p53 et son rôle dans l'apoptose

p53 est le facteur de transcription le plus étudié. Il est étroitement relié à l'arrêt du cycle cellulaire et l'apoptose en réponse aux stress cellulaires comme les dommages de l'ADN (Evan et Littlewood, 1998). Il a été décrit la première fois en 1979 et il est le premier gène suppresseur de tumeur à être identifié. La principale fonction du p53 est l'élimination des cellules en prolifération anormale pour prévenir le développement néoplasique. Une dérégulation de la fonction du p53 est retrouvée dans la grande majorité des tumeurs humaines.

Des dommages à l'ADN, une forte augmentation d'AMPc ou des radiations UV induisent la phosphorylation de la protéine p53 et de ce fait son activation (Amsterdam *et al.*, 1996; 1997) (Figure 1.17). Au niveau de l'ADN, il est bien établi que la protéine p53, suppresseur de tumeurs, joue un rôle essentiel dans la réponse suite à des cassures double-brins, en modulant l'expression de certains gènes. Le facteur de transcription p53 induit la transcription de plusieurs gènes qui sont impliqués dans sa régulation négative telle que la murine double minute (MDM2), dans le contrôle de la réplication et de la réparation de l'ADN telle que la protéine induite par les dommages à l'ADN et arrêtant la croissance (GADD45), transactive

des protéines impliquées dans l'arrêt du cycle cellulaire (p21), qui inhibent les kinases cyclines-dépendantes et provoque alors l'arrêt en G1 (Tokino et Nakamura, 2000). D'autres gènes sont ciblés par le p53 dont ceux impliqués dans l'inhibition de l'angiogénèse (la thrombospondine) et dans l'induction de l'apoptose (Bax) (Tokino et Nakamura, 2000). La p53 active la transcription de Bax (Figure 1.17), qui induit la sortie de cyt-c des mitochondries. Le cyt-c ainsi libéré va activer les cascades de caspases et induire l'apoptose (Levine, 1997; Li *et al.*, 2000). La p53 peut aussi activer la transcription du récepteur Fas et inhiber celle des protéines anti-apoptotiques telles que Bcl-2 et les IAPs (Curtin et Cotter, 2003).

Il y a trois principaux régulateurs de la p53, la protéine mutée d'ataxia-telangiectasia (ATM), la protéine similaire à Rad3 (ATR) et la kinase DNA-dépendante (DNA-PK) (Zhu et Zhang, 2003). Les ATM, ATR et DNA-PK qui appartiennent à des cascades distinctes phosphorylent la p53 (Figure 1.18) (Baatout *et al.*, 2002; Abraham, 2001) pour inhiber sa dégradation par la Mdm2 et ainsi prolonger son effet anti-prolifératif. Les ATM, ATR et DNA-PK appartiennent à la famille des phosphoinositides 3-kinase-related kinases (PIKK). Elles jouent un rôle clé dans la régulation de la prolifération cellulaire et la surveillance génomique. Les activités kinases de ATM et ATR sont induites en réponse au bris à l'ADN et elles phosphorylent plusieurs effecteurs du point de contrôle du cycle cellulaire. L'ATM phophoryle les kinases de vérification 1 et 2 (Chk1 et 2), l'antigène 1 du cancer du sein (BRCA1), p53 à la sérine 15, Mdm2. L'ATR phosphoryle la kinase de vérification 1 (Chk1) (Das et Dashnamoorthy, 2004). La DNA-PK appartient aussi à la famille des PIKK et elle est activée en réponse au bris à l'ADN. La DNA-PK phosphoryle p53 aux résidus sérine 37 et sérine 15 (Das et Dashnamoorthy, 2004; Achanta *et al.*, 2001). Enfin, la Mdm2 qui est une protéine possédant une activité E3 ubiquitine ligase dégrade la p53 afin d'assurer une autorégulation négative du cycle de la p53 (Maya *et al.*, 2001). En plus, la protéine p53 peut être phosphorylée par la p38 MAPK (Curtin et Cotter, 2003; Bulavin *et al.*, 1999; Kim *et al.*, 2002; Sanchez-

Prieto *et al.*, 2000) et JNK (Curtin et Cotter, 2003), ce qui fait le lien entre les voies des MAPK, de la p53 et de l'apoptose (Figure 1.17).

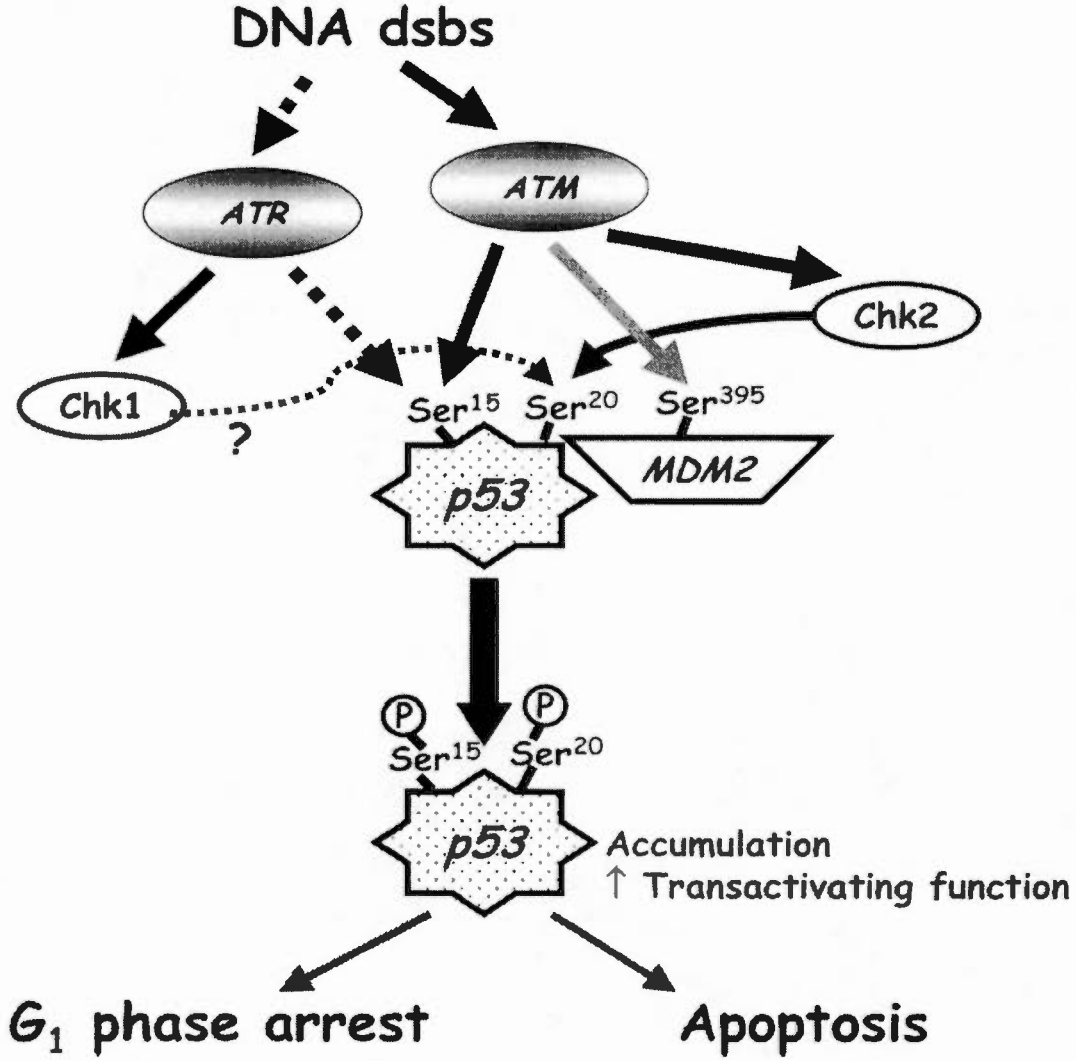


Figure 1.18: Signalisation de p53 suite aux dommages infligés à l'ADN. Les bris de double brin de l'ADN (dsbs) seront reconnus par ATR et ATM qui phosphoryleront Chk1 et 2 ainsi que la p53 à des sites bien définis. La p53 phosphorylée ne sera plus reconnue et dégradée par son inhibiteur la MDM2. En plus, la forme phosphorylée est la forme active de la protéine qui va transcrire les protéines pro-apoptotiques Bax, Bim, le récepteur FasR et son ligand Fas (Baatout *et al.*, 2002; Abraham, 2001).

1.4. PRÉSENTATION DU PROJET

1.4.1. Introduction

L'apoptose est un processus physiologique de mort cellulaire au cours duquel des mécanismes complexes sont activés pour aboutir à la destruction de la cellule. Ce « suicide cellulaire » est un événement clé en biologie car il permet l'équilibre entre prolifération et dégénérescence cellulaire dans les organismes pluricellulaires, c'est-à-dire le maintien de leur homéostasie. De plus, l'apoptose est un mécanisme « silencieux » pour l'organisme et il permet donc la régulation des populations cellulaires tout en respectant l'intégrité de l'organisme. Cette mort cellulaire régulée, induite et régie par une large variété de stimuli exogènes et endogènes, conduit à l'activation de la famille des protéases à cystéine, les caspases. Elles jouent un rôle fondamental dans le processus apoptotique.

Il y a un intérêt croissant à définir les mécanismes d'apoptose associés à la toxicité de faibles doses de différents composés chimiques (Orrenius et Zhivotovsky, 2006). Ceci inclut les polluants environnementaux tels que la toxine aquatique tributyltin (Reader *et al.*, 1999; Jurkiewicz *et al.*, 2004) et les pesticides organochlorés (Okoumassoun *et al.*, 2003) qui ont des impacts importants sur la santé humaine et d'autres espèces.

La liste des pathologies liées à un dysfonctionnement de l'apoptose est en croissance. La compréhension des mécanismes qui régulent ce type de mort cellulaire peut aider à identifier des sites d'action potentiels pour envisager différentes stratégies thérapeutiques, soient activatrices, soient inhibitrices du processus apoptotique. Le traitement de la leucémie aiguë promyélocytaire par le trioxyde d'arsenic représente un exemple d'utilisation thérapeutique d'agents inducteurs de l'apoptose (Florijn E, 1950). D'autre part, on a le traitement des lymphomes par le cyclophosphamide qui entraîne l'apoptose à des doses déterminées, et on pense que

l'effet thérapeutique de ce médicament est dû à son métabolite l'acroléine (Schwerdt *et al.*, 2006).

Dans notre recherche, on s'intéresse à l'acroléine car elle est produite par la réaction enzymatique de l'amine oxydase avec les polyamines cellulaires (spermine, spermidine) nécessaires à la division et à la prolifération cellulaire. Cette oxydation enzymatique génère aussi le peroxyde d'hydrogène qui est connu pour son potentiel apoptotique. Ainsi, ces propriétés de l'amine oxydase de générer deux produits toxiques, l'acroléine et le H_2O_2 lui confèrent des caractéristiques pertinentes dans le traitement de cancer. C'est pourquoi des études pré-cliniques sur des modèles cellulaires s'avèrent essentielles pour que l'enzymothérapie puisse être validée et peut-être utilisée de façon efficace en clinique. En plus, on s'intéresse à étudier l'acroléine car elle est un polluant ominéprésent de notre environnement et un marqueur important dans le pronostic de la maladie d'Alzheimer.

En général, on remarque que l'acroléine induit la mort cellulaire par nécrose (Rudra et Krokan, 1999) et cause une inhibition de la prolifération cellulaire (Agostinelli *et al.*, 1994, 1996; Biswal *et al.*, 2002). Toutefois, l'acroléine peut induire l'apoptose ou l'inhiber dans le même type cellulaire et ceci dépendamment de la concentration et du type cellulaire utilisé. Par exemple, une faible concentration en acroléine (<10 μM) induit l'apoptose chez les neutrophiles tandis qu'une plus grande concentration (>10 μM) l'inhibe (Finkelsetin *et al.*, 2005). Cependant, les mécanismes d'induction de l'apoptose par l'acroléine ne sont pas encore compris.

1.4.2. Modèle cellulaire

Pour pouvoir utiliser un nouveau médicament en clinique, chercher une protection contre les effets toxiques d'un polluant environnemental, les mécanismes d'action cellulaires et moléculaires doivent tout d'abord être compris. Les études *in vitro* utilisant des lignées cellulaires présentent comme avantage de générer rapidement des résultats fiables et utiles tout en permettant un meilleur contrôle des

conditions expérimentales, contrairement aux expériences sur les animaux qui présentent souvent des problèmes liés à l'hétérogénicité cellulaire. La culture cellulaire limite l'utilisation d'animaux de laboratoire qui est considérée de moins en moins justifiée par les organismes qui veillent à la protection des animaux, et elle est relativement peu coûteuse en comparaison. La plupart des cellules normales ont une durée de vie limitée (20 à 100 générations). Les cellules de rongeurs et celles de la plupart des tumeurs mises en culture continue, peuvent subir une transformation spontanée ou induite, qui leur confère un caractère immortel. Cette transformation provoque l'aneuploidie et une augmentation de la tumorigénicité. Ces lignées sont bien caractérisées et présentent une population de cellules homogènes. Ces cellules sont cultivées dans un environnement qui peut être facilement contrôlé (température, pH, oxygène, etc). Dans le cadre de cette étude, les différents types d'expérimentations ont été effectués sur des cellules d'ovaire de hamster chinois (CHO) (Ling et Thompson, 1973). Le phénotype des cellules CHO a été très bien caractérisé et ces dernières comptent parmi les types cellulaires les plus utilisés dans la recherche *in vitro*. Cependant, l'utilisation de culture cellulaire comporte des limitations. Elle ne peut refléter la complexité biologique des tissus ou organismes entiers. Les interactions cellulaires existant dans les tissus se perdent lors de la propagation sur un substrat bidimensionnel. Cependant, la culture cellulaire est utile pour étudier et comprendre les voies de signalisation cellulaire, les bases moléculaires de pathologies ou les mécanismes de toxicité. Enfin, il y a de plus en plus d'intérêt à développer les modèles de culture de cellules en trois dimensions pour la poursuite des études lorsque les mécanismes recherchés ont été caractérisés par les cultures cellulaires conventionnelles.

1.4.3. Objectifs du projet

La première hypothèse est que l'acroléine induit l'apoptose et ceci par la voie mitochondriale et / ou par la voie des récepteurs. La deuxième hypothèse est que

l'acroléine induit des dommages à l'ADN, ce qui entraînera l'activation de la p53. La p53 induira ensuite la transcription de Bax ainsi que celle du récepteur Fas, activant de ce fait la voie mitochondriale et la voie des récepteurs, respectivement. La troixième hypothèse est que la voie des récepteurs peut activer la voie mitochondriale par l'intermédiaire de la caspase-8, ainsi que les voies de p38 MAPK et JNK. L'apoptose induite par l'acroléine peut être inhibée par les voies AKT et ERK suite à l'activation des récepteurs tyrosines kinases. La quatrième hypothèse est que l'acroléine engendre un stress oxydatif, en diminuant le GSH, un puissant antioxydant intracellulaire, ainsi on examinera l'effet protecteur des modulateurs de glutathion sur l'apoptose induite par l'acroléine.

L'objectif général de cette étude est de comprendre les mécanismes moléculaires impliqués dans l'induction de l'apoptose par l'acroléine. Les objectifs spécifiques de l'étude sont de déterminer (1) l'implication de la voie mitochondriale, (2) la voie des récepteurs de mort et (3) la signalisation des MAPK et de la p53 dans l'apoptose induite par l'acroléine, et (4) le rôle du glutathion dans la protection de l'apoptose.

1.4.4. Approche expérimentale

Objectif 1 : Ce projet déterminera si l'acroléine entraîne l'apoptose et ou la nécrose. L'apoptose est révélé par le colorant Hoescht No. 33258 en microscopie à fluorescence. Le colorant Hoescht No. 33258 s'intercale dans l'ADN des cellules et fluoresce proportionnellement à la condensation de la chromatine qui est une caractéristique importante de l'apoptose. La nécrose est révélée par l'iodure de propidium qui entre dans les cellules dont les membranes sont endommagées et colore le noyau. De plus, la propriété de l'acroléine à activer la voie mitochondriale de l'apoptose, en induisant une baisse du potentiel membranaire, en libérant le cytochrome-c vers le cytosol et en translocant les protéines pro-apoptotiques Bax et Bad du cytosol vers la mitochondrie sera investiguée. La baisse de la protéine anti-

apoptotique Bcl-2 au niveau de la mitochondrie sera analysée. La détection de ces protéines se fera par la méthode de l'immunobuvardage de type Western en utilisant les anticorps spécifiques correspondants après le fractionnement sous-cellulaire. La pureté des différents compartiments (cytosol, mitochondrie, membranaire) sera vérifiée par des marqueurs spécifiques de chaque fraction cellulaire. La calnexine est spécifique aux microsomes, la GST-π1 au cytosol et la cytochrome oxydase est spécifique aux mitochondries. Les bandes de protéines seront ensuite révélées par développement sur un film. La quantification de ces bandes protéiques par un densitomètre au laser permettra de calculer leurs concentrations relatives par rapport aux cellules non exposées à l'acroléine. La baisse du potentiel membranaire mitochondriale sera analysée en cytométrie de flux par la sonde rhodamine-123. On déterminera si l'acroléine active les caspases initiatrices (ex : caspase -8 et -9) et les caspases effectrices (ex : caspases -3, -6 et -7) par fluorimétrie. Cela consiste à étudier la cinétique des réactions enzymatiques en utilisant les substrats fluorescents et spécifiques à chaque caspase. Si la caspase est activée, le substrat est clivé, et on aura augmentation de la fluorescence du fragment clivé qui sera détectée par un lecteur de microplaque à fluorescence à des longueures d'onde précises d'excitation et d'émission. Ensuite le clivage de chaque caspase sera étudié par immunobuvardage de type Western pour détecter la forme pro-caspase et le fragment clivé. En utilisant des inhibiteurs pharmacologiques des différentes caspases, on déterminera leurs implications dans l'exécution de l'apoptose. Le clivage de l'ICAD et de la PARP, deux importants substrats des caspases, sera examiné en immunobuvardage de type Western dans un lysat cellulaire total.

Objectif 2 : En infligeant des dommages à l'ADN par exposition à l'acroléine, la p53 peut induire l'activation du récepteur Fas conduisant de ce fait à l'activation de la voie des récepteurs. Ainsi, on examinera l'augmentation de l'expression du récepteur Fas et de son ligand FasL au niveau de la membrane plasmique, la translocation de FADD du cytosol vers la membrane plasmique par

immunobuvardage de type Western dans les fractions sous-cellulaires. On détectera le clivage de la pro-caspase-8 et l'apparition de son fragment par immunobuvardage de type Western, et l'activation de la caspase-8 par fluorimétrie. Enfin, l'utilisation d'inhibiteurs pharmacologiques spécifiques de la caspase-8 et du récepteur Fas déterminera l'importance de la voie des récepteurs de mort et de la voie mitochondriale dans l'induction de l'apoptose induite par l'acroléine. Ceci, en déterminant leurs effets sur l'activation des différentes caspases, le clivage de la PARP et la condensation de la chromatine par l'acroléine. Le clivage de Bid dans le cytosol en sa forme tronqué t-Bid qui se transloque à la membrane mitochondriale sera aussi détecté en immunobuvardage de type Western dans les fractions sous-cellulaires. L'externalisation des phosphatidylsérines, des phospholipides de la membrane plasmique, pour présenter l'état apoptotique de la cellule au milieu environnant pour qu'elle soit phagocytée par un macrophage, sera détectée à l'aide du cytomètre à flux par l'annexine V (Nardini *et al.*, 2002). L'annexine V couplée à un fluorochrome reconnaît la molécule de phosphatidylsérine qui se présente à la face externe de la membrane plasmique et marque ainsi la membrane par fluorescence.

Objectif 3 : Pour démontrer l'implication de la voie de signalisation des MAPKs dans l'induction de l'apoptose par l'acroléine, on analysera par immunobuvardage de type Werstern l'expression des formes phosphorylées (actives) ou non-phosphorylées de ERK1 et 2, c-jun, et p38 MAP kinase. En plus, on détectera l'activation par phosphorylation de ASK1 (Takeda *et al.*, 2003) qui est une MAPKKK (MAPK kinase kinase) qui active JNK et p38 MAPK. S'il y a phosphorylation des différentes MAPK, on utilisera des inhibiteurs de chacune des trois cascades des MAPKs afin de confirmer l'implication de différentes kinases dans l'apoptose induite par l'acroléine. Les inhibiteurs envisagés seront le PD 98059 et le U0126 qui sont des inhibiteurs de MEK (Lin *et al.*, 2004), le SB203580 qui est un inhibiteur de p38 MAPK (Liu et Hofmann, 2004) et le SP600125 qui est un inhibiteur de JNK (Naruishi *et al.*, 2003). Les effets des inhibiteurs seront analysés sur l'activation des différentes caspases, le clivage de l'ICAD et la condensation de la

chromatine par l'acroléine. Enfin, on analysera l'expression de AKT (protéine kinase B) qui est impliquée dans l'inhibition de l'apoptose par phosphorylation de Bad (Sarker *et al.*, 2003). S'il y a phosphorylation, on utilisera des inhibiteurs pharmacologiques afin de confirmer son implication dans l'apoptose induite par l'acroléine. Les inhibiteurs envisagés seront le LY294002 et le wortmannin qui sont des inhibiteurs de la voie PI3K-AKT (Hu *et al.*, 2000; Takeda *et al.*, 2004). Enfin, on examinera l'expression de la protéine suppresseur de tumeur la p53 qui est induite par des dommages à l'ADN causés par des composés toxiques tel que l'acroléine et ceci par immunobuvardage de type Western. L'activation par phosphorylation de la p53 sur les sérines -15 et -46 sera aussi détectée par immunobuvardage de type Western.

Objectif 4 : Compte tenu que l'acroléine diminue de façon marquée l'expression d'un antioxydant important, le glutathion, la capacité des thiols N-acétylcystéine (NAC) et L-2-oxothiazolidine-4-carboxylate (OTC) à détoxifier la cellule de l'acroléine et ainsi inhiber l'apoptose sera investiguée. Le NAC et l'OTC sont utilisés pour leur capacité à augmenter le glutathion et les thiols intracellulaires. La concentration du glutathion est mesurée par spectrophotométrie à une longueur d'onde précise. D'autre part, l'habileté de la diminution du glutathion par le L-buthionine sulfoximine (BSO) (inhibiteur de synthèse du glutathion) à augmenter l'induction de l'apoptose et la cytotoxicité, sera déterminée. La cytotoxicité est un test qui mesure la capacité des cellules à proliférer dans des pétris après une incubation avec un toxique. Après l'exposition, les cellules seront diluées et déposées dans des pétris. Enfin, on comptera les colonies formées après une semaine de culture.

En conclusion, on s'attend que ce projet apporte une meilleure compréhension de l'apoptose induite par l'acroléine afin d'explorer le potentiel d'utiliser la BSAO dans le traitement des cancers. En plus, les mécanismes explorés dans ce projet apporteront une meilleure compréhension de la toxicité de l'acroléine qui est impliquée dans la maladie d'Alzheimer et la toxicité du cyclophosphamide, et ainsi trouver des moyens de protection.

CHAPITRE II
RÉSULTATS EXPÉRIMENTAUX

2.1. Préface

Ce chapitre inclut quatre articles scientifiques décrivant les résultats expérimentaux de ce projet, que j'ai réalisé au cours des quatre dernières années dans le laboratoire du Dr. Diana A. Averill.

Le premier manuscrit s'intitule: "The aldehyde acrolein induces apoptosis via activation of the mitochondrial pathway, ***Biochim Biophys Acta*** 1743 (3): 255-67, 2005; par André Tanel et Diana Averill-Bates". Ce manuscrit a été rédigé par moi-même et a été révisé par le Dr. Diana Averill-Bates. Pour ce qui est des expériences de cet article, je les ai effectuées en entier. Cette étude porte sur le mécanisme de mort cellulaire par la voie mitochondriale induit par l'acroléine chez les cellules d'ovaire de hamster chinois (CHO).

Le deuxième article s'intitule: "Activation of the death receptor pathway of apoptosis by the aldehyde acrolein, ***Free Radical Biology and Medicine*** 42 (6): 798-810, 2007; par André Tanel et Diana Averill-Bates ". Ce manuscrit a été rédigé par moi-même et corrigé par Dr Diana Averill-Bates. Pour ce qui est des expériences de cet article, je les ai effectuées en entier. Cette étude porte sur le mécanisme de mort cellulaire par la voie des récepteurs de mort induite par l'acroléine chez les cellules CHO.

Le troisième article s'intitule: "p38 and ERK mitogen-activated protein kinases mediate acrolein-induced apoptosis in Chinese hamster ovary cells, ***Cell Signal*** 19 (5) : 968-977, 2007; par André Tanel et Diana Averill-Bates ". Ce manuscrit a été rédigé par moi-même et a été révisé par Dr Diana Averill-Bates. Pour ce qui est des

expériences de cet article, je les ai effectuées en entier. Cette étude porte sur l'implication de la voie des MAPKs dans l'induction de l'apoptose par l'acroléine chez les cellules CHO.

Le quatrième article s'intitule: "Inhibition of acrolein-induced apoptosis by the antioxidant N-acetylcysteine, ***J Pharmacol Exp Ther*** 321 (1): 73-83, 2007; par André Tanel et Diana Averill-Bates". Ce manuscrit a été rédigé par moi-même et a été révisé par Dr Diana Averill-Bates. Pour ce qui est des expériences de cet article, je les ai effectuées en entier. Cette étude porte sur le mécanisme de mort cellulaire par la voie mitochondriale induit par l'acroléine et la protection apportée par un prétraitement avec l'antioxydant N-acétylcystéine (NAC) chez les cellules CHO.

D'autres contributions: "Anti-tumoral effect of native and immobilized bovine serum amine oxidase in a mouse melanoma model. ***Biochem Pharmacol*** 69 (12): 1693-704, 2005. Par Averill-Bates DA, Cherif A, Agostinelli E, Tanel A et Fortier G. Ce manuscrit a été rédigé par Dr Diana Averill-Bates et les expériences ont été effectuées par Anissa Cherif et moi-même. Dr Diana Averill-Bates et Dr Guy Fortier sont les directeurs de cette étude. Dr Enzo Agostinelli a fourni l'enzyme BSAO. En ce qui concerne ma partie, j'ai effectué la mise au point du test de l'annexine V et j'ai effectué les expériences de la figure 7 et du tableau 2.

2.2. ARTICLE I

THE ALDEHYDE ACROLEIN INDUCES APOPTOSIS VIA ACTIVATION OF THE MITOCHONDRIAL PATHWAY

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Abbreviations: AFC: amino trifluorocoumarin; AMC: amino methylcoumarin; BSA: bovine serum albumin; BSAO: bovine serum amine oxidase; CAD: caspase activated DNase; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CHO: Chinese hamster ovary; Fas: fibroblast-associated; FBS: fetal bovine serum; FCCP: P-trifluoromethoxy-phenyl-hydrazone; ICAD: inhibitor of

caspase activated DNase; MEM: minimum essential medium; MOPS: 3-(N-morpholino)-propane sulfonic acid; PARP: polyADP-ribose polymerase; PBS: phosphate-buffered saline; PMSF: phenylmethylsufonyl fluoride; PTP: permeability transition pore; PVDF: polyvinylidene difluoride; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM: standard error of mean.

Keywords: Acrolein, apoptosis, caspase, mitochondria, cell, cytotoxicity.

RÉSUMÉ

L'acroléine est un aldéhyde α,β-insaturé extrêmement réactif et elle est un produit de la peroxydation lipidique. Elle est un polluant environnemental qui est impliqué dans plusieurs maladies respiratoires. L'acroléine est produite lors de la désamination oxydative par la spermine oxydase. Les produits d'oxydation des polyamines sont impliqués dans l'inhibition de la prolifération cellulaire, l'apoptose, et l'inhibition de la synthèse de l'ADN et des protéines. La présente étude explore le mécanisme de mort cellulaire induit par l'acroléine. Acroléine induit l'apoptose en entraînant la baisse du potentiel membranaire mitochondrial, la libération du cytochrome-c, l'activation de la caspase-9 initiatrice et la caspase-7 exécutrice. Par ailleurs, l'acroléine a inhibé l'activité enzymatique de la caspase-3 exécutrice sans empêcher cependant le clivage de la proenzyme. L'activation des caspases -7 et -9 a été confirmée par le clivage de leurs proenzymes. L'apoptose induite par l'acroléine a été inhibée par un inhibiteur de la caspase-9 mais pas par un inhibiteur de la caspase-3. L'induction de l'apoptose par l'acroléine a été confirmée morphologiquement par la condensation nucléaire de la chromatine et par le clivage de l'inhibiteur de la DNase activée par les caspases (ICAD). Le clivage de l'ICAD libère l'endonucléase CAD qui emmène la fragmentation de l'ADN. Ces résultats démontrent que l'acroléine induit l'apoptose par la voie mitochondriale.

ABSTRACT

Acrolein is a highly reactive α,β-unsaturated aldehyde, which is a product of lipid peroxydation. It is an environmental pollutant that has been implicated in multiple respiratory diseases. Acrolein is produced by the enzymatic oxidative deamination of spermine by amine oxidase. Oxidation products of polyamines have been involved in inhibition of cell proliferation, apoptosis, and inhibition of DNA and protein synthesis. The present study investigates the mechanism of cell death induced by acrolein. Acrolein induced apoptosis through a decrease in mitochondrial membrane potential, liberation of cytochrome-c, activation of initiator caspase-9 and activation of the effector caspase-7. However, acrolein inhibited enzymatic activity of the effector caspase-3, although cleavage of pro-caspase-3 occurred. The activation of caspases-9 and -7 was confirmed by cleavage of their pro-enzyme form by acrolein. Apoptosis was inhibited by an inhibitor of caspase-9, but not by an inhibitor of caspase-3. Induction of apoptosis by acrolein was confirmed morphologically by condensation of nuclear chromatin and by cleavage of inhibitor of caspase activated DNase (ICAD), which leads to liberation of CAD that causes DNA fragmentation. These results demonstrate that acrolein causes apoptosis through the mitochondrial pathway.

INTRODUCTION

Acrolein is a highly reactive, α,β-unsaturated aldehyde and humans are exposed to this compound in multiple situations [1]. Acrolein is one of the toxic products of endogenous lipid peroxidation (LPO) [2]. Acrolein is a major component of cigarette smoke and a constituent of automotive exhaust [3]. Industrially, acrolein is used as a herbicide as well as a starting material for acrylate polymers and in the production of acrylic acid [4]. Acrolein reacts with cysteine, histidine and lysine residues of proteins [5, 6] . Acrolein protein adducts have been demonstrated in diabetic nephropathy [7], Alzheimer's disease [8, 9] and atherosclerosis [10].

Apoptosis is a physiological cell death process which plays an important role during development and maintenance of tissue homeostasis [11, 12]. A balance between cell death and cell proliferation is required to maintain a cellular homeostatic state. Deviation from this cellular balance disrupts the normal state and can lead to human disease [13]. A family of cytosolic cysteine proteases, the caspases, stored in most cells as zymogens, play an essential role in the execution of apoptosis. The caspases are divided into apical (-2, -8, -9 and -10) and executioner subsets (-3, -6 and -7) [14]. The zymogen of caspase-9 is activated via a post-mitochondrial route [15].

The mitochondrial pathway is triggered by growth factor deprivation, ionizing radiation and some anticancer drugs such as cyclophosphamide and etoposide [16]. Cytochrome-c is released from mitochondria into the cytosol, where it interacts with dATP and apoptosis protease activating factor (Apaf1) and pro-caspase-9 in the apoptosome complex in the cytosol. This leads to conversion of pro-caspase-9 to active caspase-9 [17]. Anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) members of the Bcl-2 family are thought to control apoptosis by modulating cytochrome-c release from mitochondria [18]. Once activated, caspase-9 activates effector caspases such as caspases-3, 6 and 7 [14]. Effector caspases then cleave specific cytoplasmic,

cytoskeletal and nuclear protein substrates such as polyADP-ribose polymerase (PARP), lamins and inhibitor of caspase activated DNase (ICAD). The cell then exhibits the characteristic morphological features of apoptosis such as chromatin condensation, cell blebbing and formation of apoptotic bodies [19].

Acrolein has been shown to induce apoptosis in cell types such as human alveolar macrophages [20], human keratinocytes [21] and human bronchial epithelial cells HBE1 [22]. In contrast, acrolein inhibited neutrophil apoptosis [23] and induced oncosis/necrosis rather than apoptosis in murine proB lymphocytes [24]. Thus, acrolein-induced apoptosis appears to be cell type dependent. Most of these studies confirmed induction of apoptosis by means of endpoints occurring later in the apoptotic cascade such as DNA fragmentation, without determining the mechanisms occurring upstream of these events.

Acrolein is a metabolic product of cyclophosphamide [25] and could be involved in its anticancer action. It is also a product of the oxidation of polyamines by amine oxidases along with hydrogen peroxyde[26]. Our previous studies suggest that amine oxidase could prove to be useful in cancer treatment [27]. Targeting polyamines has emerged as a promising therapeutic strategy since they play an important role in the development and maintenance of neoplastic growth [28]. Moreover, rapidly growing tissues such as tumors have elevated levels of polyamines [29]. To take advantage of this differential effect between normal and tumor cells, toxic products such as H_2O_2 and aldehydes could be generated in situ by amine oxidases for the selective killing of tumor cells [30]. However, the molecular mechanisms involved in acrolein-induced apoptosis in cancer cells are not understood. This study investigates the ability of acrolein to activate apoptosis via the mitochondrial pathway in proliferating Chinese hamster ovary (CHO) cells which are known to induce tumors.

MATERIALS AND METHODS

Cell culture

CHO cells (AuxBl) [31] were grown in monolayer in minimum essential medium-Alpha (α-MEM) (Gibco Canada, Burlington, ON, Canada) plus 10% fetal bovine serum (FBS) (Gibco Canada) and 1% penicillin (50 units/mL)- streptomycin (50 μg/mL) (Flow Laboratories, Mississauga, ON, Canada), in tissue culture flasks (Starstedt, St Laurent, QC, Canada), in a humidified atmosphere of 5% CO_2 in a water jacketed incubator at 37°C [32]. The cells were grown to near confluence and were then incubated for 24 h with fresh culture medium. Confluent cells were then harvested using citrated phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, 0.015 M sodium citrate), washed by centrifugation (1000g, 3 min) and resuspended in α-MEM for experimental studies.

Clonogenic cytotoxicity assay

Cytotoxicity was evaluated as the ability of cells to proliferate to form macroscopic colonies after a toxic insult with acrolein, using a clonogenic cell survival assay. CHO cells (10^5/mL) were incubated with acrolein (Aldrich Chemical Co, Milwaukee, WI), in a final volume of 1.0 mL in α-MEM containing 10 % FBS for 1 h at 37°C. The cells were then washed three times by centrifugation (1000g, 2 min) to stop the incubation [32]. The cells were resuspended and diluted to the appropriate concentration with α-MEM plus FBS and plated in tissue culture dishes (60 x 15 mm), which were incubated at 37°C in an atmosphere of 5% $C0_2$ for 8 days. The dishes were then washed with PBS, fixed with 95% ethanol and stained with methylene blue before counting macroscopic colonies (>50 cells). Cytotoxicity was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells were seeded in the control plates, but where there was a loss of cell survival, cells were plated at several different

densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have previously demonstrated that, in this system, there is linearity between the number of cells plated and colonies formed over the range of 10 to 10^4 [32].

Morphological analysis of apoptosis

To visualize nuclear morphology and chromatin condensation by fluorescence microscopy [33], cells were seeded and cultured to near confluence in tissue culture dishes containing 5mL of α-MEM and 10% FBS. Cells were incubated with acrolein for 4 h or 24 h. Dishes were washed twice with PBS and Hoechst (33258) (0.06 mg/mL) was added for 15 min at 37^0C to stain apoptotic cells. The dishes were washed with PBS and propidium iodide (50 μg/mL) was added to stain the necrotic cells. Observations were made by fluorescence microscopy (Carl Zeiss Ltd, Montreal, QC) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc, Mississauga, ON). Images were analysed by Northern Eclipse software. Cells were classified using the following criteria : a) live cells (normal nuclei, pale blue chromatin with organized structure); b) membrane-intact apoptotic cells (bright blue condensed or fragmented chromatin); c) necrotic cells (red, enlarged nuclei with smooth normal structure [33]. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). A minimum of 200 cells were counted per dish.

Caspase inhibitors

Treatment of cells with inhibitors of caspases was performed on confluent cells in monolayer. The specific inhibitors used were: Caspase-3 Inhibitor V, Z-DQMD-FMK; Caspase-9 Inhibitor I, Z-LEHD-FMK and the general Caspase Inhibitor I, Z-VAD-FMK (Calbiochem, La Jolla, CA. The cells were exposed to 10 or 20 μM concentrations of inhibitors and to various concentrations of acrolein for 4 h.

Cells were analysed for apoptosis by fluorescence microscopy using Hoescht staining.

Determination of caspase activity by fluorescence spectroscopy

Freshly harvested CHO cells (0.5 x 10^6) were resuspended in α-MEM and incubated with acrolein in a final volume of 1.0 mL at 37^0C. After the appropriate time, the cells were washed three times with cold PBS by centrifugation (1000g, 3 min) to stop the incubation. The cells were resuspended in 50 µl of PBS and 25 µl were deposited into 96-well plates and lysed by freezing at -20^0C for 20 minutes. Fifty µl of reaction buffer (20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) was added and stabilized at 37^0C [34]. The kinetic reaction was started after addition of 25 µl of the appropriate caspase substrate at 37°C using a Spectra Max spectrofluorimeter (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA).

Caspase-3 activity was measured by cleavage of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Calbiochem, La Jolla, CA) to produce amino methylcoumarin (AMC) with λmax excitation at 380 nm and λmax emission at 460 nm. Caspase-7 activity was measured by cleavage of the fluorogenic substrate I MCA-VDQVDGWK(DNP)-NH_2 with λmax excitation at 325 nm and λmax emission at 395 nm. Caspase-9 activity was measured by cleavage of the substrate Ac-LEHD-AFC to produce AFC with λmax excitation at 415 nm and λmax emission at 490 nm.

Flow cytometry analysis of mitochondrial membrane potential

To measure Δψm, the fluorescent probe rhodamine 123 was used. Freshly harvested CHO cells (10^6) were resuspended in α-MEM and incubated with acrolein,

or the positive control p-trifluoromethoxy-phenyl-hydrazone (FCCP), in a final volume of 1.0 mL at 37^0C. After one hour of incubation, the cells were washed three times with cold PBS by centrifugation (1000g, 3 min) to stop the incubation. The cells were resuspended with PBS and then incubated with the fluorescent probe (800 ng/mL) for 5 minutes. Samples were washed and stored in the dark at 4°C until the time of analysis (usually within 5 min). Propidium iodide (PI) was added to cell suspensions stained with rhodamine 123 to identify dead cells. Cells were analyzed with a FACScan flow cytometer equipped with an argon laser emitting at 488 nm (Becton Dickinson, Oxford, UK). Data was acquired and analysed using Lysis II software (Becton Dickinson). Mean fluorescence intensity of 20,000 cells was calculated for each sample and corrected for autofluorescence obtained from samples of unlabeled cells. The analyser threshold was adjusted on the forward scatter (FSC) channel to exclude noise and subcellular debris. Photomultiplier settings were adjusted to detect rhodamine 123 fluorescence on the FL1 detector and PI fluorescence on the FL2 detector. In each case, the photomultiplier voltage was set so that the signal peak from non-stained cells (mostly due to autofluorescence), fell within the first decade of the logarithmic amplifier. Light scatter parameters were used to establish size gates which detect apoptotic cells, thus excluding dead cells [35]. Populations of dead cells highly fluoresce in FL-2 because they incorporate PI, thus 2 populations of cells appear on the computer screen. For each sample, only the live cells are selected and their mean fluorescence in FL-1 is analysed. Apoptotic cells, which undergo a decrease in mitochondrial membrane potential, incorporate less of the rhodamine 123 dye, therefore emitting less fluorescence on the FL-1 detector.

Subcellular fractionation and immunodetection of cytochrome-c, caspases and ICAD

Following treatment with acrolein, cells were washed in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After 30 min incubation on ice, lysates were homogenised using a hand potter (Kontes glass CO, Duall 22, Fisher, QC, Canada). Unbroken cells and nuclei were pelleted by centifugation at 2500 *g* for 10 min. The supernatant was centrifuged further at 100 000 *g* for 1 h. The resultant supernatant was designated as the cytosolic fraction, which was used for detection of cytochrome-c. For immunodetection of caspases and ICAD, whole cell lysates were used.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins was carried out according to Laemmli [36]. Proteins (30 μg) were quantified according to Bradford [37] and then solubilised in Laemmli sample buffer. The samples were boiled for 5 min at 100°C and loaded onto a 15% acrylamide gel. Electrophoresis was carried out at a constant voltage of 125 V. Cellular proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Milli-pore, Bedford, MA). The transfer buffer contained 96 mM glycine, 10 mM Tris and 10% methanol. The transfer was carried out for 1.5 h at constant amperage of 80 mA/gel. Hydrophobic or nonspecific sites were blocked overnight at 4°C with 5% powdered skim milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.1% Tween 20 (TBS-T). Membranes were washed four times for 15 min in TBS-T. The blots were probed with the primary antibody: anti-cytochrome-c (BD Biosciences Canada, Mississauga, ON), anti-caspase-3, anti-caspase-9, anti-ICAD, anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-caspase-7 (Cell Signaling Technology, Beverly, MA) in TBS-T, 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed four times for 15 min and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (1:1000) in TBS-T

containing 5% milk powder. Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-mouse, anti-rabbit and anti-goat IgG (Biosource, Camarillo, CA). PVDF membranes were washed four times for 15 min and cytochrome-c, caspases and ICAD were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA). For verification of equivalence in protein loading, the blot was probed with the anti-tubulin antibody and by coloration of the gels using Coomassie blue. Protein expression was quantified using a scanning laser densitometer, relative to β-tubulin (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Statistical differences between control and treated groups for cell survival and ICAD cleavage were determined by a two-tailed unpaired Student's *t* test. Statistical comparisons for the glutathione assay were made with one-way ANOVA which measures the linear contrast of means. An adjustment was made to limit the familywise error rate (FWE) to 5% by calculating an adjusted *p*-value which is a simulated based *p*-value obtained from the multivariate *t* distribution (number of simulations = 1000 000) [38]. Two-way ANOVA with a p-adjusted value obtained using the Bonferroni-Holm method (a stepwise method) was used to compare cells treated with acrolein versus control, using the Hoescht test. Data for caspase activity and caspase cleavage were analyzed for significant differences using a one-way ANOVA with a Bonferroni-Holm post-test correction for multiple comparisons. For experiments involving caspase inhibitors, adjusted *p*-values were obtained using one-way ANOVA followed by the Dunnett test.

Values are expressed as means ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

We established the concentrations of acrolein which were able to induce cytotoxicity in CHO cells (Fig. 1). Cytotoxicity was induced at concentrations of 50 *f*mol/cell of acrolein and higher. A concentration of about 180 to 190 *f*mol/cell (18-19 μM) was sufficient to decrease the fraction of surviving cells to 10%. Above 200 *f*mol/cell, there was a marked decline in cell survival to 5 logarithms of cell killing at 350 *f*mol/cell. It should be noted that the acrolein concentration was expressed as *f*mol/cell because the cell density was different for certain tests [39].

Subsequently, we investigated the type of cell death (i.e. apoptosis or necrosis) induced by acrolein in proliferating CHO cells. Morphological analysis demonstrates that acrolein induces both apoptosis and necrosis in cells (Fig. 2). Apoptosis is characterized by early and prominent condensation of nuclear chromatin [33]. Apoptosis induced by acrolein was revealed by condensation of chromatin with the fluorescent probe Hoescht (blue-green) whereas necrosis was revealed by the fluorescent probe PI (red). Acrolein (50 *f*mol/cell, 4h) (Fig. 2C) induced apoptosis and necrosis in 21% and 4% of cells, respectively (Fig. 2E, 2F). A higher concentration of acrolein (100 *f*mol/cell, 4h) (Fig. 2D) switched the mode of cell death from apoptosis (9%) to necrosis (20%) (Fig. 2E, 2F). It should be pointed out that induction of apoptosis occurred at lower concentrations of acrolein (≥ 30 *f*mol/cell, 4h) (Fig. 2B, 2E), relative to induction of necrosis (≥ 50 *f*mol/cell, 4h) (Fig. 2C, 2F). Very few dead cells were seen in untreated controls (Fig. 2A). When cells were exposed to acrolein for 24 h, higher levels of toxicity occurred by apoptosis and necrosis (Fig. 2E, 2F). Maximum levels of apoptosis were induced by 30 and 50 *f*mol/cell of acrolein, whereas 100 *f*mol/cell of acrolein caused essentially only necrosis.

The subsequent step was to determine whether acrolein could induce apoptosis via activation of the mitochondrial pathway. The ability of acrolein to alter

mitochondrial membrane potential and to induce liberation of cytochrome-c from the mitochondria to the cytosol was determined. Exposure of cells to acrolein (10 to 30 *f*mol/cell) for 1 h led to a decrease in rhodamine 123 fluorescence in the FL1 channel, relative to untreated control cells (Fig. 3A, 3B). This represented a decrease in mitochondrial membrane potential by 25% in the presence of 30 *f*mol/cell of acrolein, relative to controls (Fig. 3B). The magnitude of the decrease was similar to that induced by FCCP (5μM), which was used as a positive control for membrane depolarisation (Fig 3B). The mitochondrial permeability transition pore (PTP) complex is considered to play an important role in the release of pro-apoptotic proteins such as cytochrome c. Opening of the PTP can be inhibited by compounds such as cyclosporine A [40]. Indeed, cyclosporine A partially inhibited apoptosis induced by acrolein (Fig. 3C), further confirming the role of membrane depolarisation in acrolein-induced apoptosis. Acrolein (4 to 30 *f*mol/cell) caused the liberation of cytochrome-c after 1 h (Fig. 3D). Exposure to acrolein (1 to 50 *f*mol/cell) for 1 h caused activation of caspase-9, which is the initiator caspase of the mitochondrial pathway (Fig. 4A). However, after 2 h, there was no activation of caspase-9, but instead, inhibition of the enzyme. In fact, caspase-9 activity was lower than the control level for concentrations between 20 and 150 *f*mol/cell, although this was only significant at the highest dose. We found that acrolein cleaved procaspase-9 as a function of increasing concentration from 2 to 50 *f*mol/cell after a 1 h incubation (Fig. 4B, 4C). This was apparent as a decrease in the intensity of bands (Fig. 4B) for the pro-enzyme form of caspase-9 (Fig. 4C). There was a corresponding increase in the quantity of cleavage fragment of 35 kDa for caspase-9 (Fig. 4B, 4D), with increasing acrolein concentration.

We subsequently determined the ability of acrolein to activate effector caspases. However, the enzymatic activity of the major downstream effector caspase-3 was inhibited by low concentrations of acrolein (5 to 20 *f*mol/cell) (Fig. 5A). Caspase-3 activity was rapidly inhibited after exposure to acrolein for only 5 min

(Fig. 5A). Indeed, exposure of cells to 20 *f*mol/cell of acrolein for 1 h completely inhibited caspase-3 activity (Fig. 5A). Although caspase-3 activity was inhibited, procaspase-3 was cleaved (Fig. 5B, 5C) to its two fragments p11 and p17 (Fig. 5B, 5D). Cleavage of pro-caspase-3 is necessary for the formation of the active dimeric form of caspase-3 [41].

Since effector caspase-3 was not activated, we determined whether other downstream caspases could be activated, in order to explain the acrolein-induced chromatin condensation (Fig. 2C). We evaluated the effect of acrolein on activity of caspase-7, which is a downstream caspase that can cleave PARP. Thus, 10 *f*mol/cell of acrolein, which inhibited caspase-3 after 5 min of exposure (Fig. 5A), activated caspase-7 after 2 h (Fig. 6A). Similar to caspase-3, cleavage of pro-caspase-7 is necessary for the formation of the active dimeric form of caspase-7 [41]. Indeed, cleavage of procaspase-7 (Fig. 6B, 6C) to its active fragment p20 (Fig. 6B, 6D) was induced after 1 h, by 10 to 50 *f*mol/cell of acrolein. Higher concentrations of acrolein (≥100 *f*mol/cell) were needed to activate caspase-7 after 1 h, whereas lower concentrations (10 and 50 *f*mol/cell) activated the enzyme after 2 h of acrolein exposure (Fig. 6A). Higher doses of acrolein (>100 *f*mol/cell) did not activate caspase-7 after 2 h (Fig. 6A).

The subsequent step was to confirm the implication of caspase-9 in acrolein induced apoptosis. Therefore, the ability of a general inhibitor of caspases and of specific inhibitors of caspases-3 and -9 to inhibit apoptosis induced by acrolein was evaluated. The level of apoptosis was partially inhibited by the general inhibitor of caspases (Fig. 7A). The inhibitor of caspase-9 decreased cell death by apoptosis by about 70% (Fig. 7A). However, the inhibitor of caspase-3 had no effect on apoptosis induced by acrolein (Fig. 7B). Hydrogen peroxydewas used as a positive control to show that the inhibitor of caspase-3 was able to inhibit peroxide-induced apoptosis (Fig. 7C).

Acrolein (≥50 *f*mol/cell) induced cleavage of the caspase substrate ICAD, the inhibitor of caspase activated DNase (CAD), after 2 h (Fig. 8), thereby liberating CAD which can cause DNA fragmentation in the nucleus.

DISCUSSION

The new finding of the present study is that acrolein is capable of inducing the mitochondrial pathway of apoptosis involving liberation of cytochrome c and the activation of certain caspases in proliferating cells. We provide new information on the molecular mechanisms by which acrolein induces apoptosis. Induction of apoptosis by acrolein was confirmed morphologically by the condensation of nuclear chromatin after 4h, a later event in the apoptotic cascade, as well as by several earlier, upstream events associated with apoptosis at the mitochondrial and post-mitochondrial levels. At the mitochondrial level, acrolein caused a decrease in membrane potential, followed by the liberation of cytochrome-c into the cytosol, after 1h. At the post-mitochondrial level, cytochrome c is an essential component of the cytosolic apoptosome complex, along with Apaf1 and pro-caspase-9. Acrolein indeed induced the activation of initiator caspase-9 after 1h and the effector caspase-7 after 1 to 2h. Caspase activation was followed by cleavage of ICAD after 2h. This leads to the liberation of the protease CAD, which can cause later events such as chromatin condensation and fragmentation of DNA in the nucleus.

Activation of these caspases was confirmed by the cleavage of their pro-enzyme forms and the generation of appropriate cleavage fragments, as well as by the increase in their enzymatic activities. The role of caspase-9 was also confirmed using a specific caspase-9 inhibitor. At present, there is no specific inhibitor of caspase-7 available commercially.

The implication of the PTP and cytochrome-c release in acrolein induced apoptosis was assessed using cyclosporine A. Although the mechanisms of cytochrome c release are not completely understood, it is considered that cytochrome c can be released from mitochondria via the PTP as well as by formation of channels on the membrane involving Bax [42]. The inhibition of apoptosis by cyclosporine A supports a role for PTP opening and release of cytochrome c in acrolein-induced

apoptosis. The partial inhibition by cyclosporine A suggests that acrolein could also induce apoptosis by alternative mechanisms to the mitochondrial pathway and/or that cytochrome c can be released by several different mechanisms.

Our findings show that caspase-3 is not essential for the execution phase of acrolein-induced apoptosis in proliferating cells. This was demonstrated by the lack of activation of its enzymatic activity and the lack of inhibition of nuclear chromatin condensation by a specific inhibitor of caspase-3. It was reported that MCF-7 cells, which lack caspase-3, can still undergo apoptosis [41]. Indeed, acrolein-induced apoptosis in CHO cells appears to be mediated through activation of the effector caspase-7. In addition to caspase-3, caspase-9 is able to directly cleave pro-caspase-7, leading to subsequent activation of caspase-7 [43]. In our study, cleavage of ICAD is likely to be mediated by caspase-7 [44]. It is often difficult to distinguish between the involvement of caspase-3 and -7 in apoptosis since they share similar protein (PARP, ICAD) [19] and peptide (Ac-DEVD-AMC) substrates [45].

A surprising finding in our study was that pro-caspase-3 was indeed cleaved in cells, yet the enzymatic activity of caspase-3 was markedly inhibited by acrolein. The cleavage of pro-caspase-3 is likely to result from the proteolytic action of the active caspase-9 [46]. Even though pro-caspase-3 undergoes cleavage to generate caspase-3 in cells, enzymatic activity appears to be immediately inhibited. This could arise through direct alkylation of its active site cysteine residue by acrolein. The inhibition of caspase-3 by acrolein is in agreement with other studies. Inhibition of caspase-3 activity occurred in neutrophils after 2 to 8 h of treatment with 10 μM acrolein [23]. Activities of caspases-3, -8 and -9 were inhibited 12 h following a 30 min exposure to acrolein (5 to 40 μM) in murine proB lymphocytes [24]. For each of the caspases-3, -7 and -9, cleavage of the pro-enzyme form into cleavage fragments occurred in CHO cells. However, all of the caspase enzymes contain a nucleophilic active-site cysteine residue and potentially they could all be inhibited by acrolein [23, 47]. Acrolein appears to be causing some activation of caspases-7 and -9, while at the

same time inhibiting their activity. It appears that caspases-7 and -9 are not resistant to inactivation by acrolein, but rather, they may be less susceptible to inactivation than caspase-3. Although acrolein appears to eventually inhibit enzymatic activity of each of these 3 caspases, caspases-9 and -7 were sufficiently activated to trigger caspase-dependent downstream events such the ICAD cleavage and chromatin condensation. The fact that acrolein completely inhibits caspase-3, whereas caspases-7 and -9 undergo some level of activation in CHO cells could also be explained by more restricted access of acrolein to the active site cysteine residues of caspases-7 and -9. It was reported that the major structural differences among the caspases occur at the active sites, whereas the rest of the structures are very similar. These structural differences at the active sites could affect access to the active site as well as interactions with other molecules and could explain the differences in the substrate specificities among the different caspases [41].

It has recently been reported that proteolytic cleavage is neither required nor sufficient for activation of monomeric initiator caspases such as caspase-9 [48]. Dimerization, rather than cleavage, appears to be the most important step for caspase-9 activation and formation of an active site. However, cleavage of the prodomain stabilises the active form of caspase-9. There are several similarities between activation of caspases-7 and –9. Interestingly, the caspase-9 dimer only contains one active site. The catalytic apparatus in the other domain is disabled due to steric clashes. This inactive domain is almost identical to that of the zymogen form of caspase-7, indicating that some structural similarities exist between caspases-7 and -9. Also, identical conformational changes leading to formation of the active sites was reported for caspases-7 and –9, even though they are activated by different mechanisms: proteolysis versus dimerisation, respectively [48]. However, interdomain cleavage by initiator caspases is needed for activation of executioners such as caspases-3 and –7 [48].

In general, different studies have reported variable findings in that acrolein can either cause or inhibit apoptosis, or cause predominantly necrosis rather than apoptosis. For example, acrolein (25 μM) caused apoptosis in isolated human alveolar macrophages, detected by morphological changes and DNA fragmentation after 24 h [20]. Exposure to 50 μM acrolein for 24 h induced atypical apoptosis in primary cultures of human keratinocytes [21]. Acrolein stimulated apoptosis in the human lung epithelial cell line HBE1, as indicated by externalisation of phosphatidylserine and DNA fragmentation 24 h after a 30 min exposure to 10 to 25 μM acrolein [22]. In human neutrophils, however, acrolein (25 μM) inhibited the constitutive pathway of apoptosis [23]. In proB lymphoid cells, the cell death pathway was predominantly oncosis/necrosis, with only low levels of apoptosis occurring at lower doses (<10 μM) [24]. These variable results could be due to differences in biochemical factors determining cell death pathways in different cell types, which include non-proliferating primary cell cultures and cells involved in immune responses, as well as proliferating cancer cell lines. Furthermore, different methodologies were used in these studies, such as longer exposures to acrolein for 24 h or 48 h versus shorter exposures to acrolein for 30 to 60 min, with or without a 24 h-recovery period. In some cases, including the present work, cells were exposed to acrolein in medium containing serum [20], whereas other studies exposed cells in serum-free medium [21, 22, 23, 24]. Acrolein can bind to serum proteins, which would likely decrease the concentration available to interact with cells. This may slow or modify the onset of toxicity leading to apoptosis, rather than necrosis. Necrosis appeared to be the predominant form of cell death when cells were exposed to acrolein in serum-free medium [24]. In our study acrolein did induce necrosis, but at higher doses than those which caused apoptosis.

One of our interests in exploring mechanisms of acrolein toxicity in proliferating cells lies in the context of anticancer treatment. We reported that acrolein and H_2O_2 both contributed to cytotoxicity induced by spermine and bovine

serum amine oxidase (BSAO, EC 1.4.3.6) in CHO cells [49]. Since tumor tissues contain elevated levels of polyamines, targeting polyamines could be a promising therapeutic strategy [28]. Toxic products such as H_2O_2 and acrolein could be generated *in situ* by amine oxidases for the selective killing of tumor cells [49]. Furthermore, the enzyme could also act by depleting polyamines which are necessary for tumor growth. BSAO has been successfully immobilized in biocompatible polyethylene glycol hydrogels, which could be useful for *in vivo* stability and delivery of the enzyme to tumors [50].

In conclusion, this study clearly demonstrates that acrolein can cause cell death by both apoptosis and necrosis. A small elevation in acrolein concentration switched the mechanism of cell death from apoptosis to necrosis. Acrolein induced apoptosis through the mitochondrial pathway, involving cytochrome c release from mitochondria and activation of caspases. However, this does not rule out the possibility that acrolein could induce apoptosis by alternative or complementary mechanisms. Apoptosis could also occur via death receptor pathways without the involvement of cytochrome c. Furthermore, a cross-talk pathway exists between the death receptor and mitochondrial pathways, which also leads to cytochrome c release. Future studies will investigate the role of death receptor and cross-talk pathways in acrolein-induced apoptosis. The present findings may be important in explaining the pharmacological action and/or toxic side effects of cyclophosphamide, which has acrolein as one of its metabolites, as well as the toxicity of environmental exposures to low doses of acrolein in proliferating cells.

Figure 1: Induction of cytotoxicity by acrolein

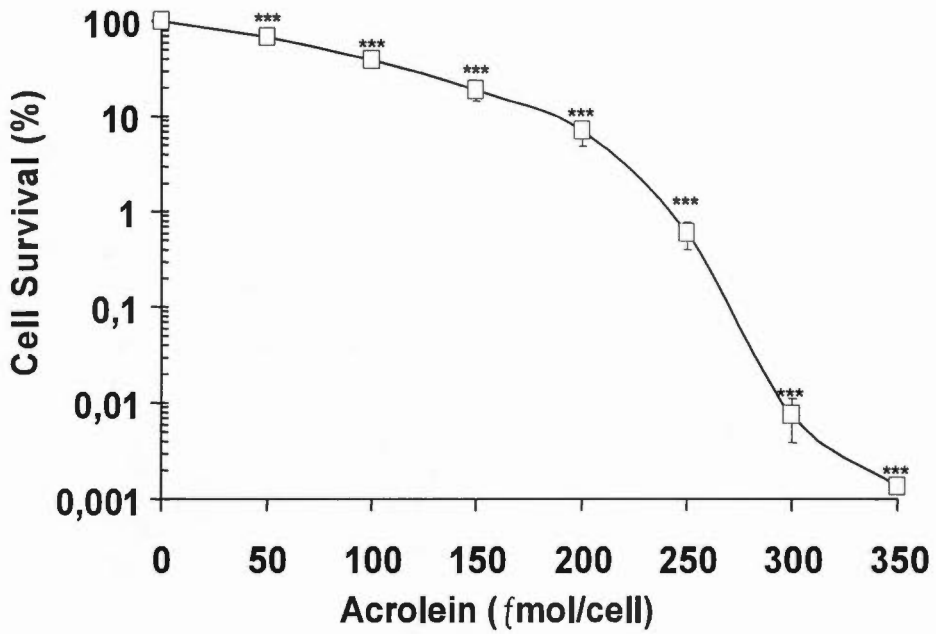


Figure 2: Morphological analysis of apoptosis and necrosis in cells following exposure to acrolein

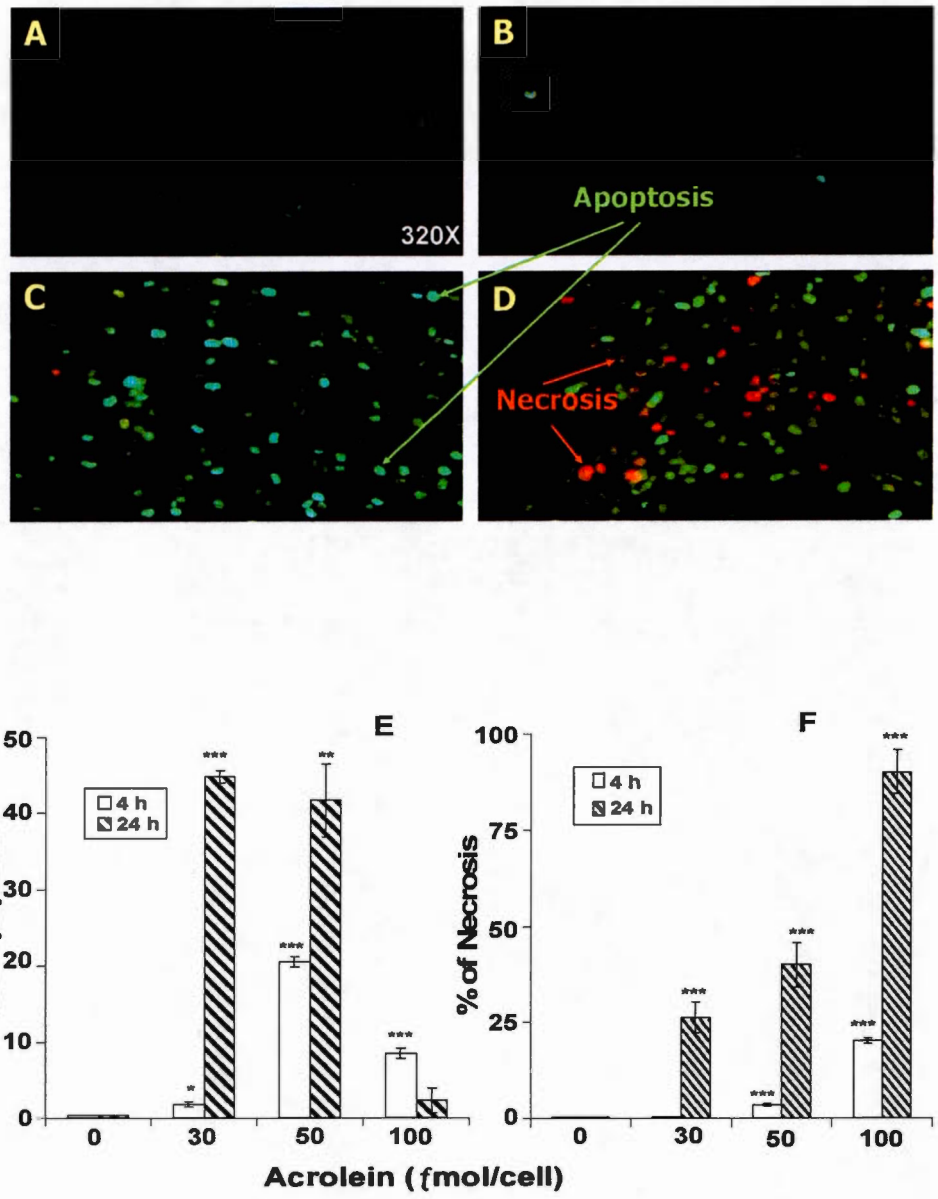
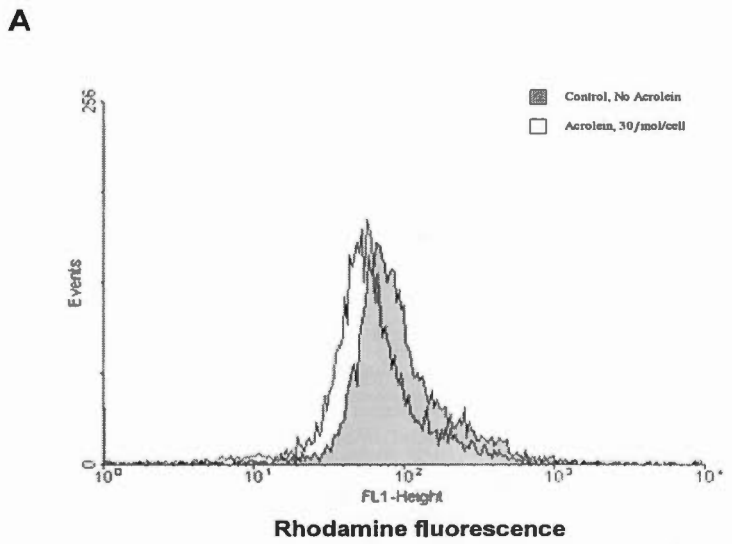
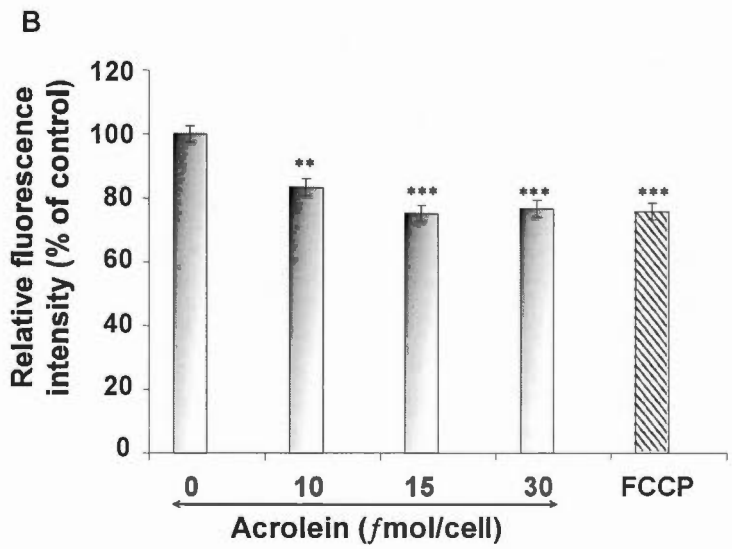
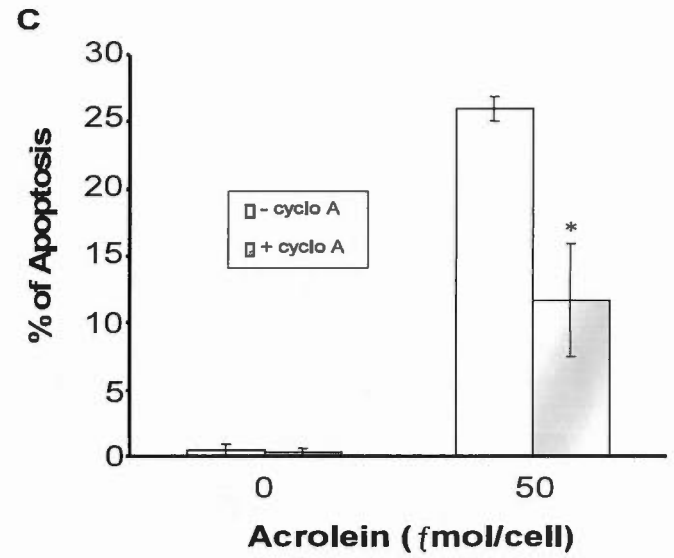


Figure 3: Acrolein induces depolarization of the mitochondrial membrane and liberation of cytochrome-c






C
30
25
20
15
10
5
0
% of Apoptosis
- cyclo A
+ cyclo A
*
0
50
Acrolein (fmol/cell)

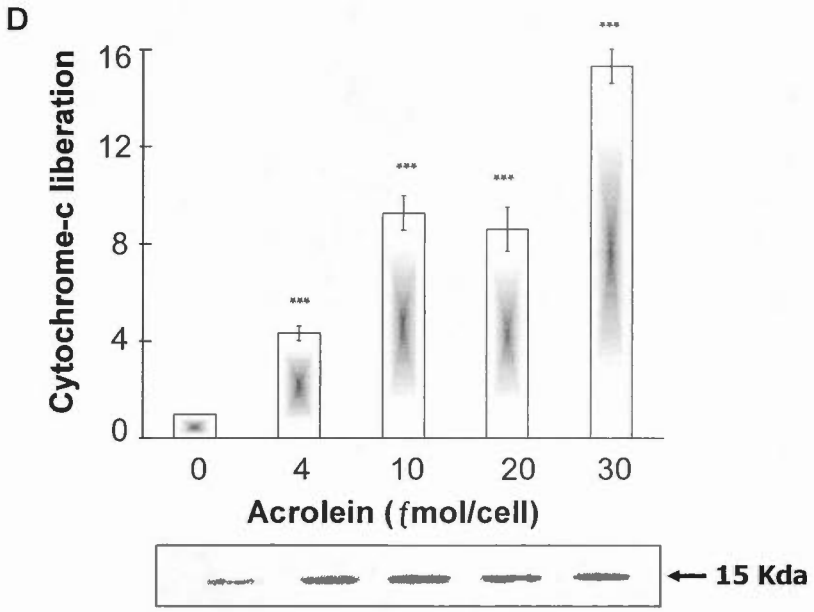
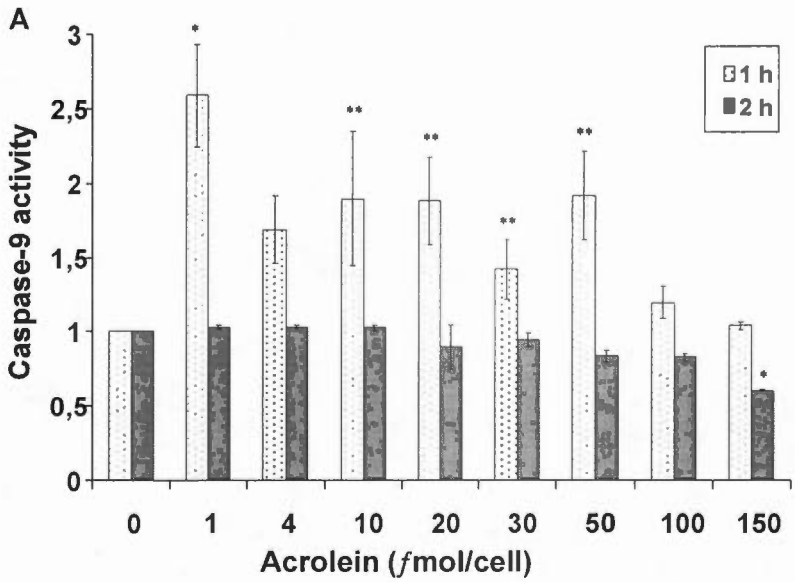
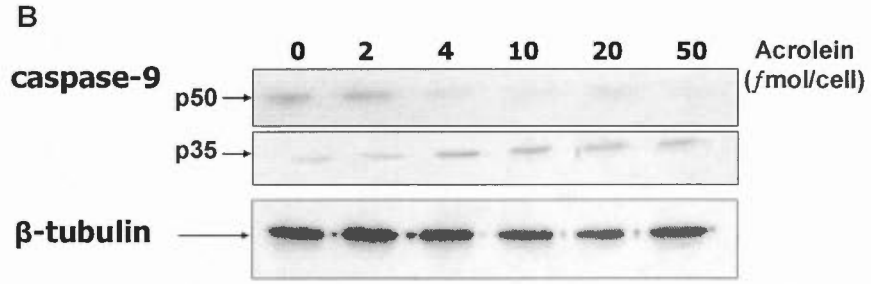

D
16
12
8
4
0
Cytochrome-c liberation
***
***
***
***
0
4
10
20
30
Acrolein (fmol/cell)
15 Kda

Figure 4: Activation of initiator caspase-9 and cleavage of procaspase-9 by acrolein




B
0
2
4
10
20
50
Acrolein
(fmol/cell)
caspase-9
p50
p35
β-tubulin

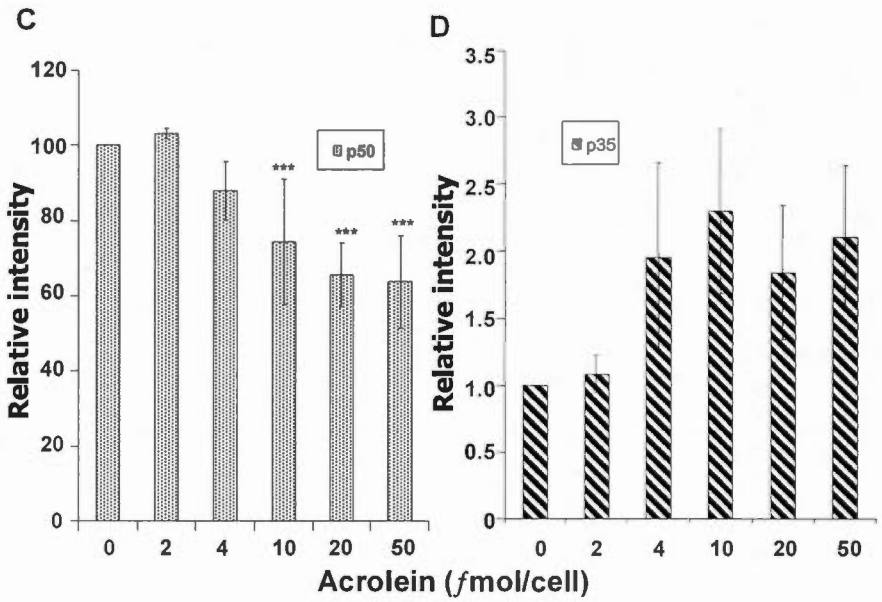
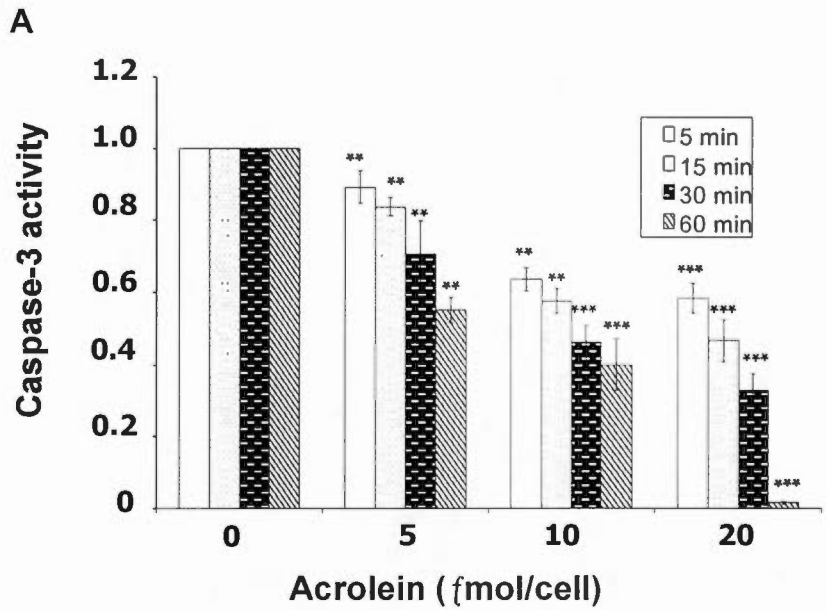
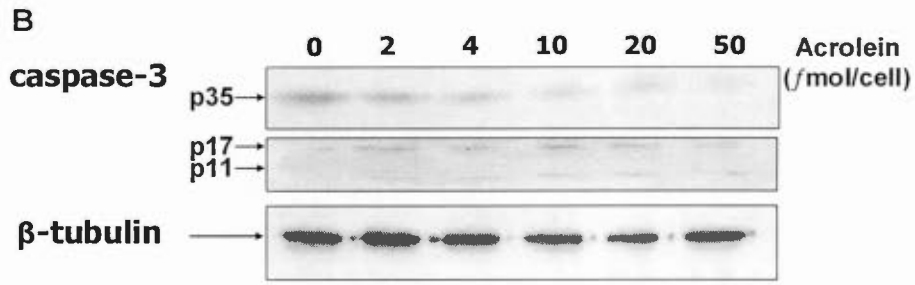

C
D
Relative intensity
p50
p35
***
0
2
4
10
20
50
Acrolein (fmol/cell)

Figure 5: Acrolein cleaves procaspase-3 but inhibits enzymatic activity of caspase-3




B
0
2
4
10
20
50
Acrolein
(fmol/cell)
caspase-3
p35
p17
p11
β-tubulin

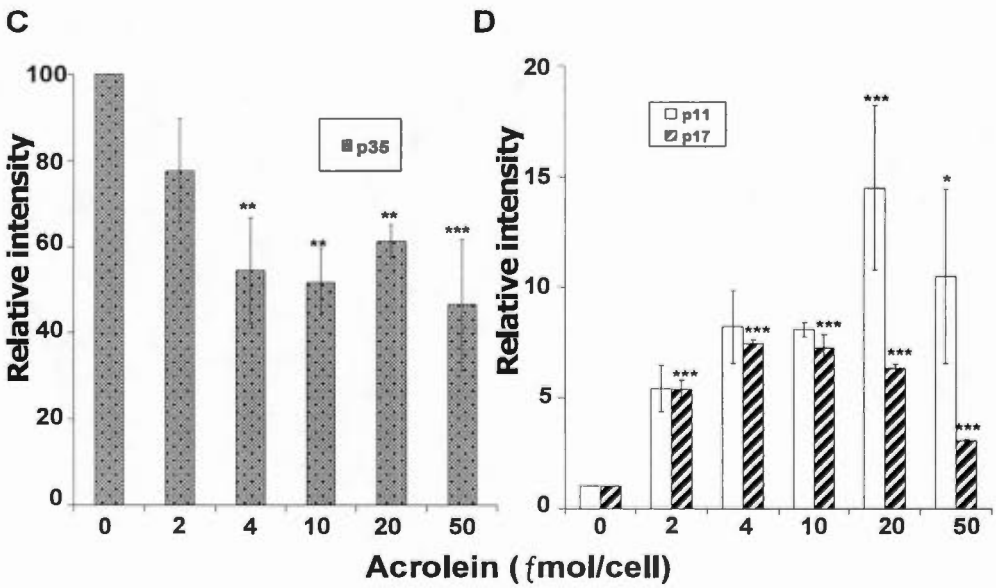
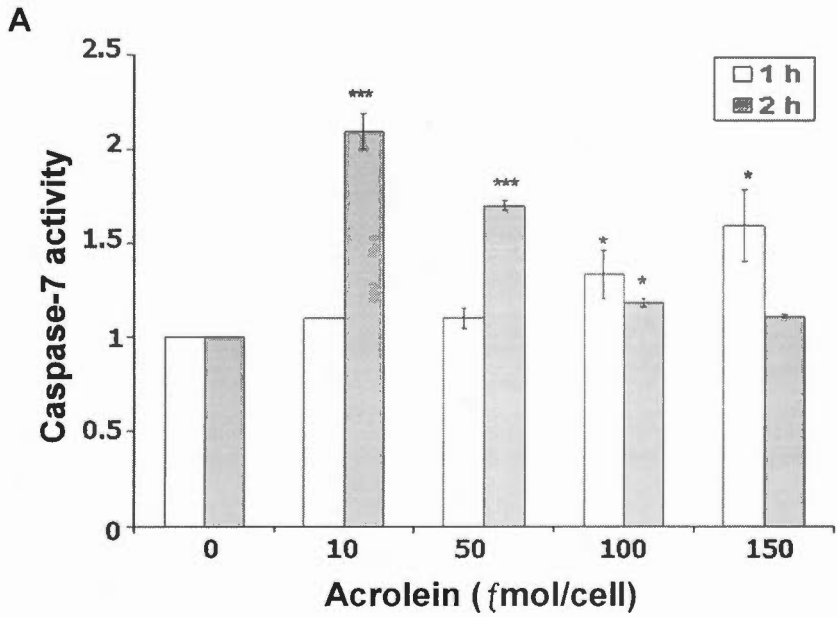
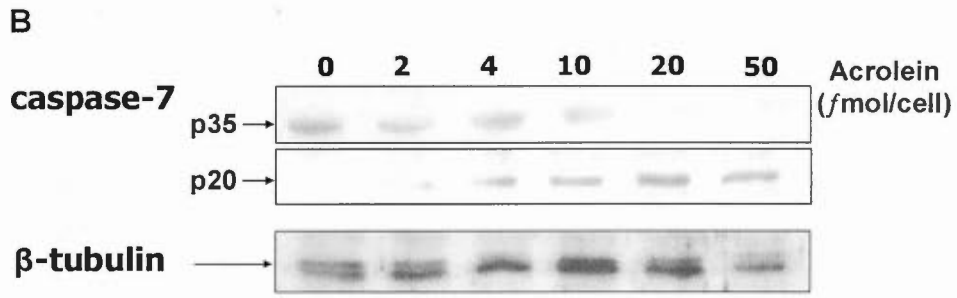

C
D
Relative intensity
p35
p11
p17
Acrolein (fmol/cell)

Figure 6: Acrolein activates caspase-7 and cleaves procaspase-7




B
0
2
4
10
20
50
Acrolein
(fmol/cell)
caspase-7
p35
p20
β-tubulin

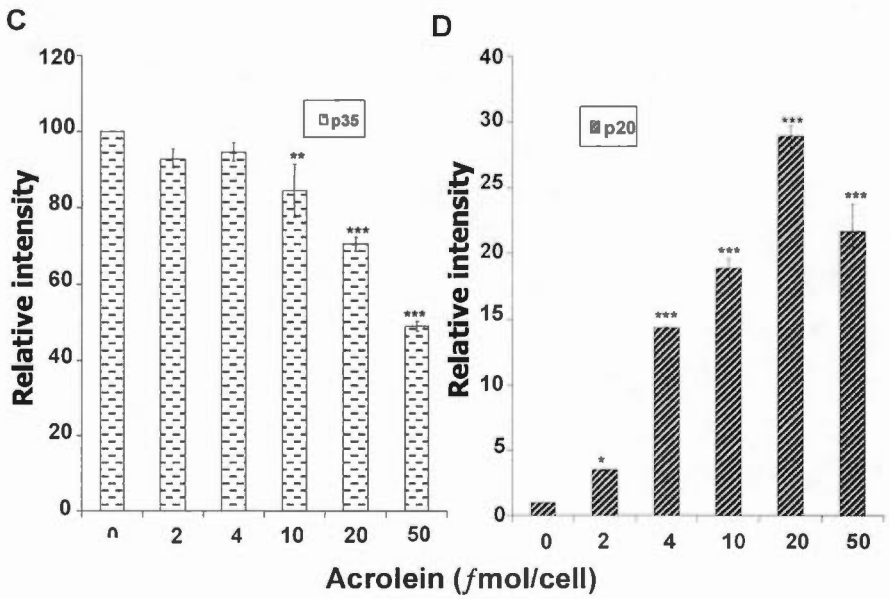

C
D
Relative intensity
p35
p20
0
2
4
10
20
50
Acrolein (fmol/cell)

Figure 7: Inhibition of apoptosis induced by acrolein by a specific inhibitor of caspase-9

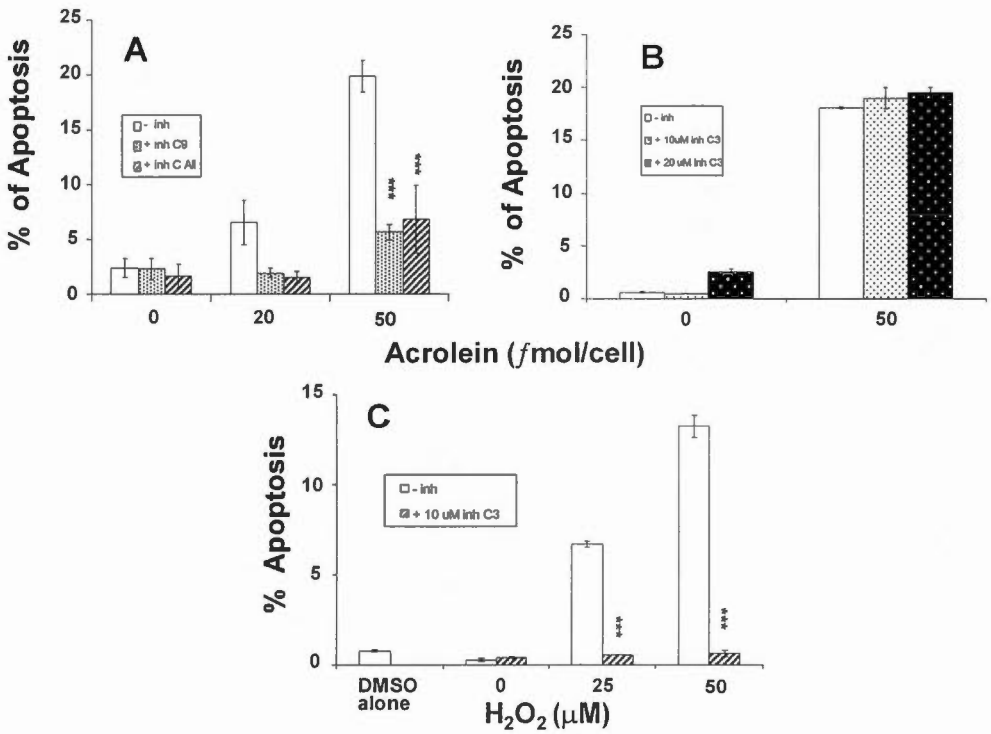


Figure 8: Acrolein causes cleavage of ICAD

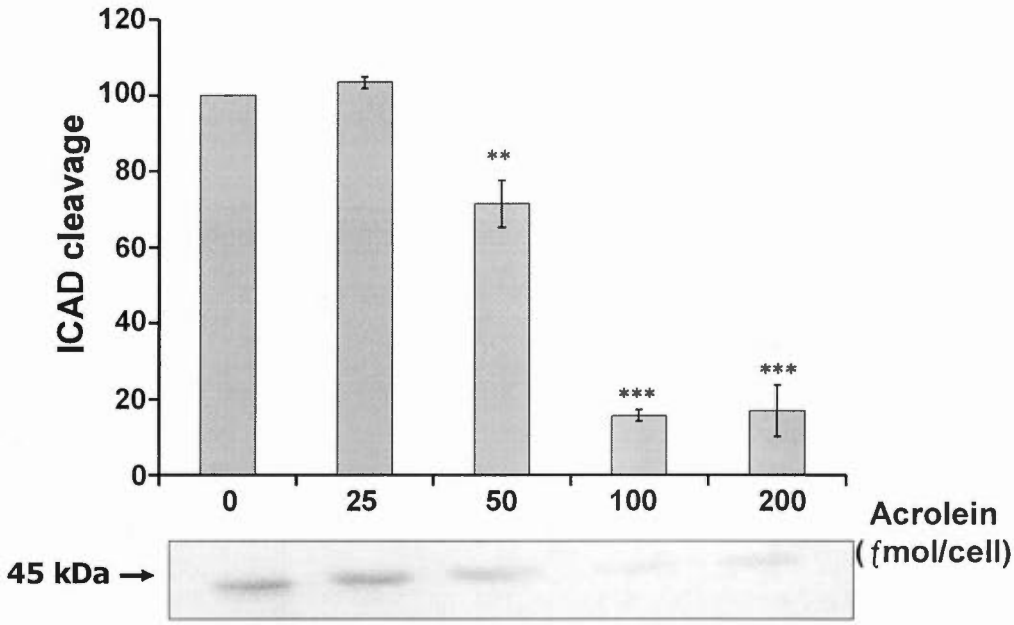


FIGURE LEGENDS

Fig. 1. Induction of cytotoxicity by acrolein. Cytotoxicity was assessed by a clonogenic cell survival assay. CHO cells (10^5/mL) were exposed to acrolein (50 to 350 *f*mol/cell) for 1 h at 37^0C in 1 mL of α-MEM containing 10% FBS. Acrolein was then removed and cells were incubated in culture dishes for 8 days to allow the formation of macroscopic colonies (see Methods). The control value represents 10^5 cells and this was normalized to represent 100% cell survival. There is a significant difference between cells treated with acrolein, relative to untreated controls, $p < 0.001$ (***). Data represent means and SEM from four independent experiments performed with multiple estimations per point. When not shown, error bars lie within the symbols.

Fig. 2. Morphological analysis of apoptosis and necrosis in cells following exposure to acrolein. Cells ($0.3x10^6$) were seeded and cultured for two days to near confluence in tissue culture dishes containing α-MEM and 10% FBS at 37^0C. Cells were incubated with different concentrations of acrolein: A) 0, B) 30, C) 50 and D) 100 *f*mol/cell, for 4 h. Cells were stained with Hoechst and PI and visualised by fluorescence microscopy (magnification 320x). The fractions of (E) apoptotic and (F) necrotic cells following treatment with acrolein for 4 h and 24 h are given relative to total cells. A minimum of 600 cells were counted per dish. Data represent means and SEM from four or five independent experiments performed with multiple estimations per point. $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 3. Acrolein induces depolarization of the mitochondrial membrane and liberation of cytochrome-c. (A) CHO cells (10^6/mL) were incubated with acrolein (30 *f*mol/cell) (grey) for 1 h at 37^0C in α-MEM and 10% FBS, relative to untreated control cells (white), and then analysed by flow cytometry for rhodamine 123 (800ng/mL) fluorescence in channel FL1. (B) Data represent means and SEM of relative fluorescence intensity of rhodamine 123 from seven independent experiments. $p < 0.01$ (**) or $p < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein or FCCP and the control. The absolute data value for the untreated control cells from 7 experiments was 39 ± 3 (mean ± SEM) relative fluorescence units. The control value was designated as 100% and other data values in FCCP- and acrolein-treated cells were normalised to this point. (C) Cells were incubated with acrolein, with or without 5 μM cyclosporine A, for 4 h, and then stained with Hoechst and PI. A minimum of 600 cells were counted per dish. Data represent means and SEM from four independent experiments performed with multiple estimations per point. $p < 0.05$ (*) indicates a statistically significant difference between treatment with acrolein with or without cyclosporine A. (D) Cells (10^6 /mL) were incubated with acrolein (4 to 30 *f*mol/cell) for 1 h in α-MEM containing 10% FBS. Immunodetection of cytochrome-c was carried out by SDS-PAGE, using β–tubulin as a loading control (not shown). A representative gel is shown from four independent experiments. Expression of cytochrome-c was relative to the untreated control, designated as 1. Data represent means and SEM for gels from four independent experiments. $p < 0.001$(***) indicates a statistically significant difference between acrolein treatment and control.

Fig. 4. Activation of initiator caspase-9 and cleavage of procaspase-9 by acrolein. (A) CHO cells ($0.5x10^6$ /mL) were incubated with acrolein (1 to 150 *f*mol/cell) for 1 or 2 h in α-MEM with 10% FBS. Caspase-9 activity was measured in cell lysates using the fluorescent substrate Ac-LEHD-AFC. Caspase-9 activity was expressed

relative to the untreated control, designated as 1. Data represent means and SEM from eight independent experiments performed with multiple estimations per point. $p < 0.05$ (*) or $p < 0.01$ (**) indicates a statistically significant difference between acrolein treatment and the control. (B) Cells (10^6 /mL) were incubated with acrolein (2 to 50 *f*mol/cell) for 1 h in α-MEM containing 10% FBS. Immunodetection of procaspase-9 and its cleavage fragment (35 kDa) was carried out by SDS-PAGE, using β–tubulin as a loading control. A representative gel is shown from four independent experiments. Densitometric analysis of expression of (C) procaspase-9 and (D) the cleavage fragment are relative to the untreated control. $p < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 5. Acrolein cleaves procaspase-3 but inhibits enzymatic activity of caspase-3. (A) CHO cells ($0.5x10^6$ /mL) were incubated with acrolein (5 to 20 *f*mol/cell) for 5, 15, 30 and 60 min in α-MEM containing 10% FBS. Caspase-3 activity was measured in cell lysates using the fluorescent substrate Ac-DEVD-AMC. Caspase-3 activity was expressed relative to the untreated control, designated as 1. Data represent means and SEM from seven independent experiments performed with multiple estimations per point. $p < 0.01$ (**) or $p < 0.001$ (***) indicates a statistically significant difference between acrolein treatment and the control. (B) Cells (10^6 /mL) were incubated with acrolein (2 to 50 *f*mol/cell) for 1 h in α-MEM containing 10% FBS. Immunodetection of procaspase-3 and its cleavage fragments (11 and 17 kDa) was carried out by SDS-PAGE, using β–tubulin as a loading control. A representative gel is shown from four independent experiments. Densitometric analysis of expression of (C) procaspase-3 and (D) the cleavage fragments are relative to the untreated control. $p < 0.05$ (*), $p < 0.005$ (**) or $p < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 6. Acrolein activates caspase-7 and cleaves procaspase-7. (A) CHO cells ($0.5x10^6$ /mL) were incubated with acrolein (10 to 150 *f*mol/cell) for 1 and 2 h in α-MEM containing 10% FBS. Caspase-7 activity was measured in cell lysates using the fluorescent substrate MCA-VDQVDGWK-(DNP)-NH2. Caspase-7 activity was expressed relative to the untreated control, designated as 1. Data represent means and SEM from seven independent experiments performed with multiple estimations per point. $p < 0.05$ (*) or $p < 0.001$ (***) indicates a statistically significant difference between acrolein treatment and the control. (B) Cells (10^6 /mL) were incubated with acrolein (2 to 50 *f*mol/cell) for 1 h in α-MEM containing 10% FBS. Immunodetection of procaspase-7 and its cleavage fragment (20 kDa) was carried out by SDS-PAGE, using β–tubulin as a loading control. A representative gel is shown from four independent experiments. Densitometric analysis of expression of (C) procaspase-7 and (D) the cleavage fragment are relative to the untreated control. $p < 0.05$ (*), $p < 0.005$ (**) or $p < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 7. Inhibition of apoptosis induced by acrolein by a specific inhibitor of caspase-9. Treatment with (A) 10 μM of caspase-9 inhibitor I, Z-LEHD-FMK, 10 μM of general caspase inhibitor I, Z-VAD-FMK, (B) 10 or 20 μM of caspase-3 inhibitor V, Z-DQMD-FMK and (C) 10 μM of caspase-3 inhibitor V, Z-DQMD-FMK (Calbiochem, La Jolla, CA) was performed on confluent cells in monolayer. Cells were incubated with (A, B) acrolein (20 and 50 *f*mol/cell) or (C) H_2O_2 (25 and 50 μM) for 4 h with inhibitors present. The fraction of apoptotic cells (Hoechst) is given relative to total cells. A minimum of 600 cells was counted per dish. Data represent means and SEM from five independent experiments performed with multiple estimations per point. (A, C) $p < 0.001$ (***) indicates a statistically significant difference between cells exposed to acrolein with and without caspase inhibitors.

Fig. 8. Acrolein causes cleavage of ICAD. CHO cells (10^6 /mL) were incubated with acrolein (25 to 200 *f*mol/cell) for 1 h in α-MEM containing 10% FBS. Immunodetection of ICAD was carried out using SDS-PAGE. Expression of ICAD was relative to the untreated control, designated as 100%. Data represent means and SEM from four independent experiments performed with multiple estimations per point. $p < 0.01$ (**) or $p < 0.001$(***) indicates a statistically significant difference between acrolein treatment and the control.

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2.3. ARTICLE II

ACTIVATION OF THE DEATH RECEPTOR PATHWAY OF APOPTOSIS BY THE ALDEHYDE ACROLEIN

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Dedication: This paper is dedicated to the memory of Professor Francine Beaudoin-Denizeau, and to her endless energy and enthusiasm in the pursuit of scientific excellence in biochemistry and environmental toxicology (March 25, 2004).

Abbreviations: AFC: amino trifluorocoumarin; AMC: amino methylcoumarin; BSA: bovine serum albumin; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CHO: Chinese hamster ovary; DISC: death-inducing signalling complex; FADD: Fas associating protein with death domain; Fas: fibroblast-associated; FasL: Fas ligand; FasR: Fas receptor, FBS: fetal bovine serum; 4-HNE: 4-hydroxynonenal; ICAD: inhibitor of caspase activated DNase; MEM: minimum essential medium; MOPS: 3-(N-morpholino)-propane sulfonic acid; PARP: polyADP-ribose polymerase; PBS: phosphate-buffered saline; PMSF: phenylmethylsufonyl fluoride; PS: phosphatidylserine; PVDF: polyvinylidene difluoride; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM: standard error of mean. TNF-R: tumor necrosis factor receptor.

Keywords: Acrolein, death receptor, apoptosis, caspase

RÉSUMÉ

Les aldéhydes hyperactifs comme l'acroléine sont des composés polluants majeurs de l'environnement et y constituent un grand risque. L'acroléine est un produit de peroxidation lipidique impliquée dans plusieurs pathophysiologies incluant les maladies neurodégénératives et respiratoires. Bien que l'acroléine induit l'apoptose dans plusieurs types cellulaires, les mécanismes biochimiques impliqués demeurent inconnus. Cette étude explore la voie des récepteurs de mort dans l'induction de l'apoptose par l'acroléine. L'exposition des cellules ovariennes de hamster chinois à l'acroléine a entraîné la translocation de l'adapteur de domaine de mort de Fas (FADD) à la membrane plasmique et l'activation de la caspase-8 initiatrice. Kp7-6, un antagoniste du récepteur Fas, a bloqué les évènements apoptotiques en aval de la caspase-8, comme l'activation de la caspase-7 exécutrice et la condensation de la chromatine nucléaire. Acroléine active la voie de *crosstalk* entre la voie de signalisation des récepteurs de mort et celle de la mitochondrie en clivant la protéine Bid en sa forme tronquée t-bid qui s'est transloqué à la membrane mitochondriale pour stimuler cette voie. L'inhibition spécifique du récepteur Fas ou de la caspase-8 a inhibé partiellement l'activation de la caspase-9 par l'acroléine. Ces résultats démontrent que l'acroléine active la voie du récepteur Fas en amont de la voie mitochondriale. La caspase-9 est demeurée active malgré l'inhibition du récepteur Fas et de la caspase-8 suggérant que l'acroléine peut induire la voie mitochondriale indépendamment de la voie des récepteurs. Ces résultats nous emmènent à mieux comprendre le mécanisme de toxicité de l'acroléine, un polluant omniprésent de notre environnement.

ABSTRACT

Reactive alpha,beta-unsaturated aldehydes such as acrolein are major components of common environmental pollutants. As a toxic by-product of lipid peroxidation, acrolein has been implicated as a possible mediator of oxidative damage to cells and tissues in a wide variety of disease states, including atherosclerosis, neurodegenerative and pulmonary diseases. Although acrolein can induce apoptotic cell death in various cell types, the biochemical mechanisms are not understood. This study investigates the implication of the death receptor pathway in acrolein-induced apoptosis. Exposure of Chinese hamster ovary cells to acrolein caused translocation of adaptor protein Fas–associated with death domain to the cytoplasmic membrane and caspase-8 activation. Kp7-6, an antagonist of Fas receptor activation, blocked apoptotic events downstream of caspase-8, such as caspase-7 activation and nuclear chromatin condensation. Acrolein activated the crosstalk pathway between the death receptor and mitochondrial pathways. Bid was cleaved to truncated-bid, which was translocated to mitochondria. Activation of the mitochondrial pathway by acrolein was confirmed by caspase-9 activation. Inhibition of activation of either the Fas receptor or caspase-8 partially decreased acrolein-induced caspase-9 activation. These findings indicate that acrolein activates the Fas receptor pathway, which occurs upstream from the mitochondrial pathway. Caspase-9 activation still occurred despite inhibition of the Fas receptor pathway, suggesting that acrolein could trigger the mitochondrial pathway independently of the receptor pathway. These findings improve our understanding of mechanisms of toxicity of the reactive aldehyde acrolein, which has widespread implications in multiple disease states which appear to be mediated by oxidative stress and lipid peroxidation.

Keywords: Acrolein, death receptor, apoptosis, caspase, mitochondria

INTRODUCTION

Acrolein is a highly reactive, α,β-unsaturated aldehyde and humans are exposed to this compound in multiple contexts [1]. It is a ubiquitous environmental pollutant which is present in food. Acrolein is found in the vapours of overheated cooking oil and severe human toxic exposures have been reported [2]. It is used industrially as a starting material for acrylate polymers and in the production of acrylic acid, and as a herbicide [3]. Acrolein is also a metabolic product of the widely used anticancer drug cyclophosphamide and has been implicated in its toxic side effects [4].

As well as being a ubiquitous environmental pollutant, acrolein is one of the toxic aldehyde by-products of endogenous lipid peroxidation, together with 4-hydroxy-2-nonenal [5; 6]. Lipid peroxidation is a deleterious chain reaction occurring mainly in biological membranes, resulting from oxidative stress. Acrolein also reacts with glutathione [7; 8]. In fact, acrolein causes more rapid and severe depletion of this important cellular antioxidant when compared to the well-known oxidant H_2O_2 [9]. Furthermore, acrolein and its glutathione adduct, glutathionylpropionaldehyde, were shown to cause formation of oxygen radicals [10], which may be responsible for induction of lipid peroxidation by acrolein. Together, these studies show that acrolein causes an oxidative redox imbalance.

Oxidative stress and lipid peroxidation have been implicated in a variety of human disease states. Since acrolein is an exogenous product of lipid peroxidation and can itself induce lipid peroxidation, this reactive aldehyde may have a possible role as a mediator of oxidative damage to cells and tissues. Indeed, acrolein has been implicated in the development of multiple disease states involving oxidative stress, such as atherosclerosis [6; 11], various lung diseases including chronic obstructive pulmonary disease (COPD) [12], colon carcinogenesis [13], diabetic nephropathy [14], head trauma during aging [15] and Alzheimer's disease [16]. Acrolein has been

used as a biomarker to evaluate oxidative stress-induced damage to retina [17] and to erythrocytes [18], as well as in Alzheimer's disease [19; 20].

Acrolein is a component of smoke and is generated during forest and house fires and is a constituent of automobile exhaust. It is a major component of cigarette smoke and appears to contribute to its toxicity [21; 3]. Acrolein may contribute to cigarette smoke-induced inflammatory processes in the lung by increasing neutrophil recruitment and reducing neutrophil clearance by apoptosis [21]. It increases the expression of mucin gene transcripts (MUC5AC and MUC5B), which leads to excessive mucus secretion [22; 23], a hallmark in the pathogenesis of several airway diseases including COPD [24], asthma and cystic fibrosis [25].

Apoptosis is a normal physiological process that plays an essential role in development and the maintenance of homeostasis in multicellular organisms [26; 27]. A balance between cell death and cell proliferation is required to maintain a cellular homeostatic state. Deviation from this cellular balance disrupts the normal state and can lead to human disease [28; 29]. Apoptosis has many important repercussions in human health. Cell accumulation via insufficient apoptosis can contribute to conditions such as cancer, inflammation and autoimmune disease. In contrast, excessive apoptosis can play an important role in neurodegeneration, AIDS, eye disorders, osteoporosis and heart failure [30; 31]. Apoptotic cell death has been implicated in the mechanisms of toxicity of different chemicals and environmental pollutants [32], such as the aquatic toxin tributyltin [33; 34] and the organochlorine pesticide heptachlor [35], which have important implications in human health as well as that of other species.

Two of the major pathways of apoptosis are the death receptor and mitochondrial pathways. There are several death receptors located at the cytoplasmic membrane of cells, which are involved in the induction of apoptosis, such as Fas (CD95, APO-1) and tumor necrosis factor receptor (TNF-R) [36]. The Fas receptor mediates apoptotic signalling after binding of its natural ligand, Fas (FasL) [37]. FasL

binding induces Fas receptor trimerization. This is followed by the recruitment of a cytoplasmic adaptor protein, Fas-associated death domain (FADD) [38] and pro-caspase 8, to the cytoplasmic side of the membrane, to form a death-inducing signalling complex (DISC). The initiator caspase-8 then becomes auto-activated [39], thereby initiating the cysteine protease cascade by direct activation of effector caspases such as caspases-7 and -3. Caspase-8 can also activate the mitochondrial pathway of apoptosis through cleavage of the proapoptotic protein bid to generate its truncated form, t-bid. T-bid then translocates to the mitochondrial membrane and interacts with the proapoptotic protein Bax to induce mitochondrial membrane permeabilization and activation of caspase-9 and the mitochondrial pathway [40]. Downstream events in the apoptotic cascade include the caspase-mediated cleavage of protein substrates such as polyADP-ribose polymerase (PARP) and inhibitor of caspase activated DNase (ICAD). The cell then exhibits the characteristic morphological features of apoptosis such as chromatin condensation, cytoskeletal changes, nuclear membrane breakage, cell blebbing and formation of apoptotic bodies [31; 41]. Apoptotic bodies are then phagocytozed by macrophages or neighboring cells, thus avoiding inflammatory damage to adjacent tissues.

Given the widespread exposure of humans and other species to acrolein, as well as its implication in multiple disease states involving oxidative stress, it is important to understand its mechanisms of toxicity. Acrolein has been shown to induce apoptosis in several different cell types [42; 43; 44], however, the molecular mechanisms and biochemical pathways involved are not well understood. Recently, acrolein was shown to activate the mitochondrial pathway of apoptosis in Chinese hamster ovary (CHO) cells [45]. With the aim of advancing our knowledge of this ubiquitous toxic compound, the present study investigates whether acrolein can cause apoptosis through activation of the death receptor signalling pathway.

MATERIALS AND METHODS

Cell culture

CHO cells (AuxBl) [46] were grown in monolayer in minimum essential medium-Alpha (α-MEM) plus 10% fetal bovine serum (FBS) (Gibco Canada) and 1% penicillin (50 units/ml)-streptomycin (50 μg/ml) (Flow Laboratories, Mississauga, ON, Canada), in tissue culture flasks (Sarstedt, St Laurent, QC, Canada), in a humidified atmosphere of 5% CO_2 in a water jacketed incubator at 37°C [47]. The cells were grown to near confluence and then incubated for 24 h with fresh culture medium. Confluent cells were then harvested using citrated phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, 0.015 M sodium citrate), washed by centrifugation (1000g, 3 min) and resuspended in α-MEM plus 10% FBS for experimental studies.

Determination of apoptotic cell death by Annexin V-FITC staining

Externalized phosphatidylserine (PS) on the outer surface of the cytoplasmic membrane becomes labelled by fluorescein-labelled Annexin V (BD Biosciences Canada, Mississauga, ON, Canada), which has a high affinity for PS-containing phospholipid bilayers [48]. CHO cells (1×10^6/ml) were incubated for 2 h with acrolein or hydrogen peroxide, and then washed twice with PBS and resuspended in 1 ml of binding buffer (10 mM Hepes/NaOH, pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$). Five hundred μl of cell suspension were then incubated with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) for 10 min at room temperature in the dark. The population of annexin V-positive cells was evaluated by flow cytometry. Data were collected using a FACS scan equipped with an argon laser emitting at 488 nm and analyzed using Lysis II software (Becton Dickinson, Oxford, UK).

Morphological analysis of apoptosis by fluorescence microscopy

Cells were incubated with acrolein for 4 h in tissue culture dishes containing 5ml of α-MEM and 10% FBS. Dishes were washed twice with PBS and Hoechst (33258) (0.06 mg/ml) was added for 15 min at 37^0C to stain apoptotic cells. The dishes were washed with PBS and PI (50 μg/ml) was added to stain necrotic cells. Observations were made by fluorescence microscopy (Carl Zeiss Ltd, Montreal, QC, Canada) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc, Mississauga, ON, Canada). Images were analysed by Northern Eclipse software. Cells were classified using the following criteria: a) live cells (normal nuclei, pale blue chromatin with organized structure); b) membrane-intact apoptotic cells (bright blue condensed or fragmented chromatin); c) necrotic cells (red, enlarged nuclei with smooth normal structure [49]. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). A minimum of 600 cells was counted per dish.

Treatment of cells with inhibitors of caspase-8 and Fas receptor

Confluent cells in monolayer were pretreated with the following irreversible inhibitors: Caspase-8 Inhibitor II, Z-IETD-FMK and Fas/FasL antagonist Kp7-6 (Calbiochem, La Jolla, CA, USA). The cells were exposed to a 10 μM concentration of the inhibitor Z-IETD-FMK or to 1 mM of Kp7-6 and then to various concentrations of acrolein for 1 or 4 h.

Determination of caspase activity by fluorescence spectroscopy

Freshly harvested CHO cells (0.5×10^6) were incubated with acrolein in 1.0 ml of α-MEM plus 10% FBS at 37^0C. After the appropriate time, cells were washed three times with cold PBS by centrifugation (1000g, 3 min), resuspended in 50 μl of PBS and 25 μl and were deposited into 96-well plates and lysed by freezing at -20^0C for 20 min. Fifty μl of reaction buffer (20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[(3-

cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) was added and stabilized at 37^0C [50]. The kinetic reaction was started after addition of 25 µl of the appropriate caspase substrate (Calbiochem) at 37°C using a spectrofluorimeter (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA, USA) [45].

Caspase-8 activity was measured by cleavage of the fluorogenic substrate Z-IETD- amino-4-trifluoromethylcoumarin (caspase-8 substrate II) to produce 7-amino-4-trifluoromethylcoumarin (AFC) with λmax excitation at 415 nm and λmax emission at 490 nm. Caspase-9 activity was measured by cleavage of the substrate Ac-LEHD-AFC to produce AFC. Caspase-7 activity was measured by cleavage of the fluorogenic substrate I MCA-VDQVDGWK(DNP)-NH_2 with λmax excitation at 325 nm and λmax emission at 395 nm.

Subcellular fractionation and immunodetection of Fas receptor (FasR), Fas ligand (FasL), FADD, caspase-8, bid and PARP

Following treatment with acrolein, cells were washed in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 µM aprotinin, 10 µM pepstatin A, 10 µM leupeptin, 25 µM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] [51]. After 30 min incubation on ice, lysates were homogenised using a hand potter (Kontes glass CO, Duall 22, Fisher, QC, Canada). Unbroken cells and nuclei were pelleted by centifugation at 2500 *g* for 10 min. The supernatant was centrifuged at 15 000 g for 20 min and the resulting pellet was designated as the mitochondrial fraction, which was used for the detection of t-bid [34]. A further centrifugation of the supernatant fraction at 100 000 *g* for 1 h resulted in a pellet designated as the microsomal fraction for the detection of FasR and FADD, whereas the supernatant was designated as the cytosolic fraction, which

was used for the detection of bid. Whole cell lysates were used for immunodetection of FasL, caspase-8 and PARP.

Separation of cellular proteins (30 μg) [52] was carried out by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [53], using a 10% (PARP) or a 15% (FADD, cytochrome-c oxidase, GST-π1, caspase-8, FasR, FasL, calnexin, bid) acrylamide gel [45]. Cellular proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Millipore, Bedford, MA) [45]. Membranes were probed with the primary antibodies: anti-FasR, anti-FADD (Stressgen, San Diego, CA, USA), anti-caspase-8, anti-bid, anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-FasL (Caltag Laboratories, Burlingame, CA, USA). Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-mouse, anti-rabbit and anti-goat IgG (1:1000) (Biosource, Camarillo, CA, USA). Proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA, USA) and films (Fuji medical X-ray film, Düsserldorf, Germany) were scanned with a Laser Scanning Densitometer (Alpha Innotech Corp., San Leandro, CA, USA). Purity of mitochondrial, cytosolic and microsomal fractions was verified using antibodies to cytochrome-c oxidase (Santa Cruz Biotechnology), $GST\pi_1$ (Calbiochem) and calnexin (BD Biosciences Canada), respectively. Caspase-8, PARP and FasL expression in whole cell lysates was quantified using IPGEL software, relative to β-tubulin loading controls.

Statistical analysis

Statistical differences between control and treated groups were determined using a one-way ANOVA which measures the linear contrast of means. An adjustment was made to limit the familywise error rate (FWE) to 5% by calculating an adjusted *p*-value which is a simulated based *p*-value obtained from the multivariate *t* distribution (number of simulations = 1 000 000) [54]. Then, the Bonferroni-Holm

method (a stepwise method) was used to control the FWE. For comparison between a treatment with acrolein in presence or absence of an inhibitor, statistical differences were determined by a two-tailed unpaired Student's *t* test.

Values are expressed as means ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Induction of apoptosis by acrolein

The induction of apoptosis by acrolein was evaluated by Annexin V labelling of externalised PS on cell membranes (Fig. 1). There was a 2.5 to 3-fold increase in Annexin V positive cells following exposure to 20 and 30 *f*mol/cell of acrolein (Fig. 1B, 1C), compared to untreated controls (Fig. 1A). Hydrogen peroxide also increased Annexin V positive cells and was used as a positive control (Fig 1D, 1E). It should be noted that the acrolein concentration was expressed as *f*mol/cell because the cell density was different for certain tests [55].

Acrolein induces translocation of FADD, cleavage of procaspase-8 and activation of initiator caspase-8

The ability of acrolein to activate the death receptor pathway of apoptosis was investigated in CHO cells. The adaptor protein FADD, which allows the Fas receptor and procaspase-8 to form the membrane DISC complex, was recruited to the cytoplasmic membrane after 30 to 60 min (Fig. 2). The translocation of FADD to the membrane (Fig. 2A, 2B) resulted in a corresponding reduction in levels of FADD in the cytosol (Fig. 2A, 2C), as well as activation of caspase-8, an initiator caspase (Fig. 3A). The cleavage of the inactive pro-enzyme form is a major step in generation of the active forms of caspases. Exposure of cells to acrolein resulted in cleavage of procaspase-8 as a function of increasing concentration from 20 to 30 *f*mol/cell after 30 or 60 min (Fig. 3B, 3C, 3D). This was apparent as a decrease in the intensity of the band (Fig. 3B, 3C) for the pro-enzyme form of caspase-8 (55 kDa) and a corresponding increase in the quantity of the 20 kDa cleavage fragment (Fig. 3B, 3D).

Blockade of Fas receptor activation partially inhibits acrolein-induced apoptosis

The next step was to determine whether activation of caspase-8 by acrolein was dependent on Fas receptor signalling. First of all, we confirmed that CHO cells indeed express the Fas receptor on their cell membranes (Fig. 4A). Furthermore, exposure of cells to acrolein at apoptosis-inducing concentrations (10 to 50 *f*mol/cell) did not affect the level of Fas receptor expression (Fig. 4A, 4B). There was, however, an increase in Fas ligand (FasL) expression when cells were exposed to acrolein (Fig. 4C, 4D).

The subsequent step was to determine the ability of an inhibitor of Fas receptor activation to block acrolein-induced caspase-8 activation and other apoptotic events downstream of caspase-8. Kp7-6 is an irreversible inhibitor of Fas receptor activation. It can bind to the Fas receptor and FasL, therefore preventing the FasL from forming a stable complex with the Fas receptor [56]. Once caspase-8 is activated, it directly processes downstream caspases such as caspases-3, -6 and -7. It was previously reported that acrolein can activate caspase-7, but causes inhibition of caspase-3 in these cells [45]. Downstream events from caspase-8 that were investigated in the apoptotic cascade included the effector caspase-7 and chromatin condensation in the nucleus. Kp7-6 effectively inhibited activation of initiator caspase-8 by acrolein (Fig. 5A) and partially inhibited activation of effector caspase-7 (Fig. 5B).

Morphological analysis demonstrates that acrolein induces both apoptosis and necrosis in cells (Fig. 6B). Apoptosis was revealed by condensation of chromatin with Hoescht 33258 (blue fluorescence) whereas necrosis was revealed using PI (red fluorescence). Kp7-6 also inhibited acrolein-induced nuclear chromatin condensation by about 60% (Fig. 6D, 6E). The Fas receptor inhibitor did not affect control cells (Fig. 5A, 5B, 6C, 6E, 8B).

Acrolein activates the crosstalk pathway

Once procaspase-8 is autoactivated at the DISC to become a mature active enzyme, two distinct downstream pathways have been identified for activation of apoptosis. In one route, caspase-8 directly processes downstream effector caspases such as caspases-3, -6 and -7. Through an alternative route, caspase-8 can activate a crosstalk pathway between the death receptor and mitochondrial pathways, by the cleavage of bid, a pro-apoptotic member of the Bcl-2 family. Exposure of cells to acrolein for 30 or 60 min caused cleavage of bid (Fig. 7) to form t-bid, which translocates to the mitochondria. This was apparent as a decrease in levels of bid in the cytosol with increasing acrolein concentration (Fig. 7A, 7B) and an increase in the level of the cleavage fragment t-bid in the mitochondrial fraction (Fig. 7A, 7C). Once localised to the mitochondrial membrane, t-bid can activate the mitochondrial death pathway. This occurs by the release of cytochrome-c from mitochondria and activation of another initiator caspase, caspase-9.

Caspase-8 is a major upstream caspase in acrolein-induced apoptosis

The subsequent step was to determine the role of caspase-8 in activation of the mitochondrial pathway in acrolein-induced apoptosis. To achieve this, the ability of an irreversible inhibitor of caspase-8, Z-IETD-FMK, was used to assess the effect of caspase-8 activation on acrolein-induced caspase-9 activation, a downstream event in the mitochondrial pathway. Acrolein induced the activation of caspase-9, which was partially decreased by the caspase-8 inhibitor (Fig. 8A). The activation of caspase-9 by acrolein was also partially diminished by inhibition of Fas receptor activation by Kp7-6, confirming the role of the Fas receptor upstream of the mitochondrial pathway (Fig. 8B). The role of initiator caspase-8 was investigated in the activation of other downstream apoptotic events, such as cleavage of the caspase substrate PARP and chromatin condensation in the nucleus. Exposure of cells to acrolein led to cleavage of the DNA repair enzyme PARP (116 kDa), to form a 85 kDa fragment, which was also diminished by the caspase-8 inhibitor (Fig. 9). Z-IETD-FMK also decreased acrolein-induced chromatin condensation, a late stage event in apoptotic cell death,

by about 60% (Fig. 10D, 10E). The caspase-8 inhibitor did not affect control cells (Fig. 8A, 9, 10C, 10E).

DISCUSSION

This study shows for the first time that acrolein can induce apoptosis in proliferating cells through activation of a death receptor pathway. Induction of apoptosis by acrolein was confirmed by annexin V labelling of externalised PS and morphologically by condensation of nuclear chromatin. Exposure to acrolein led to recruitment of FADD and procaspase-8 to the cytoplasmic membrane, resulting in caspase-8 processing. Once activated, the initiator caspase-8 processed the downstream effector caspase-7. We confirmed that the Fas receptor was involved in acrolein-induced apoptosis, by increased expression of FasL by acrolein and also with Kp7-6, an inhibitor of Fas receptor activation. Kp7-6 inhibited downstream apoptotic events in the death receptor pathway, such as activation of caspases-8 and –7, and chromatin condensation in the nucleus.

It was previously reported that acrolein can activate the mitochondrial pathway of apoptosis in CHO cells [45]. This was apparent as a decrease in mitochondrial membrane potential, liberation of cytochrome-c from mitochondria into the cytosol and the subsequent activation of initiator caspase-9. It is possible that acrolein could activate the mitochondrial pathway directly, and/or by death receptor signalling through the crosstalk pathway involving caspase-8 mediated cleavage of the proapoptotic protein Bid. Our findings support both of these scenarios. The crosstalk pathway was indeed activated by acrolein and the post-mitochondrial activation of caspase-9 was partially decreased by an inhibitor of caspase-8. The fact that there was only partial inhibition of caspase-9 activation suggests that the mitochondrial pathway could also be stimulated by acrolein independently of the death receptor pathway. This is also supported by the partial inhibition of acrolein-induced chromatin condensation by Z-LEHD-FMK, an inhibitor of caspase-9 [45]. The caspase-8 inhibitor, Z-IETD-FMK, also caused partial inhibition of chromatin

condensation, suggesting that acrolein can activate both the death receptor and mitochondrial pathways directly.

Acrolein was shown to mediate apoptosis via the effector caspase-7, rather than through caspase-3 [45]. Several studies have reported that acrolein inhibits activity of the major effector caspase-3 [45; 57; 58], probably through inhibition of its active site cysteine residue. The effector caspase-7 can be activated by both of the initiator caspases, caspase-8 and caspase-9 [59]. Both of the effector caspases-3 and -7 share similar protein substrates and are able to cleave the caspase substrate PARP [41; 60].

The concentrations (10-50 fmol/cell or 10-50 μM) used in this study appear to be relevant to in vivo levels of acrolein. The physiological level of acrolein in the serum of a normal human was estimated to reach 50 μM [61]. Higher levels of acrolein were reported for certain disease states. Acrolein is estimated to reach 80 μM in the respiratory tract lining fluids as a result of cigarette smoke [43]. In our study, exposure to 10-50 μM acrolein induced apoptotic cell death after relatively short exposure times of 1 to 4 h. Exposure to these concentrations, or even lower concentrations of acrolein for much longer times, such as days (as likely occurs in vivo), would therefore be expected to cause widespread cell death. Furthermore, it would be expected that acrolein concentrations in vivo would increase with time after injury, since lipid peroxidation is a chain reaction process, and in addition, dead cells would liberate acrolein from the cytosol into the extracellular environment.

The modes of cell death induced by different stresses and toxic compounds are dependent on dose and depend on the susceptibility of individual cell types [32]. Acrolein-induced apoptosis is clearly cell type dependent. In neutrophils, low doses of acrolein were shown to inhibit caspase-8 activity [57; 58]. Different studies have reported variable findings in that acrolein can either cause or inhibit apoptosis, or cause predominantly necrosis/oncosis rather than apoptosis. For example, acrolein (25 μM) caused apoptosis in isolated human alveolar macrophages, detected by

morphological changes and DNA fragmentation after 24 h [42]. Acrolein activated the mitochondrial pathway of apoptosis in CHO cells [45]. Acrolein stimulated apoptosis in the human lung epithelial cell line HBE1, as indicated by externalisation of phosphatidylserine and DNA fragmentation 24 h after a 30 min exposure to 10 to 25 μM acrolein [43]. Exposure to 50 μM acrolein for 24 h induced atypical apoptosis in primary cultures of human keratinocytes [44]. In human neutrophils, however, acrolein (25 μM) inhibited the constitutive pathway of apoptosis [21]. In proB lymphoid cells and human neutrophils, the cell death pathway was predominantly oncosis/necrosis, with only low levels of apoptosis occurring at lower doses (<10 μM) [57; 58]. These variable results could be due to differences in biochemical factors determining cell death pathways in different cell types, which include non-proliferating primary cell cultures and cells involved in immune responses, as well as proliferating cancer cell lines.

ATP appears to play an active role in determining the type of cell death occurring under various metabolic conditions [62]. Depletion of cellular ATP levels by an ATP synthase inhibitor, oligomycin, mediated the switch from apoptosis to necrosis in leukemic cells [63]. Cells treated with known apoptosis inducers, staurosporin (STP) and anti-CD95, died exclusively by necrosis when pretreated with oligomycin. Apoptotic characteristics were not observed when cells were depleted of ATP below a critical level, but were restored upon glucose addition. Several studies have reported the inhibitory effect of acrolein on ATP production. Exposure to 10 uM acrolein for 24 h in rat pneumocyte II L2 cells [64], and for 4 h in myocytes [65], led to decreased ATP levels. Intracellular ATP concentrations were also decreased in murine FL5.12 proB lymphocytes, reaching 0% of control 24 h after a 30 min exposure to 40 μM acrolein [57] and in mitochondria of neuronal PC12 cells 24 h after exposure to 10 μM acrolein [66]. The type of cell death in proB lymphocytes and PC12 cells under these conditions was mainly necrosis. To our knowledge, ATP levels have not been determined under conditions where acrolein induces apoptosis.

The antioxidant glutathione appears to have an important role in the detoxification of acrolein [67]. This reaction can be spontaneous or catalyzed by the detoxification enzyme glutathione S-transferase (GST) [68] via the reaction of Michael addition [69]. Moreover, acrolein can also be metabolized by the rat liver aldo-keto reductase [70] and by aldehyde dehydrogenase [71].

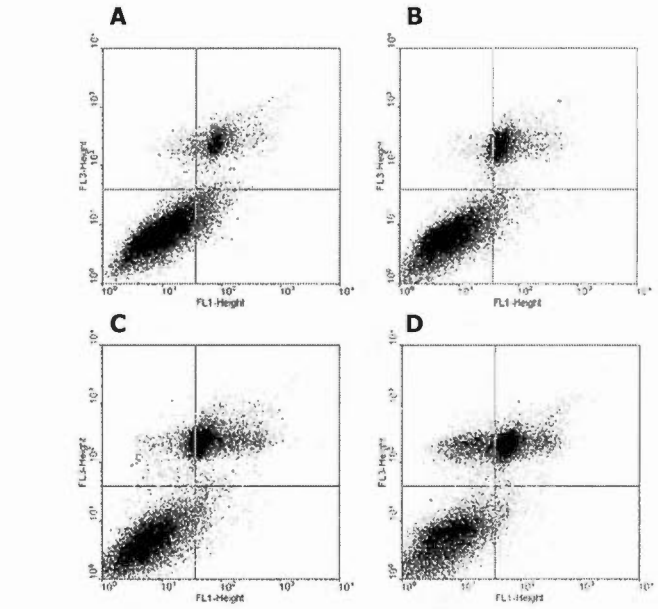
Acrolein is generated as a product of the enzymatic action of amine oxidases on naturally occurring polyamines such as spermine and spermidine [7; 72]. Polyamines play an essential role in cellular proliferation and differentiation [73]. The reason for the generation of a highly toxic product as acrolein from such a basic and important physiological process is certainly intriguing and is not presently known.

In conclusion, this is the first detailed study to demonstrate that the mechanism of acrolein-induced apoptosis is mediated by the Fas receptor pathway as well as the mitochondrial pathway in proliferating cells. These findings are relevant to the toxicity of acrolein in many contexts, including the pharmacological action and/or toxic side effects of the anticancer agent cyclophosphamide, the regulation of cellular proliferation and tumor growth by polyamines, Alzheimer's disease, as well as the toxicity of environmental exposures to low doses of acrolein.

ABBREVIATIONS

AFC: amino trifluorocoumarin; AMC: amino methylcoumarin; BSA: bovine serum albumin; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CHO: Chinese hamster ovary; DISC: death-inducing signalling complex; FADD: Fas associating protein with death domain; Fas: fibroblast-associated; FasL: Fas ligand; FasR: Fas receptor, FBS: fetal bovine serum; ICAD: inhibitor of caspase activated DNase; MEM: minimum essential medium; MOPS: 3-(N-morpholino)-propane sulfonic acid; PARP: polyADP-ribose polymerase; PBS: phosphate-buffered saline; PMSF: phenylmethylsufonyl fluoride; PS: phosphatidylserine; PVDF: polyvinylidene difluoride; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM: standard error of mean. TNF-R: tumor necrosis factor receptor.

Figure 1: Acrolein induces externalization of phosphatidylserine



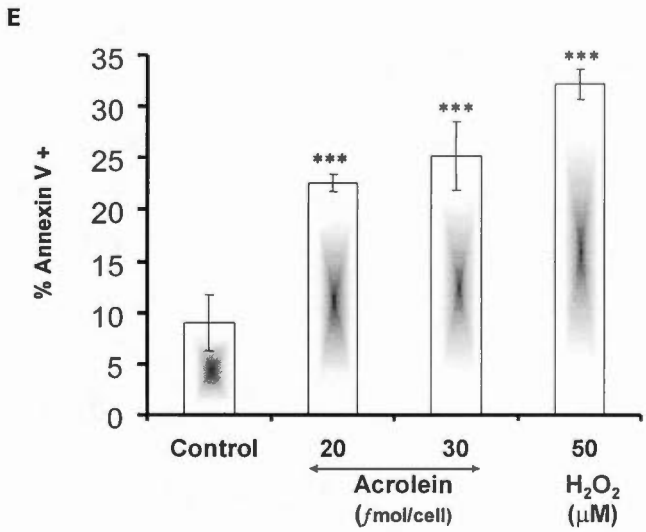
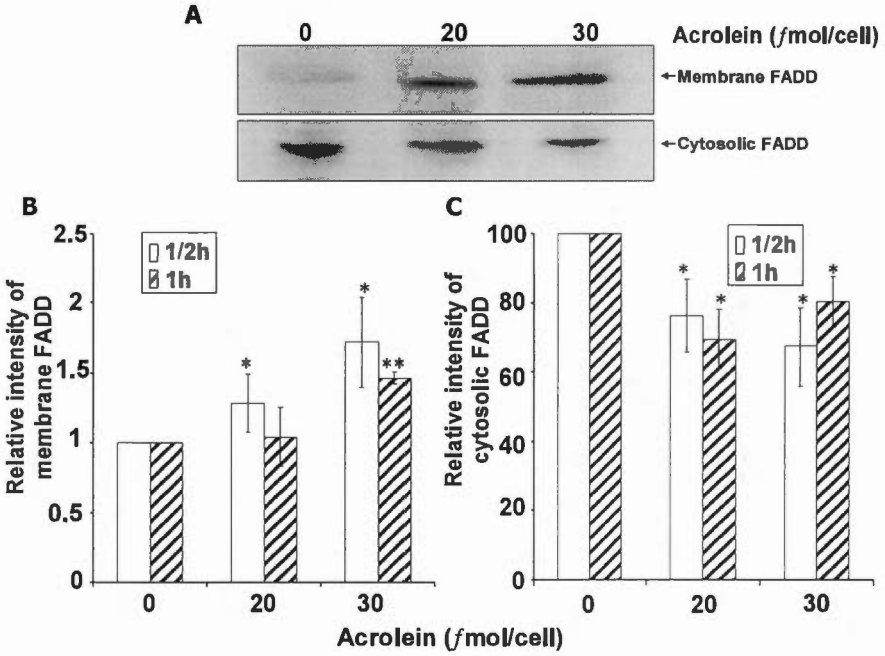


Figure 2: Acrolein induces translocation of FADD



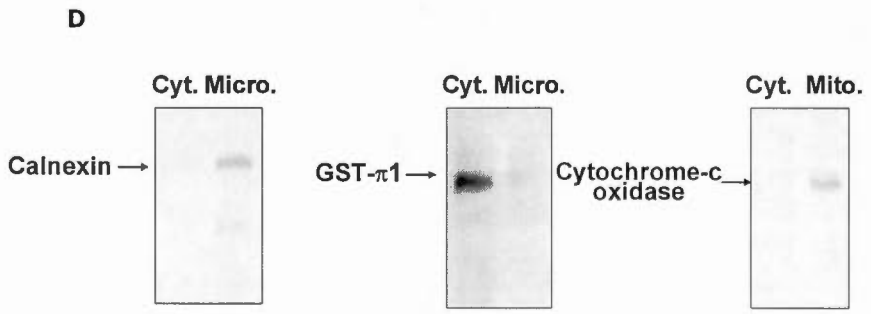


Figure 3: Activation of initiator caspase-8 by acrolein

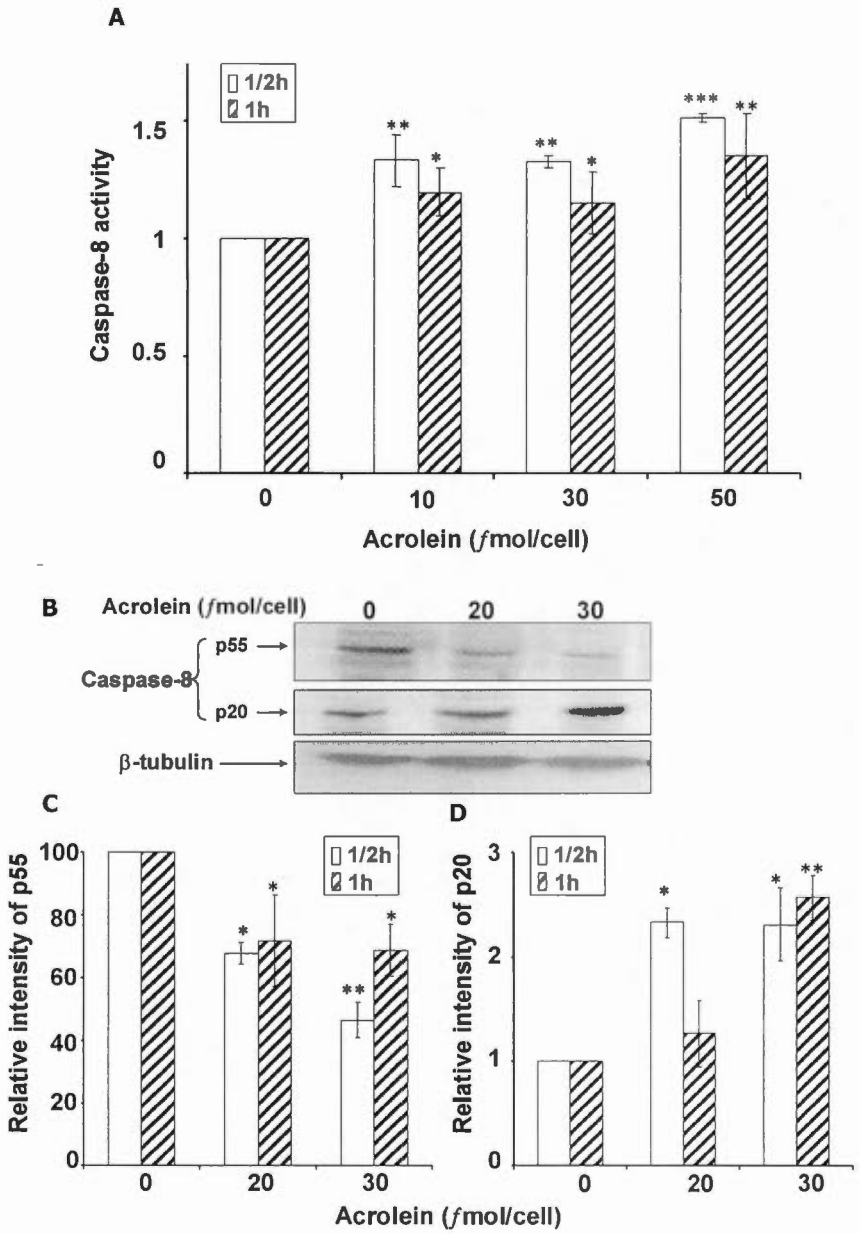


Figure 4: FasL expression is increased by acrolein whereas FasR expression is not affected

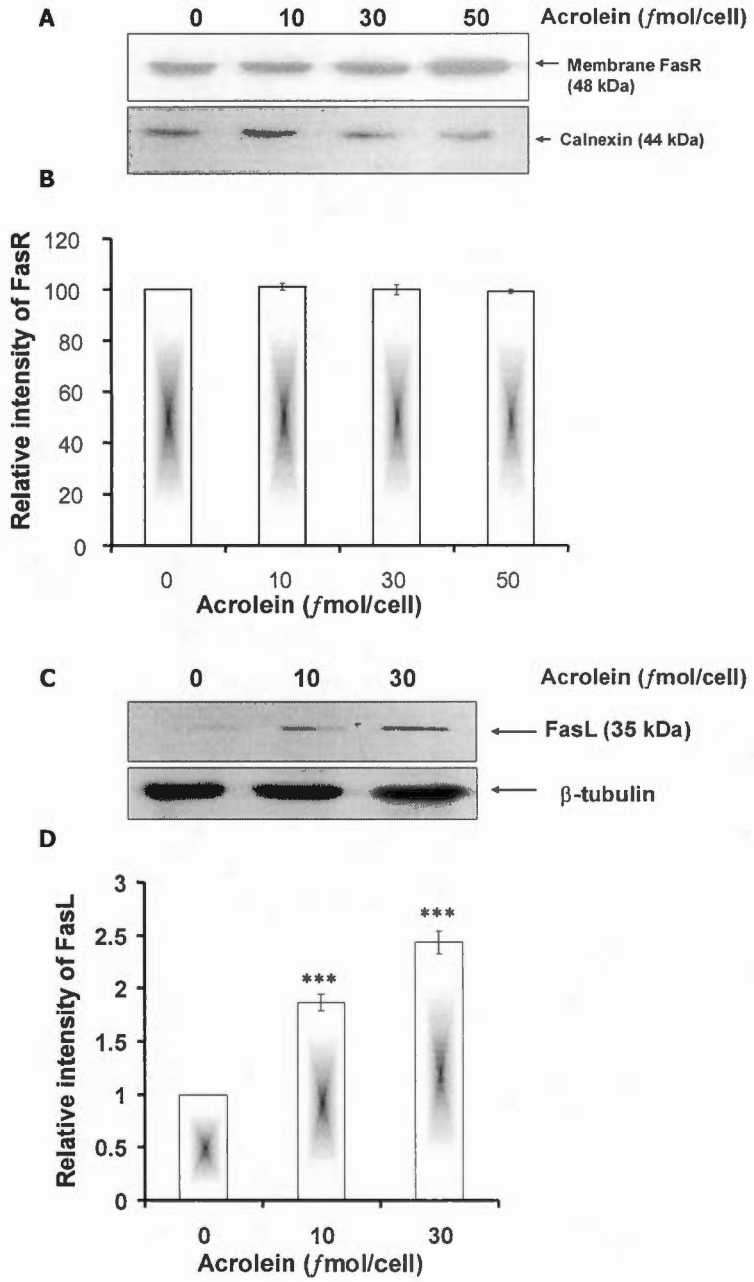


Figure 5. Activation of caspase-8 and caspase-7 by acrolein is decreased by an inhibitor of Fas receptor

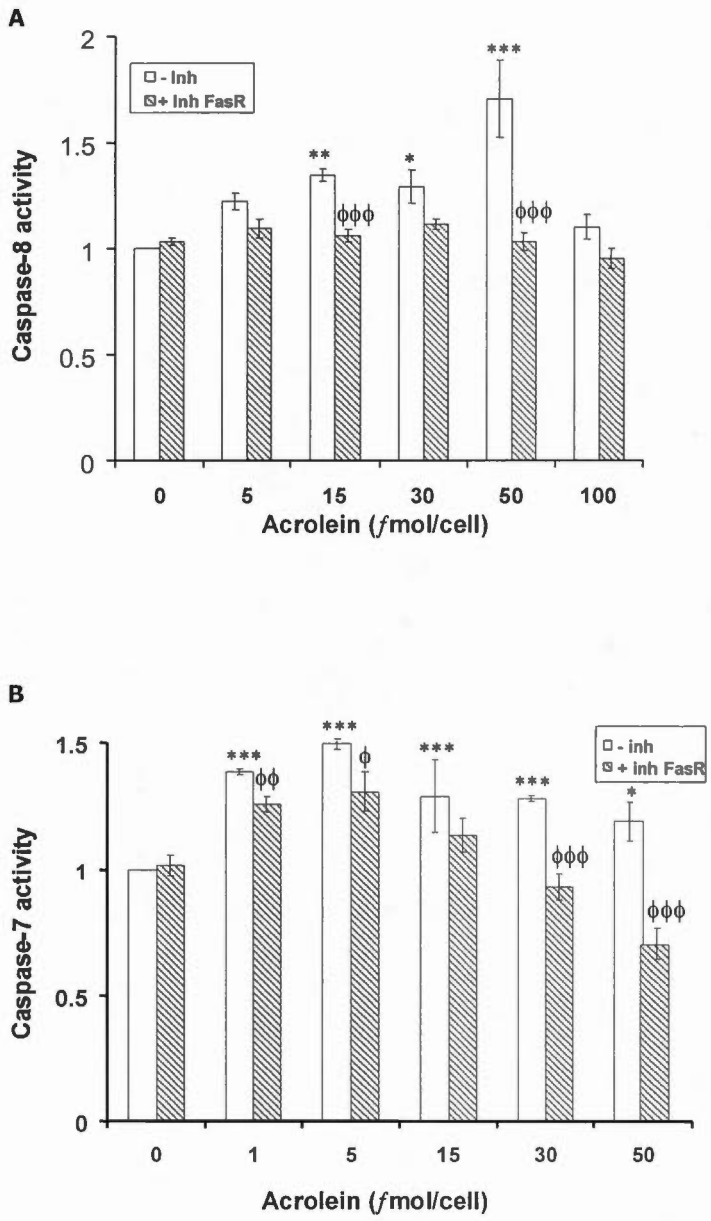


Figure 6: An inhibitor of Fas receptor decreases apoptosis induced by acrolein

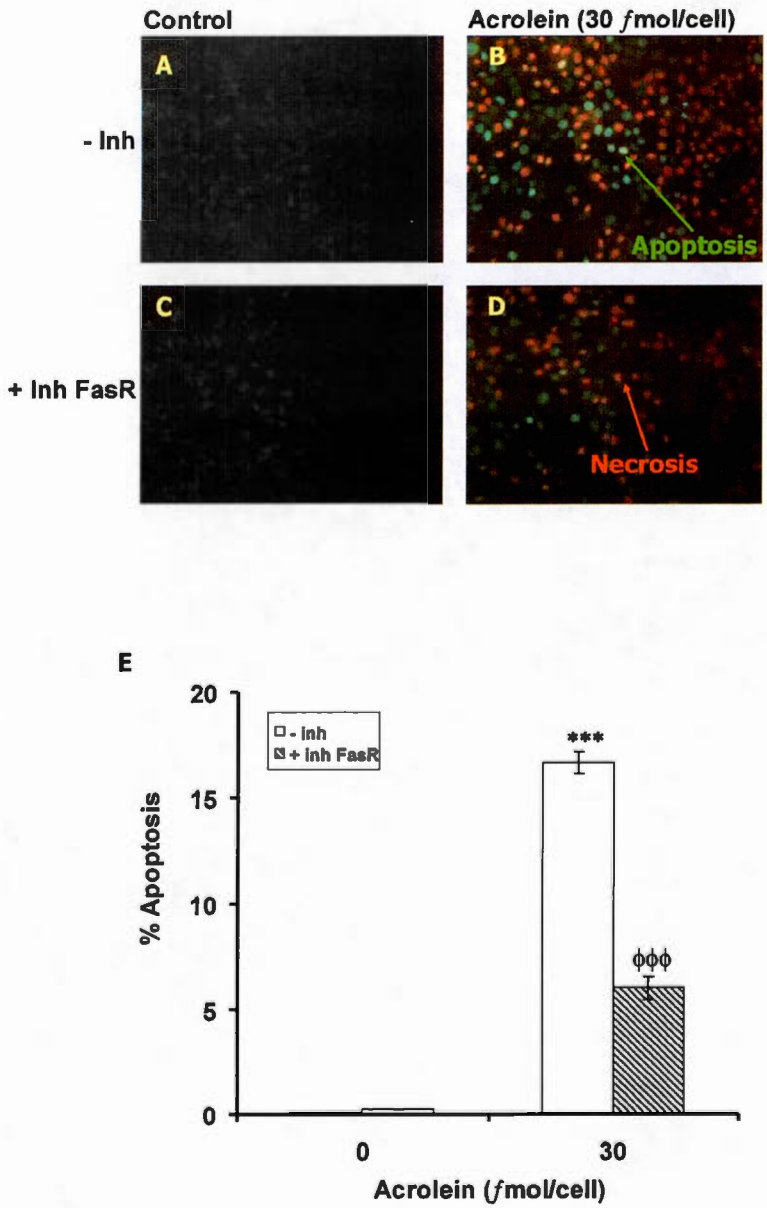


Figure 7: Acrolein induces cleavage of bid to t-bid

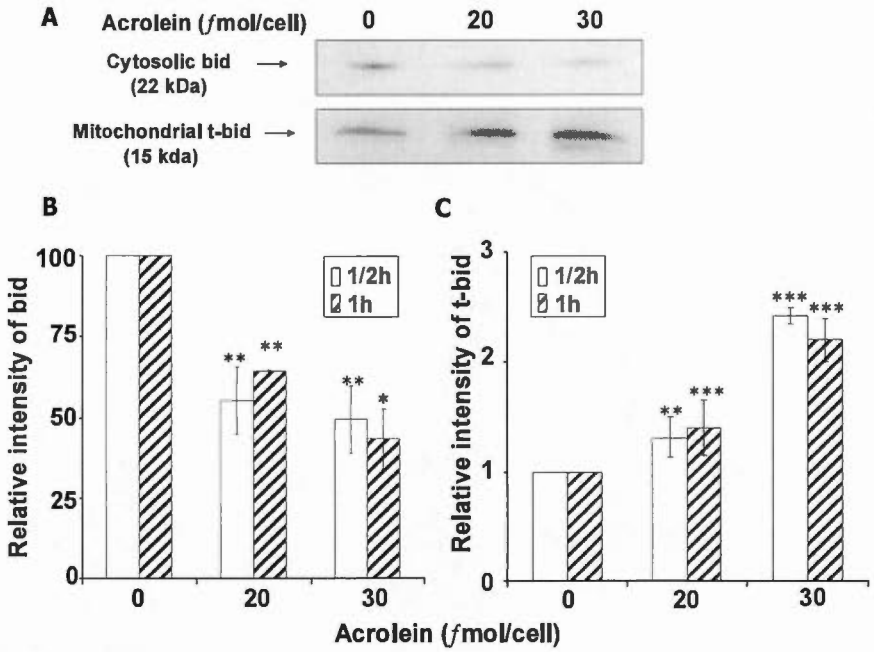


Figure 8: Activation of caspase-9 by acrolein is decreased by inhibitors of caspase-8 and FasR

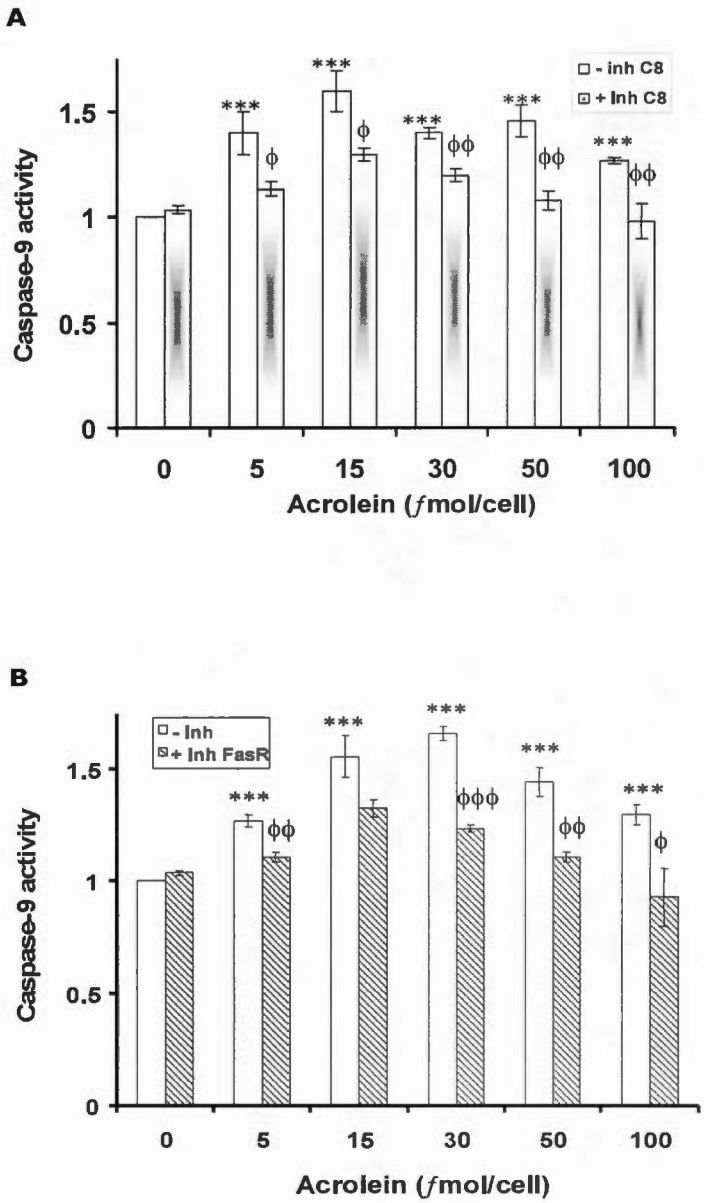


Figure 9: Cleavage of PARP by acrolein is inhibited by an inhibitor of caspase-8

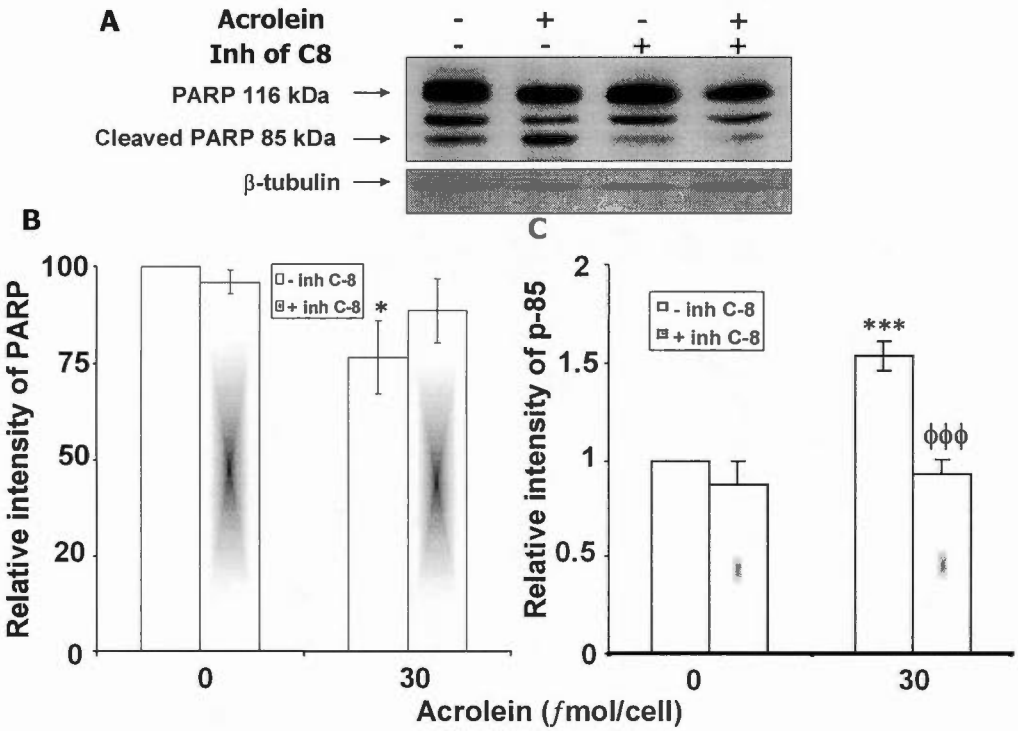
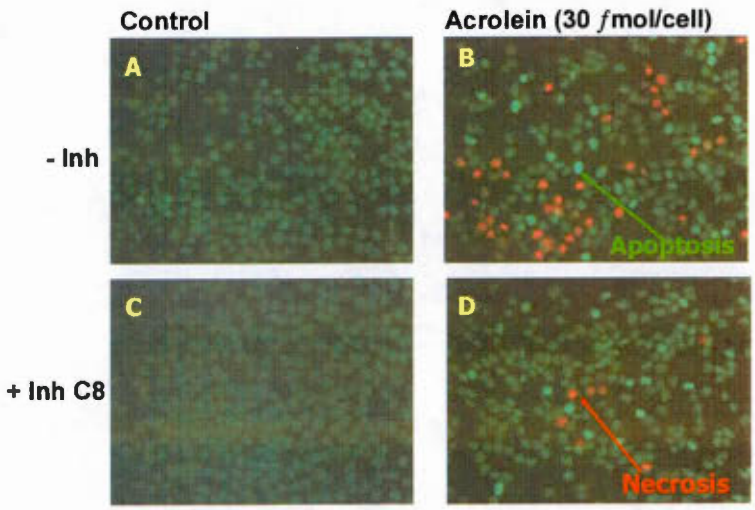


Figure 10: Inhibition of caspase-8 decreases acrolein-induced chromatin condensation



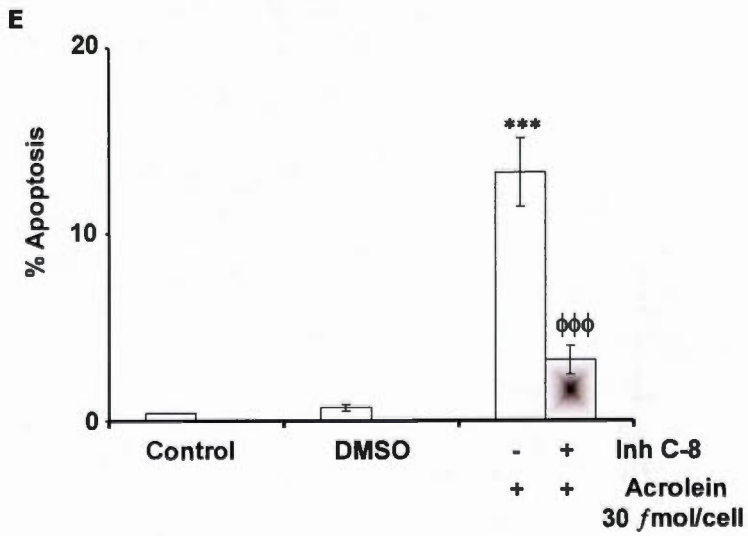


FIGURE LEGENDS

Fig. 1 Acrolein induces externalization of phosphatidylserine. Apoptosis was analysed by flow cytometry using Annexin V-FITC in CHO cells following treatment with acrolein. Cells (10^6/ml) were either (A) untreated controls, or they were treated with (B) 20 *f*mol/¢ and (C) 30 *f*mol/¢ acrolein or (D) 50 μM hydrogen peroxide for 2 h. Cells were subsequently stained with Annexin V-FITC (x-axis) and PI (y-axis). Twenty thousand cells were then analyzed using a FACS scan to determine the percentage of Annexin V^+-labelled cells. One representative experiment is shown from six independent experiments. (E) $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein or H_2O_2 and the control.

Fig. 2. Acrolein induces translocation of FADD. CHO cells (10^6 /ml) were incubated for 1 h with different concentrations of acrolein (20 and 30 *f*mol/cell) in α-MEM containing 10% FBS. Immunodetection of FADD (28 kDa) in membrane and cytosolic fractions was carried out by Western blotting, using calnexin as a loading control for the membrane fraction and GSTπ-1 for the cytosolic fraction. (A) A representative blot is shown from four independent experiments. Densitometric analyses of the expression of (B) membrane FADD and (C) cytosolic FADD are relative to the untreated control. $P<0.05$ (*) or $P<0.01$ (**) indicates a statistically significant difference between treatment with acrolein and the control. (D) Purity of mitochondrial, cytosolic and microsomal fractions was verified using antibodies to cytochrome-c oxidase, $GST\pi_1$ and calnexin, respectively.

Fig. 3. Activation of initiator caspase-8 by acrolein. (A) CHO cells ($0.5x10^6$/ml) were incubated with acrolein (10 to 50 *f*mol/cell) for 1/2 h or 1 h in α-MEM with 10% FBS. Caspase-8 activity was measured in cell lysates using the fluorescent substrate Z-IETD-AFC. Caspase activity was expressed relative to the untreated

control, designated as 1. Data represent means and SEM from eight independent experiments performed with multiple estimations per point. (B) Cells (10^6/ml) were incubated with acrolein (20 and 30 *f*mol/cell) for 1 h in α -MEM containing 10% FBS. The immunodetection of procaspase-8 (55 kDa) and its cleavage fragment (20 kDa) was carried out by Western blotting, using β-tubulin as a loading control. A representative blot is shown from four independent experiments. Densitometric analyses of the expression of (C) procaspase-8 and (D) the cleavage fragment are relative to the untreated control. $P<0.05$ (*) or $P<0.01$ (**) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 4. FasL expression is increased by acrolein whereas FasR expression is not affected. CHO cells (10^6 /ml) were incubated for 1 h with different concentrations of acrolein (10 to 50 *f*mol/cell) in α-MEM containing 10% FBS. Immunodetection of membrane FasR (48 kDa) and FasL (35 kDa) was carried out by Western blotting, using calnexin and β-tubulin , respectively, as loading controls. Representative blots are shown for (A) FasR and (C) FasL from four independent experiments. Densitometric analyses of the expression of (B) FasR and (D) FasL are relative to the untreated control. (E) $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 5. Activation of caspase-8 and caspase-7 by acrolein is decreased by an inhibitor of Fas receptor. Pretreatment of cells with 1 mM of Kp7-6 (Fas/FasL antagonist) was performed for 1h before addition of acrolein. (A) CHO cells ($0.5x10^6$/ml) were then incubated with acrolein (5 to 100 *f*mol/cell) for 1 h (caspase-8 assay) or (B) with acrolein (1 to 50 *f*mol/cell) for 2 h (caspase-7 assay) in α-MEM with 10% FBS. Activities of caspases-8 and -7 were measured in cell lysates using the fluorescent substrates Z-IETD-AFC and MCA-VDQVDGWK(DNP)-NH_2, respectively. Caspase activity was expressed relative to the untreated control,

designated as 1. Data represent means and SEM from eight independent experiments performed with multiple estimations per point. $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ), $P<0.01$ (ϕϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without Fas receptor inhibitor.

Fig. 6. An inhibitor of Fas receptor decreases apoptosis induced by acrolein. Confluent cells in monolayer were pre-incubated for 1 h with (C, D) or without (A, B) 1 mM of Kp7-6 and then incubated with (A, C) 0 or (B, D) 30 *f*mol/cell of acrolein for 4 h, with inhibitor present (C, D). Cells were stained with Hoechst 33258 and PI and visualised by fluorescence microscopy (magnification 320x). The fraction of (E) apoptotic cells (blue Hoechst 33258 fluorescence) is given relative to total cells. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without Fas receptor inhibitor.

Fig. 7. Acrolein induces cleavage of bid to t-bid. CHO cells (10^6 /ml) were incubated for 1 h with different concentrations of acrolein (20 and 30 *f*mol/cell) in α-MEM containing 10% FBS. Immunodetection of cytosolic bid (22 kDa) and mitochondrial t-bid (15 kDa) was carried out by Western blotting, using GSTπ-1 as a loading control for the cytosolic fraction and cytochrome-c oxidase for the mitochondrial fraction. (A) A representative blot is shown from four independent experiments. Densitometric analyses of the expression of (B) cytosolic bid and (C) mitochondrial t-bid are relative to the untreated control. $P<0.05$ (*), $P<0.01$ (**) or

$P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 8. Activation of caspase-9 by acrolein is decreased by inhibitors of caspase-8 and FasR. Pretreatment of cells with (A) 10 μM of Caspase-8 Inhibitor II, Z-IETD-FMK, or (B) 1 mM of Kp7-6 was carried out for 1h before addition of acrolein. (A, B) CHO cells ($0.5x10^6$/ml) were then incubated with acrolein (5 to 100 *f*mol/cell) for 1 h in α-MEM with 10% FBS. Caspase-9 activity was measured in cell lysates, either with or without inhibitor pretreatment, using the fluorescent substrate Ac-LEHD-AFC. Caspase-9 activity was expressed relative to the untreated control, designated as 1. Data represent means and SEM from eight independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ), $P<0.01$ (ϕϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without caspase-8 inhibitor or Fas receptor inhibitor.

Fig. 9. Cleavage of PARP by acrolein is decreased by an inhibitor of caspase-8. (A) Cells (10^6/ml) were incubated with acrolein for 2 h and immunodetection of PARP (116 kDa) and its cleavage fragment (85 kDa) was carried out by Western blotting, using β-tubulin as a loading control. A representative blot is shown from four independent experiments. Densitometric analyses of the expression of (B) PARP and (C) the cleavage fragment are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without caspase-8 inhibitor.

Fig. 10. Inhibition of caspase-8 decreases acrolein-induced chromatin condensation. Confluent cells in monolayer were pre-incubated for 1 h with (C, D) or without (A, B) 10 μM of Caspase-8 Inhibitor II, Z-IETD-FMK, before addition of acrolein. Cells were then incubated with (A, C) 0 or (B, D) 30 *f*mol/cell of acrolein for 4 h, either with or without the caspase-8 inhibitor present (C, D). (E) The fraction of apoptotic cells (Hoechst) is given relative to total cells. Necrotic cells were visualised using PI (red fluorescence). Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.001$ ($\phi\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without caspase-8 inhibitor.

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2.4. ARTICLE III

P38 AND ERK MITOGEN-ACTIVATED PROTEIN KINASES MEDIATE ACROLEIN-INDUCED APOPTOSIS IN CHINESE HAMSTER OVARY CELLS

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Abbréviations: AFC: amino trifluorocoumarin; BSA: bovine serum albumin; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CAD: caspase activated DNase; CHO: Chinese hamster ovary; ERK:

extracellular signal regulated kinase; FBS: fetal bovine serum; 4-HNE: 4-hydroxynonenal; ICAD: inhibitor of caspase activated DNase; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MEK: mitogen-activated protein kinase kinase; MEM: minimum essential medium; MOPS: 3-(N-morpholino)-propane sulfonic acid; PARP: polyADP-ribose polymerase; PBS: phosphate-buffered saline; PI3-K: phosphoinositide 3-kinase; PKB: protein kinase B; PMSF: phenylmethylsufonyl fluoride; PVDF: polyvinylidene difluoride; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM: standard error of mean; Wt: wortmannin.

RÉSUMÉ

L'acroléine, qui est un aldéhyde α,β-insaturé généré par la peroxidation lipidique, peut affecter les tissus et les cellules et causer plusieurs pathologies. L'augmentation des aldéhydes joue un rôle important dans la pathogenèse de plusieurs maladies notamment l'Alzheimer, l'athérosclérose et le diabète. Quelques études ont démontré que l'acroléine active les MAPKs et d'autres ont rapporté que l'acroléine induit l'apoptose. Cependant, aucune étude n'a examiné le lien entre l'activation des MAPKs et l'apoptose induite par l'acroléine. Ici, on montre pour la première fois que l'apoptose induite par l'acroléine est dépendante des MAPK. Une heure d'exposition à l'acroléine a induit une forte phosphorylation de ERK, p38 et le substrat de JNK, c-jun chez les cellules CHO. L'inhibition de la condensation de la chromatine induite par l'acroléine à l'aide de l'inhibiteur de ERK, le U126, et l'inhibiteur de la p38, le SB203580, démontre clairement l'implication de ces deux voies dans l'apoptose induite par l'acroléine. En plus, le U0126 et le SB203580 ont inhibé l'activation des caspases-7 et -9 et le clivage de l'ICAD induites par l'acroléine. D'autre part, les voies de JNK et AKT semble être impliquées dans la survie contre l'agression par l'acroléine, puisque des inhibiteurs pharmacologiques de ces deux voies, le SP600125, le LY294002 et le Wortmannin ont changé le type de mort de l'apoptose en nécrose. Enfin, l'acroléine a entraîné la phosphorylation de la p53, une protéine responsable de la transcription de plusieurs gènes impliqués dans l'apoptose dont ceux de Bax et du ligand Fas. Ces résultats fournissent de nouvelles informations sur l'apoptose induite par l'acroléine qui pourraient être utiles pour la compréhension de plusieurs pathologies ainsi que les effets des expositions environnementales impliquant l'acroléine.

ABSTRACT

Acrolein, which is a highly reactive α,β-unsaturated aldehyde generated by lipid peroxidation, can affect cells and tissues and cause various disorders. Increased levels of unsaturated aldehydes play an important role in the pathogenesis of a number of human diseases such as Alzheimer's disease, atherosclerosis and diabetes. Acrolein is a highly ubiquitous toxic environmental pollutant. Because of human exposure, there is a need for investigating the mechanisms involved in acrolein toxicity at the cellular and molecular levels. Acrolein can induce cell death by apoptosis, although the mechanisms are not entirely clear. The present study investigates whether mitogen-activated protein kinases (MAPKs) play a role in activation of apoptosis by acrolein. Our findings show that acrolein-mediated apoptosis is in fact MAPK-dependent in Chinese hamster ovary cells. The MAP family kinases, including ERK and p38 kinase, and the transcription factor c-Jun were all activated by phosphorylation after 1 h exposure to acrolein. Phosphorylation of ERK and p38 kinases and their blockade by an ERK inhibitor, U0126, or a p38 inhibitor, SB203580, respectively, suggested that activation of apoptosis by acrolein is ERK- and p38-dependent. Thus, blockade of ERK and p38 inhibited chromatin condensation, caspase-7 and -9 activation as well as ICAD cleavage induced by acrolein. JNK and AKT kinases seem to be implicated in survival pathways against acrolein insult, since their respective inhibitors, SP600125 and LY294002/Wortmannin switched the mode of cell death from apoptosis to total necrosis. Finally, acrolein induced phosphorylation of the pro-apoptotic factor p53 which is responsible for transcription of pro-apoptotic factors such as Bax and Fas ligand. These results provide new information demonstrating the implication of MAPKs and AKT in acrolein-induced apoptosis, and this information may be useful for understanding the pathogenesis of a number of tissue diseases and environmental toxicity in response to acrolein.

Keywords: Acrolein, MAPK, ASK1, AKT, apoptosis, caspase

INTRODUCTION

Acrolein, an unpleasant and troublesome by-product of overheated organic matter, occurs as a ubiquitous pollutant in the environment, arising from incomplete combustion of plastic materials, forest fires, cigarette smoking and overheated cooking oils [Beauchamp *et al.*, 1985]. Acrolein is also a metabolite formed in the biotransformation of allyl compounds and the widely used anticancer drug cyclophosphamide. In addition, acrolein is used industrially as a starting material for acrylate polymers and in the production of acrylic acid, and as a herbicide [Ghilarducci and Tjeerdema, 1995]. Since acrolein was identified as one of the "unnatural" components of tobacco smoke [Johnstone and Plimmer, 1959], a number of reports have appeared describing the damaging effects of acrolein on the tracheal ciliatory movement [Kensler and Battista, 1963] and the pulmonary wall [Izard and Liberman, 1978].

Acrolein is one of the toxic by-products of endogenous lipid peroxidation, resulting from oxidative stress, together with the aldehyde 4-hydroxy-2-nonenal (HNE) [Adams and Klaidman, 1993; Uchida 1999]. Oxidative stress has been implicated in chronic neurodegenerative disorders such as Alzheimer's disease [Calingasan *et al.*, 1999]. Lipid peroxidation is increased in Alzheimer's disease [Ramassamy *et al.*, 1999] and it has been suggested that aldehyde by-products such as acrolein and 4-HNE could be involved in causing cellular and tissue damage. Acrolein protein adducts have been demonstrated in Alzheimer's disease [Calingasan *et al.*, 1999], diabetic nephropathy [Suzuki and Miyata, 1999] and atherosclerosis [Uchida *et al.*, 1998]. These pathophysiologies have all been associated with increased lipid peroxidation and alkylation of protein residues.

Its high reactivity indeed makes acrolein a dangerous substance for the living cell. It has been shown that acrolein reduces the colony-forming efficiency of mammalian cells, forms cyclic adducts with nucleosides *in vitro* and is a potent mutagen [Esterbauer *et al.*, 1991]. Among all of the α,β-unsaturated aldehydes,

acrolein is by far the strongest electrophile and therefore, shows the highest reactivity with nucleophiles, such as the sulfhydryl group of cysteine, imidazole group of histidine and amino group of lysine [Esterbauer *et al.*, 1991].

Apoptosis is a naturally occurring process that plays an essential role in development and the maintenance of homeostasis in multicellular organisms [Saraste and Pulkki, 2000; Hale *et al.*, 1996]. Apoptosis has many important repercussions in human health. Cell accumulation via insufficient apoptosis can contribute to conditions such as cancer, inflammation and autoimmune disease. In contrast, excessive apoptosis can play an important role in neurodegeneration, AIDS, eye disorders, osteoporosis and heart failure [Fadeel *et al.*, 1999; Wilson, 1999].

A family of cytosolic cysteine proteases, the caspases, play an essential role in the execution of apoptosis. The caspases are divided into apical (-2, -8, -9 and -10) and executioner subsets (-3, -6 and -7) [Salvesen and Dixit, 1997]. The zymogens or pro-enzymes of apical caspases (-8 and -10) are recruited by specific adaptor molecules at the cytosolique face of death receptors, whereas that for caspase-9 is activated via a post-mitochondrial route [Denecker *et al.*, 2001]. Downstream events in the apoptotic cascade include the caspase-mediated cleavage of cytoplasmic, cytoskeletal and nuclear protein substrates such as polyADP-ribose polymerase (PARP), lamins, gelsolins, fodrins and inhibitor of caspase activated DNase (ICAD). The cell then exhibits the characteristic morphological features of apoptosis such as chromatin condensation, cytoskeletal changes, nuclear membrane breakage, cell membrane blebbing and formation of apoptotic bodies [Wilson, 1999; Earnshaw, 1995]. Apoptotic bodies are then phagocytozed by macrophages or neighboring cells, thus avoiding inflammatory damage to adjacent cells and tissues.

While numerous candidate genes playing important roles in the signaling of apoptosis have been identified, the mechanisms and order in which they interact to transduce signals are poorly understood. Several lines of evidence have suggested that members of the mitogen-activated protein kinase (MAPK) family are involved in

apoptotic signaling, as well as in control of growth and differentiation [Marshall, 1994; Waskiewicz and Cooper, 1995; Fanger *et al.*, 1997]. To date, three MAPK (serine/threonine kinases) cascades that converge on extracellular signal-regulated protein kinase (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38 MAP kinase (p38) have been well characterized. These MAPKs are regulated by distinct stimuli [Huot *et al.*, 1997]. ERK is predominantly activated by growth factors or mitogens leading to cell differentiation, growth, and survival. On the other hand, JNK and p38 are preferentially activated in response to various cytotoxic stresses, including tumor necrosis factor (TNF)-α, hydrogen peroxide (H_2O_2), UV light, X-rays, heat shock and growth factor- or serum-withdrawal, resulting in inflammation and apoptosis [Huot *et al.*, 1997; Verheij *et al.*, 1996].

Apoptosis signal-regulating kinase (ASK) 1 is a MAP kinase kinase kinase (MAPKKK), which is an upstream factor that activates the SEK1-JNK and MKK3/MKK6-p38 signaling cascades [Ichijo *et al.*, 1997]. Overexpression of ASK1 in epithelial cells in low serum conditions induced cell death with apoptotic features and ASK1-K709R, a kinase-inactive mutant of ASK1, reduced TNF-α-induced apoptosis, suggesting that ASK1 is a pivotal component in the mechanism of cytokine- and stress-induced apoptosis [Hayakawa et al., 2006; Ichijo *et al.*, 1997; Tobiume *et al.*, 1997].

AKT is a proto-oncogene which is activated by the phosphatidylinositol 3-kinase (PI3-K) survival pathway. Therefore, AKT promotes survival of cancer cells and tumor growth. AKT inhibits apoptosis by phosphorylating a number of downstream targets. This includes phosphorylation of the pro-apoptotic protein Bad and its degradation by the protein 14-3-3, therefore removing its inhibitory effect on the cell survival factor Bcl-X_L at the mitochondrial membrane [Franke *et al.*, 1997; Bellacosa *et al.*, 1998]. Thus, the inhibition of AKT activation induces apoptosis of cancer cells.

p53 is a tightly regulated transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress, such as DNA damage [Evan and Littlewood, 1998]. First described in 1979, and initially believed to be an oncogene, p53 was the first tumour suppressor gene to be identified. p53 functions to eliminate and inhibit the proliferation of abnormal cells, thereby preventing neoplastic development. Abrogation of the negative growth regulatory functions of p53 occurs in many, perhaps all, human tumours. The p53 signaling pathway is in 'standby' mode under normal cellular conditions. It is now clear that a number of phosphorylation sites on p53 are altered after DNA damage, and such phosphorylation events have been shown to result in alterations in p53 that make it more stable and more active [Voudsen, 2000]. As a transcription factor, phosphorylated p53 induces transcription of proapoptotic proteins such as Bax and Fas ligand and represses those of antiapoptotic proteins such as Bcl-2 [Bras *et al.*, 2005].

Given the widespread exposure of humans and other species to acrolein, it is important to improve our understanding of its mechanisms of toxicity. Acrolein has been shown to induce apoptosis [Tanel and Averill-Bates, 2005; Li *et al.*, 1997; Nardini *et al.*, 2002; Takeuchi *et al.*, 2001], as well as activation of MAPKs [Ranganna *et al.*, 2002; Takeuchi *et al.*, 2001; Misonou *et al.*, 2005; Pugazhenthi *et al.*, 2006; Finkelstein *et al.*, 2001; Deshmukh *et al.*, 2004]. However, the implication of MAPK proteins in the activation of acrolein-induced apoptosis has not been investigated. Since acrolein is a DNA damaging agent [Kailasam and Rogers, 2006], it could likely cause activation of p53. With the aim of advancing our knowledge about this toxic compound, the present study investigates whether acrolein-induced apoptosis is mediated by MAPK proteins and / or the p53 transcription factor.

MATERIALS AND METHODS

Cell culture

CHO cells (AuxBl) [Ling and Thompson, 1974] were grown in monolayer in minimum essential medium-Alpha (α-MEM) (Gibco Canada, Burlington, ON) plus 10% fetal bovine serum (FBS) (Gibco Canada) and 1% penicillin (50 units/mL)-streptomycin (50 μg/mL) (Flow Laboratories, Mississauga, ON), in tissue culture flasks (Sarstedt, St Laurent, QC), in a humidified atmosphere of 5% CO_2 in a water jacketed incubator at 37°C [Bates and Mackillop, 1986]. The cells were grown to near confluence and then incubated for 24 h with fresh culture medium. Confluent cells were then harvested using citrated phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate, 0.015 M sodium citrate), washed by centrifugation (1000g, 3 min) and resuspended in α-MEM plus 10% FBS for experimental studies.

Inhibitor treatment

To investigate the effect of MAPK inhibitors on acrolein-induced apoptosis, confluent cell cultures were preincubated for 1 h with one of the following inhibitors prior to addition of acrolein: 5 μM SB203580 (p38 inhibitor) [Deschesnes *et al.*, 2001; Meloche *et al.*, 2000], 10 μM U0126 (MEK1/2 activity inhibitor) [Favata *et al.*, 1998], 50 μM PD98059 (inhibitor of MEK1/2) [Daoud *et al.*, 2005], 50 μg/l Ly294002 (PI3-K inhibitor) [Meloche *et al.*, 2000], 100 ηM Wortmannin (PI3-K inhibitor) [Meloche *et al.*, 2000], or 10 μM SP600125 (JNK inhibitor). All of the inhibitors were purchased from Calbiochem (La Jolla, CA) and they were present during acrolein incubation.

Western Blot analysis

Following treatment with acrolein, cells were washed in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in lysis buffer B (20

mM 3-(N-morpholino) propanesulfonic acid, pH 7, 10% glycerol, 80 mM b-glycerophosphate, 5 mM EGTA, 0.5 mM EDTA, 1 mM sodium vanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1% Triton X-100, 1 mM dithiothreitol) and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] [Guay *et al.*, 1997]. After a 2 h incubation on ice, unbroken cells and nuclei were pelleted by centifugation at 2500 *g* for 10 min. The supernatant was used for the detection of proteins.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins was carried out according to Laemmli [Laemmli, 1970]. Proteins (50 μg) were quantified according to Bradford [Bradford, 1976] and then solubilised in Laemmli sample buffer. The samples were boiled for 5 min at 100°C and loaded onto a 10% (ASK1) or 15% acrylamide gel (all other proteins). Electrophoresis was carried out at a constant voltage of 125 V. Cellular proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Millipore, Bedford, MA) [Tanel and Averill-Bates, 2005]. The transfer buffer contained 96 mM glycine, 10 mM Tris and 10% methanol. The transfer was carried out for 1.5 h at constant amperage of 80 mA/gel. Hydrophobic or nonspecific sites were blocked overnight at 4°C with 5% powdered skim milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.1% Tween 20 (TBS-T). Membranes were washed four times for 15 min in TBS-T. The blots were probed with the primary antibodies: anti-c-jun, anti-phospho-c-jun, anti-AKT, anti-phospho-AKT, anti-ERK1, anti-ERK2, anti-phospho-ERK, anti-p38, anti-phospho-p38 and anti-ASK1 (Stressgen, San Diego, CA), anti-ICAD (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53, anti-phospho-p53 (S15) and anti-phospho-p53 (S46) (Calbiochem, La Jolla, CA) in TBS-T, 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed four times for 15 min and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (1:1000) in

TBS-T containing 5% milk powder. Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-mouse, anti-rabbit and anti-goat IgG (Biosource, Camarillo, CA). PVDF membranes were washed four times for 15 min, proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA) and films (Fuji medical X-ray film, Düsserldorf, Germany) were scanned with a Laser Scanning Densitometer (Alpha Innotech Corp., San Leandro, CA). Protein expression in whole cell lysates was quantified using IPGEL software, relative to β-tubulin loading controls.

Morphological analysis of apoptosis

To visualize nuclear morphology and chromatin condensation by fluorescence microscopy [Lee and Shacter, 1999], cells were seeded and cultured to near confluence in tissue culture dishes containing 5mL of α-MEM and 10% FBS. Where appropriate, cells were pretreated for 1 h with a specific MAPK inhibitor and then incubated with acrolein for 4 h with inhibitor present. Dishes were washed twice with PBS and Hoechst (33258) (blue fluorescence) (0.06 mg/mL) was added for 15 min at 37^0C to stain apoptotic cells [Tanel and Averill-Bates, 2005]. The dishes were washed with PBS and propidium iodide (red fluorescence) (50 μg/mL) was added to stain the necrotic cells. Observations were made by fluorescence microscopy (Carl Zeiss Ltd, Montreal, QC, Canada) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc, Mississauga, ON). Images were analysed by Northern Eclipse software. Cells were classified using the following criteria: a) live cells (normal nuclei, pale blue chromatin with organized structure); b) membrane-intact apoptotic cells (bright blue condensed or fragmented chromatin); c) necrotic cells (red, enlarged nuclei with smooth normal structure [Lee and Shacter, 1999]. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). A minimum of 600 cells was counted per dish.

Determination of caspase activity by fluorescence spectroscopy

Freshly harvested CHO cells (0.5 x 10^6) were resuspended in α-MEM plus 10% FBS and incubated with acrolein in a final volume of 1.0 mL at 37^0C. After the appropriate time, the cells were washed three times with cold PBS by centrifugation (1000g, 3 min) to stop the incubation. The cells were resuspended in 50 µl of PBS and 25 µl were deposited into 96-well plates and lysed by freezing at -20^0C for 20 min. Fifty µl of reaction buffer (20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) was added and stabilized at 37^0C [Stennicke and Salvesen, 1997]. The kinetic reaction was started after addition of 25 µl of the appropriate caspase substrate (Calbiochem) at 37°C using a spectrofluorimeter (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA) [Tanel and Averill-Bates, 2005]. Caspase-9 activity was measured by cleavage of the substrate Ac-LEHD-AFC to produce 7-amino-4-trifluoromethylcoumarin (AFC) with λmax excitation at 415 nm and λmax emission at 490 nm. Caspase-8 activity was measured by cleavage of the fluorogenic substrate Z-IETD-amino-4-trifluoromethylcoumarin (caspase-8 substrate II) to produce AFC. Caspase-7 activity was measured by cleavage of the fluorogenic substrate I MCA-VDQVDGWK(DNP)-NH_2 with λmax excitation at 325 nm and λmax emission at 395 nm.

Statistical analysis

Statistical differences between control and treated groups were determined using a one-way ANOVA which measures the linear contrast of means. An adjustment was made to limit the familywise error rate (FWE) to 5% by calculating an adjusted *p*-value which is a simulated based *p*-value obtained from the multivariate *t* distribution (number of simulations = 1 000 000) [Westfall et al., 1999]. Then, the

Bonferroni-Holm method (a stepwise method) was used to control the FWE. For comparison between a treatment with acrolein in presence or absence of an inhibitor, statistical differences were determined by a one-way ANOVA followed by Dunnet bilateral adjustment. For Fig. 7 a two-tailed unpaired Student's *t* test was used.

Values are expressed as means ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Acrolein-induced apoptosis is mediated by activation of MAPKs

It was reported that acrolein can activate MAPKs but their role in induction of apoptosis is not known. Therefore, this study investigates the possible implication of MAPK activation in acrolein-induced apoptosis. The changes in phosphorylation of these kinases were examined following acrolein treatment in CHO cells by Western blot analysis using phospho-specific antibodies. Treatment of cells with acrolein (30, 50 *f*mol/cell) for 1 h caused phosphorylation of both p38 (Fig. 1A, 1C) and ERK MAPKs (Fig. 1D, 1F). The expression of p38 (Fig. 1A, 1B) and ERK1/2 (Fig. 1D, 1E) in acrolein-treated cells did not change from the control. Although several publications have demonstrated that activation of the JNK and c-jun pathway leads to cell death in brain ischemia [Whitfield *et al.*, 2001; Domanska-Janik *et al.*, 2004], the role of c-jun expression and activation by extracellular stimuli such as acrolein has not been studied. Thus, we examined by Western blot analysis the phosphorylation of the downstream JNK substrate c-jun by using anti-c-jun and phospho-c-jun antibodies, respectively. Exposure to acrolein caused phosphorylation of c-jun (Fig. 1G, 1I), whereas expression of c-jun (1G, 1H) was unchanged.

Apoptosis signal regulating-kinase (ASK1) is activated in response to various cytotoxic stresses including TNF, Fas and reactive oxygen species such as hydrogen peroxide. It is an important upstream activator of JNK and p38. Since, p38 and c-jun were phosphorylated by acrolein, we assessed whether acrolein could activate ASK1. Indeed, there was a significant increase in expression of ASK1 upon exposure of cells to acrolein for 1 h (Fig. 1K).

Given that several MAPKs were activated by phosphorylation following exposure to acrolein, we subsequently determined whether there is a link between MAPKs and induction of apoptosis. Therefore, we examined whether inhibition of these kinases would result in inhibition of acrolein-induced apoptosis. Exposure of

cells to 50 *f*mol/cell of acrolein for 4 h caused both necrosis and chromatin condensation (Fig. 2B), a later event in the apoptotic cascade, relative to untreated controls (Fig. 2A). Pretreatment of cells with SB203580, a p38 inhibitor (Fig. 2C), or U0126, an inhibitor of ERK (Fig. 2D), significantly reduced the number of acrolein-induced apoptotic cells from 29.26 ± 8.12 to 5.04 ± 0.91 and 7.82 ± 1.67, respectively (Fig. 2G). Moreover, SB203580 and U0126 afforded protection against necrotic cell death induced by acrolein (Fig. 2C, 2D, 2H). PD98059, another ERK inhibitor, also reduced acrolein-induced apoptosis (Fig. 2E, 2G), but to a lesser extent than the ERK inhibitor U0126. However, inhibition of ERK with PD98059 did not affect acrolein-induced necrosis (Fig. 2H). When cells were pretreated with the JNK inhibitor, SP600125, acrolein-induced cell death was switched from apoptosis to total necrosis (Fig. 2F, 2G, 2H). Together, these findings suggest that acrolein-induced activation of MAPKs is linked to the induction of apoptosis by this toxic compound.

Acrolein-induced caspase activation is mediated by MAPKs

We subsequently investigated whether there could be a possible link between the MAPK signaling cascade and other apoptotic events such as caspase activation, during acrolein-induced apoptosis. It was shown that caspase-3 is involved in MAPK-mediated apoptosis caused by stress-inducing agents such as cisplatin [Hershberger *et al.*, 2002]. It was previously reported that caspases-9 and -7 are involved in acrolein-induced apoptosis, while caspase-3 was inhibited [Tanel and Averill-Bates, 2005]. Exposure of cells to 50 *f*mol/cell of acrolein caused activation of initiator caspases-9 (Fig. 3A) and -8 (Fig. 3B), and executor caspase-7 (Fig. 3C), relative to untreated controls. Pretreatment of cells with a p38 inhibitor, SB203580, the ERK inhibitors, U0126 and PD98059, or a JNK inhibitor, SP600125 inhibited activation of the initiator caspase-9 (Fig. 3A), as well as the effector caspase-7 (Fig. 3C). Furthermore, all of the four inhibitors inhibited cleavage of the caspase substrate ICAD by acrolein (Fig. 3D, 3E). Caspase-8 activation by acrolein was not inhibited by any of the

MAPK inhibitors (Fig. 3B). These results suggest that acrolein-induced caspase activation is mediated in part by p38, JNK and ERK MAPKs.

Acrolein induces activation of the AKT survival pathway

Given that acrolein activates the MAPKs, which are involved in cellular stress responses, we subsequently determined whether acrolein could affect the survival pathways in cells. We examined whether acrolein could affect the AKT signaling pathway. Indeed, exposure of cells to acrolein (30, 50 *f*mol/cell) resulted in a significant increase in AKT phosphorylation (Fig. 4A, 4C), whereas expression of total AKT was not affected (Fig. 4A, 4B). It is likely that inhibition of the AKT survival pathway could increase the susceptibility of cells to acrolein-induced cell death. The pharmacological inhibitors of the PI3K/AKT signaling pathway, Ly294002 (Fig. 5C) and Wortmannin (Fig. 5D), inhibited acrolein-induced apoptosis (chromatin condensation) and switched the mode of cell death from apoptosis (Fig. 5B, 5E) to near total necrosis (Fig. 5C, 5D, 5F). Moreover, Ly294002 and Wortmannin inhibited caspase-9 (Fig. 6A) and -7 activation (Fig. 6C) by acrolein. In contrast, these inhibitors didn't affect caspase-8 activation by acrolein (Fig. 6B). Ly294002 and Wortmannin also inhibited cleavage of ICAD by acrolein (Fig. 6D, 6E). These findings suggest that the activation of AKT by acrolein may be at least in part the result of a defensive response by the cell which activates a survival pathway to protect against a cytotoxic aggression.

Activation of p53 during acrolein-induced apoptosis

The role of p53 as a transcription factor has been extensively described, as have many transcriptional target genes implicated in apoptosis, which include Bcl-2 proteins such as Bax and Bcl-2 [Attardi *et al.*, 2000]. The phosphorylation status of p53 plays an important role in the pro-apoptotic activity of this protein. Thus, the ability of acrolein to phosphorylate p53 was investigated. Immunoblots using anti-

phospho Ser^{15}p53 and an anti-phospho Ser^{46}p53 antibodies indicated that acrolein (50 *f*mol/cell) caused p53 phosphorylation (Fig.7) under apoptotic conditions.

DISCUSSION

This study shows for the first time that acrolein-induced apoptosis in proliferating cells is in part MAPK-dependent. Acrolein-induced apoptosis was described previously in various cell types [Tanel and Averill-Bates, 2005; Li *et al.*, 1997; Nardini *et al.*, 2002; Takeuchi *et al.*, 2001]. Activation of MAPKs by acrolein was also reported in various cell types [Ranganna *et al.*, 2002; Takeuchi *et al.*, 2001; Misonou *et al.*, 2005; Pugazhenthi *et al.*, 2006; Finkelstein *et al.*, 2001; Deshmukh *et al.*, 2004]. However, previous studies did not address any possible links between activation of MAPK proteins in acrolein-induced apoptosis. This study clearly shows that acrolein-induced apoptosis occurred through activation of the ASK1/p38, and ERK signaling pathways as upstream events for activation of apoptotic molecules such as caspases -9 and -7, cleavage of ICAD and condensation of nuclear condensation.

Acrolein was shown to mediate apoptosis via the initiator caspase-9 and the effector caspase-7, rather than through caspase-3, in CHO cells [Tanel and Averill-Bates, 2005]. Several studies have reported that acrolein inhibits activity of the major effector caspase-3 [Tanel and Averill-Bates, 2005; Kern and Kehrer, 2002; Finkelstein *et al.*, 2005], probably through inhibition of its active site cysteine residue. The effector caspase-7 can be activated by both of the initiator caspases, caspase-8 and caspase-9 [Slee *et al.*, 1999]. Both of the effector caspases -3 and -7 share similar protein substrates and therefore caspase-7 is able to cleave caspase substrates such as ICAD and liberate CAD, which is responsible for chromatin condensation during the execution phase of apoptosis [Houde *et al.*, 2004].

Three distinct MAPKs, JNK, p38, and ERK have been characterized and reported to be involved in apoptosis in many different paradigms of cellular toxicity. Although MAPKs play an important role in apoptosis, this is the first study to show that they play a role in acrolein-induced apoptosis. Acrolein was able to activate the JNK substrate, c-jun, by phosphorylation. However, inhibition of JNK activity by

SP600125 blocked acrolein-induced apoptosis, but instead, the mode of cell death was switched to necrosis. In addition, SP600125 inhibited acrolein-induced activation of caspases-7 and -9 as well as ICAD cleavage. This can be expected since caspases are not implicated in cell death by necrosis. Our findings show that JNK is activated, but appears to be implicated in a cell survival role under acrolein aggression. Others studies showed that JNK is involved in apoptosis of lung carcinoma cells [Chuang *et al.*, 2000] and murine macrophage cells [Kim and Sharma, 2004], while it was not associated with apoptosis in HT4 neuronal cells [Rockwell *et al.*, 2004] where p38 plays a major role. Moreover, JNK is also important for the development and survival of macrophages [Himes *et al.*, 2006]. Thus, the function of JNK in apoptosis is controversial since it can play a role both as an activator of apoptosis or in survival pathways.

The MAPK p38 also appears to be implicated in the activation of acrolein-induced apoptosis. Acrolein induced activation of p38 by phosphorylation in cells and SB203580, a p38 kinase inhibitor, inhibited acrolein-induced apoptosis through inhibition of chromatin condensation. Furthermore, SB203580 inhibited activation of caspases-9 and -7 by acrolein and cleavage of the caspase substrate ICAD. There was no increase in cell death by necrosis, as was the case for JNK inhibition, but rather a decrease. These findings indicate that p38 plays a pro-apoptotic role in acrolein-induced apoptosis. Although stress stimuli usually activate p38, which can lead to apoptosis in some cellular models, it seems to be irrelevant in others. Activation of p38 was required for apoptosis induced by cadmium, trophic factor withdrawal and ischemia [Galan *et al.*, 2000; Kummer *et al.*, 1997; Mackay and Mochly-Rosen, 1999]. On the other hand, SB203580 inhibition of p38 was unable to prevent apoptosis induced by UV and *S*-nitrosoglutathione [Franklin *et al.*, 1998; Callsen and Brune, 1999], which highlights the contradictory correlation of p38 with apoptosis. Similar contradictory results were obtained in a recent study in HT4 neuronal cells, where inhibition of p38, but not JNK, blocked apoptosis induced by cadmium

[Rockwell *et al.*, 2004]. This is consistent with our study, where inhibition of p38, but not JNK, blocked acrolein-induced apoptosis. These discrepancies suggest the existence of marked differences in regulation of the stress responses depending on cell types, albeit it is difficult to explain these differences at the moment. Whether JNK and / or p38 is implicated in apoptosis could also depend on the particular cellular insult in a given cell type.

In addition to activation by a variety of growth factors, cytokines, and mitogens, ERKs are also known to be activated by cytotoxic insults. This study shows that ERK seems to be implicated in induction of apoptosis by acrolein. Acrolein induced phosphorylation of ERK and the inhibition of its activity by U0126 and PD98059, inhibited acrolein-induced apoptosis, characterized by decreased chromatin condensation. Moreover, U0126 and PD98059 inhibited activation of caspase-9 and -7 and cleavage of ICAD by acrolein. PD 98059 and U0126 are MEK inhibitors, and both are useful for probing the role of MEK in various biological processes, particularly those involving activation of the downstream substrate ERK. Both inhibitors are noncompetitive with respect to the downstream MEK substrates adenosine triphosphate (ATP) and ERK [Favata *et al.*, 1998]. Although the binding sites for PD 98059 and U0126 on MEK1 appear to overlap, their mechanisms of action differ [Dudley *et al.*, 1995; Favata *et al.*, 1998]. U0126 is a direct inhibitor of MEK activity, whereas PD 98059 inhibits MEK activation. Therefore, U0126 is a much stronger inhibitor of the ERK pathway [Dudley *et al.*, 1995; Favata *et al.*, 1998]. Researchers can exploit the differences and similarities of these two compounds. For example, if an effect is inhibited by both of these structurally and mechanistically distinct inhibitors, a much stronger argument can be made for MEK playing a role, than if only a single inhibitor was used. Furthermore, the selectivity of PD 98059 for MEK1 over MEK2 compared to the lack of preference for either by U0126 can be exploited to probe the relative roles of the two kinases. Cellular ERK activation either inhibits or causes no effect on apoptosis in some cell types [Chuang

et al., 2000; Galan *et al.*, 2000], but enhances apoptosis in others [Lee *et al.*, 2006; Ruffels *et al.*, 2004; Yang *et al.*, 2004]. Even in the same cell type, the role of ERK activation is likely to differ, depending on cellular insults. In SH-SY5Y human neuroblastoma cells, for example, hydrogen peroxide induced apoptosis which was inhibited by an ERK inhibitor [Ruffels *et al.*, 2004], while cadmium caused no changes in ERK activation in the same cell line [Kim *et al.*, 2005]. This suggests that different cytotoxic insults are likely to be linked to different signaling molecules.

Among the MAPK kinase kinases (MAPKKKs), ASK1 is known to be activated in response to various cytotoxic stresses, including reactive oxygen species (ROS). Acrolein causes severe depletion of the antioxidant glutathione [Kern and Kehrer, 2002], which could lead to an increase in cellular oxidative stress. Indeed, acrolein caused an increase in intracellular reactive oxygen species in brain mitochondria [Luo and Shi, 2005]. Thiol compounds such as glutathione and N-acetylcysteine were shown to reduce acrolein-induced toxicity [Nunoshiba and Yamamoto, 1999]. ASK1 can induce activation of the MAPKs, JNK and p38, as well as apoptosis [Tobiume *et al.*, 2001]. Our findings clearly show that ASK1 was induced under conditions where acrolein can cause apoptosis. ASK1 could be responsible for activation of its downstream targets p38 and JNK in acrolein-induced apoptosis.

AKT (protein kinase B or PKB) has been identified as a downstream target of growth factor receptor activation. It is probably the most characterized kinase known to promote cell survival and to play a significant role in glucose metabolism [Faber *et al.*, 2006]. AKT was phosphorylated by acrolein and its inhibition by the inhibitors, LY294002 and Wortmannin, switched the mode of cell death from apoptosis to necrosis. LY294002 and Wortmannin are both inhibitors of the lipid-modifying enzymes known as phosphoinositide 3-kinases (PI3-K), and both are useful for probing the role of the PI3-K/AKT pathway in biological processes [Stein and Waterfield, 2000; Fruman, 1998]. Moreover, these inhibitors significantly decreased

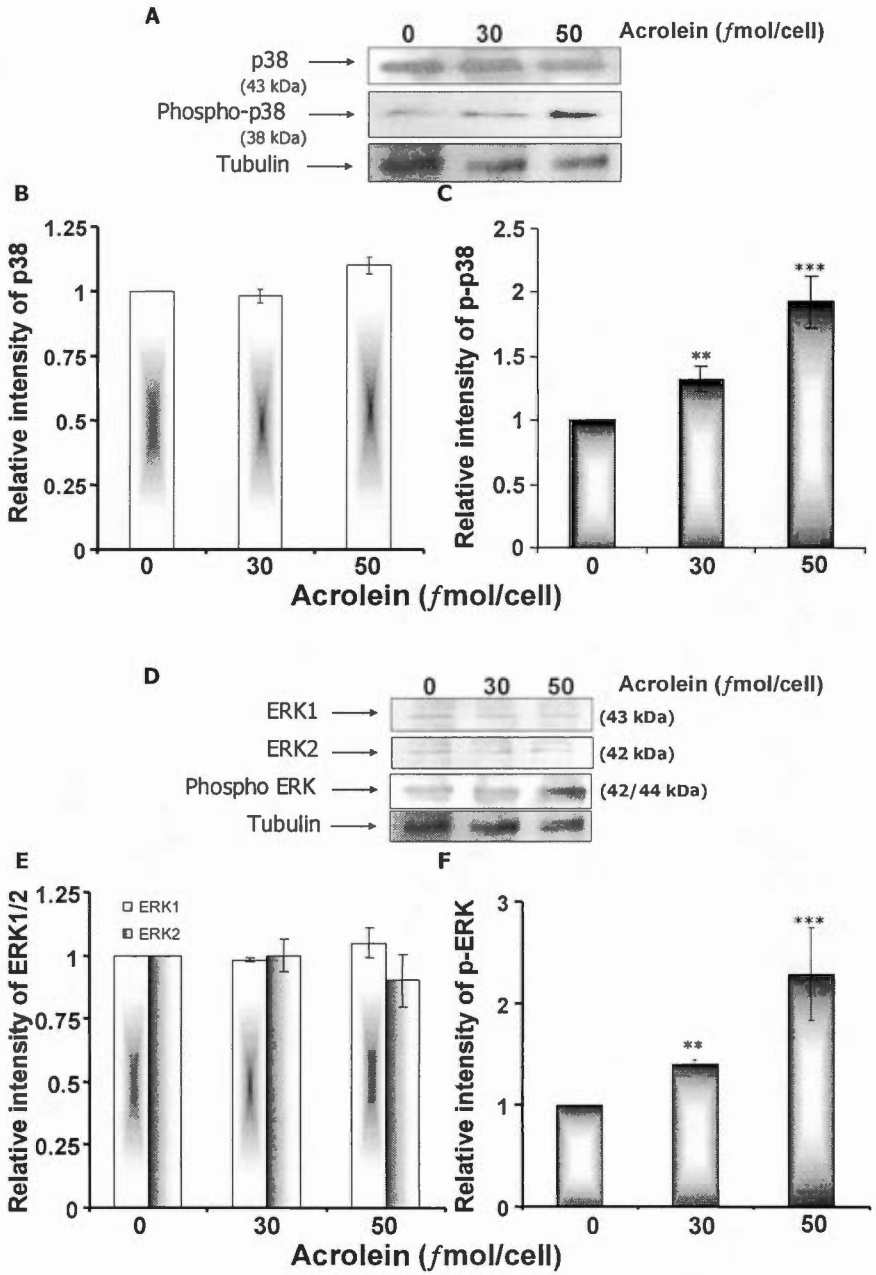
activation of caspases-9 and -7, as well as ICAD cleavage induced by acrolein. Activation of AKT by phosphorylation prevents the pro-apoptotic action of the Bcl-2 family protein Bad at the mitochondrial membrane, by promoting its degradation. Bad regulates apoptosis upstream from mitochondrial-mediated caspase-9 activation. These results show that AKT is implicated in the cell survival response against an acrolein insult. Akt/PKB is activated in response to many different growth factors, hormones, and external stresses such as heat shock and osmolarity [Datta *et al.*, 1999; Lawlor and Alessi, 2001; Talapatra and Thompson, 2001; Downward, 1998; Brazil and Hemmings, 2001].

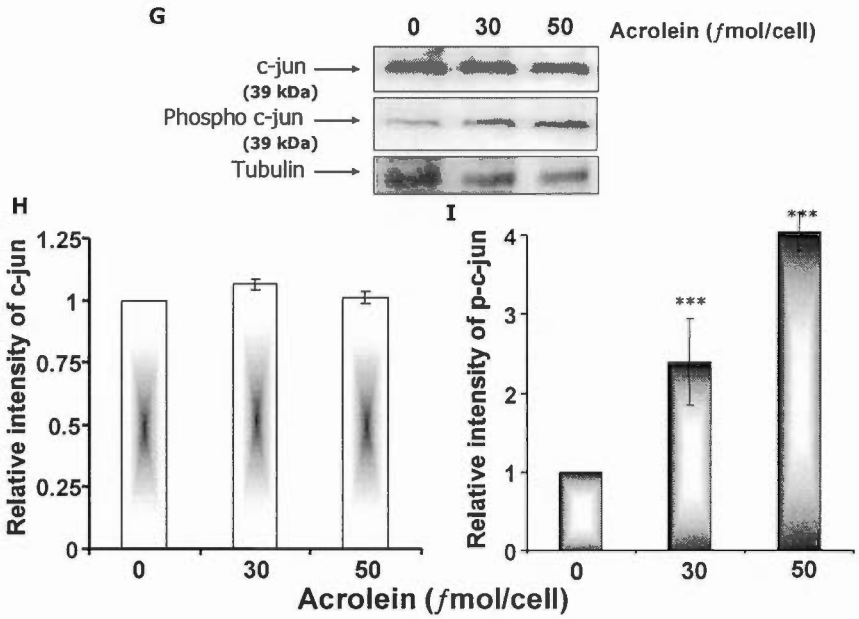
The last novel aspect of this study is that acrolein, which is a DNA damaging agent [Esterbauer *et al.*, 1991; Canman *et al.*, 1998; Bulavin *et al.*, 1999], can induce phosphorylation of p53. Our data documented for the first time this posttranslational modification of p53 at p53 $Ser^{15}P$ and p53 Ser^{46} in acrolein-treated cells. The p53 tumor suppressor protein is activated and phosphorylated at different serine residues such as serine-15 and serine-46 in response to various DNA damaging agents such as UV radiation [Bulavin *et al.*, 1999]. Phosphorylation of p53 on Ser^{15} reduces binding of the mdm2 oncogene product to p53 in vitro [Shieh *et al.*, 1997], and binding of mdm2 to p53 promotes rapid degradation of p53 by targeting it for proteolytic degradation, thereby potentially controlling p53 protein levels [Haupt *et al.*, 1997]. Effectively, acrolein induced p53 phosphorylation at serine-15, and as expected, serine-46 was also phosphorylated, since activated p38 has been shown to target serine-46 [Bulavin *et al.*, 1999]. The downstream events mediated by p53 take place by two major pathways: cell cycle arrest and apoptosis. As a transcription factor, phosphorylated p53 induces transcription of proapoptotic proteins such as Bax and Fas ligand and represses those of antiapoptotic proteins such as Bcl-2 [Bras *et al.*, 2005].

In conclusion, the present study identified p53, p38 and ERK as key molecules involved in acrolein-induced apoptosis and that CHO cells appear to use

the AKT/PKB and the JNK MAPK survival pathways to overcome cell death induced by acrolein. These findings are relevant to the toxicity of acrolein in many contexts, including the pharmacological action and/or toxic side effects of the anticancer agent cyclophosphamide, the regulation of cellular proliferation and tumor growth by polyamines, as well as the toxicity of environmental exposures to low doses of acrolein.

Figure 1: Acrolein induces activation of p38 and ERK MAPKs, the JNK substrate c-jun and ASK1




G
0
30
50
Acrolein (fmol/cell)
c-jun
(39 kDa)
Phospho c-jun
(39 kDa)
Tubulin
H
Relative intensity of c-jun
1.25
1
0.75
0.5
0.25
0
0
30
50
Acrolein (fmol/cell)
I
Relative intensity of p-c-jun
4
3
2
1
0
***
***
0
30
50

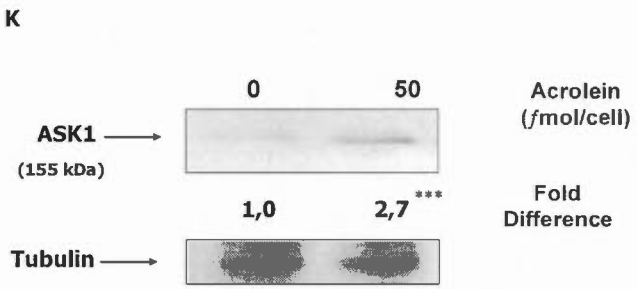
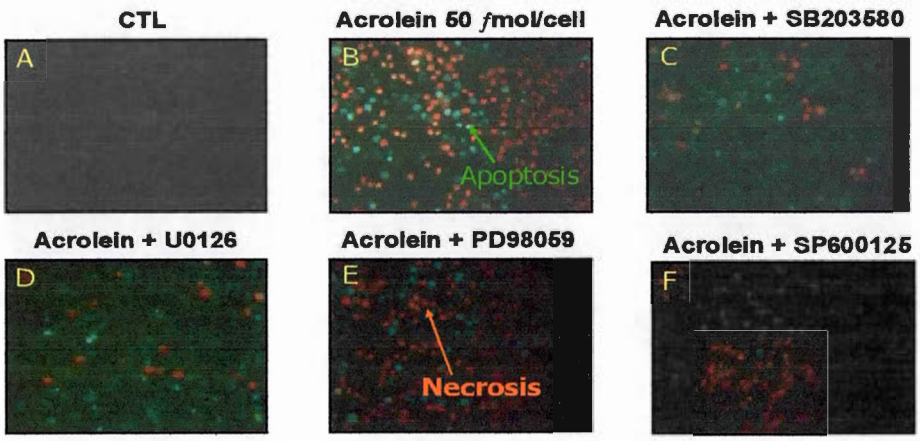

K
0
50
Acrolein
(fmol/cell)
ASK1
(155 kDa)
1,0
2,7
***
Fold
Difference
Tubulin

Figure 2: Inhibition of p38 and ERK MAPKs decreases acrolein-induced chromatin condensation



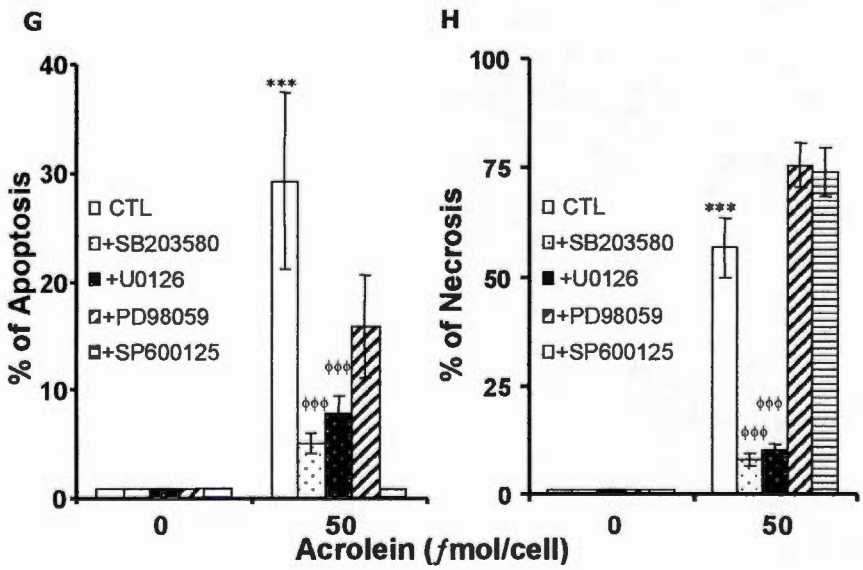
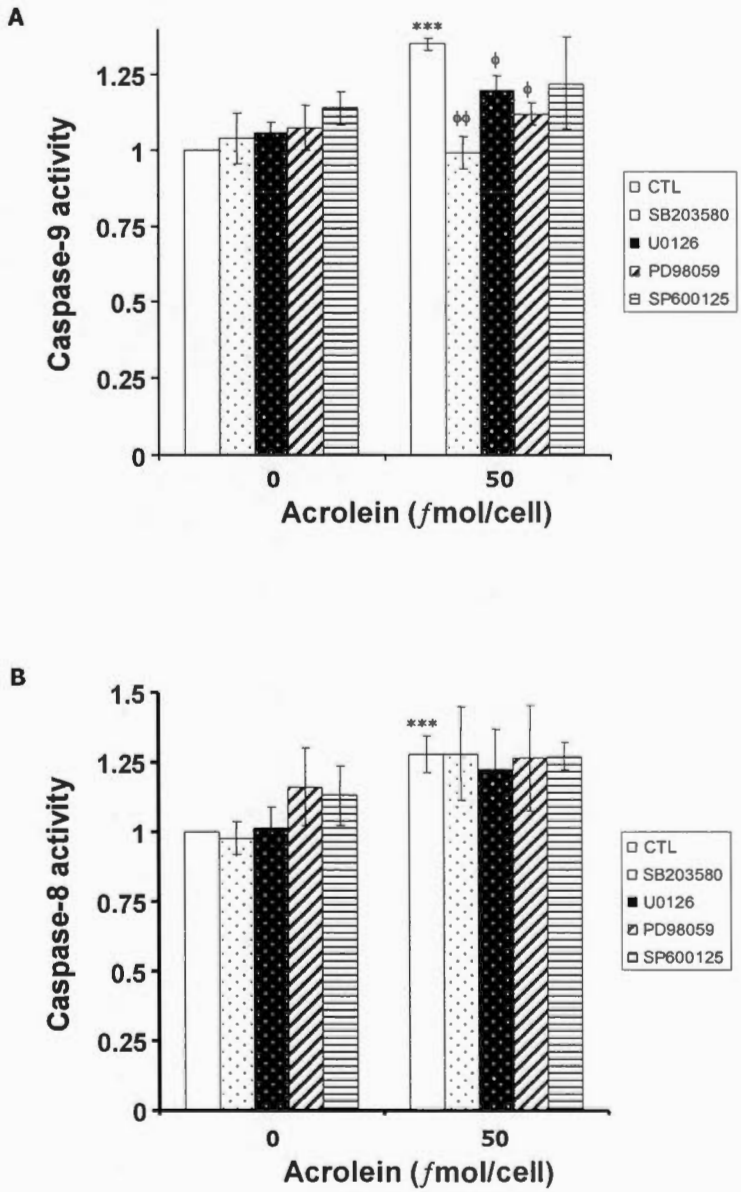


Figure 3: Acrolein-induced activation of caspase-9 and caspase-7 and cleavage of ICAD are decreased by inhibitors of p38 and ERK MAPKs



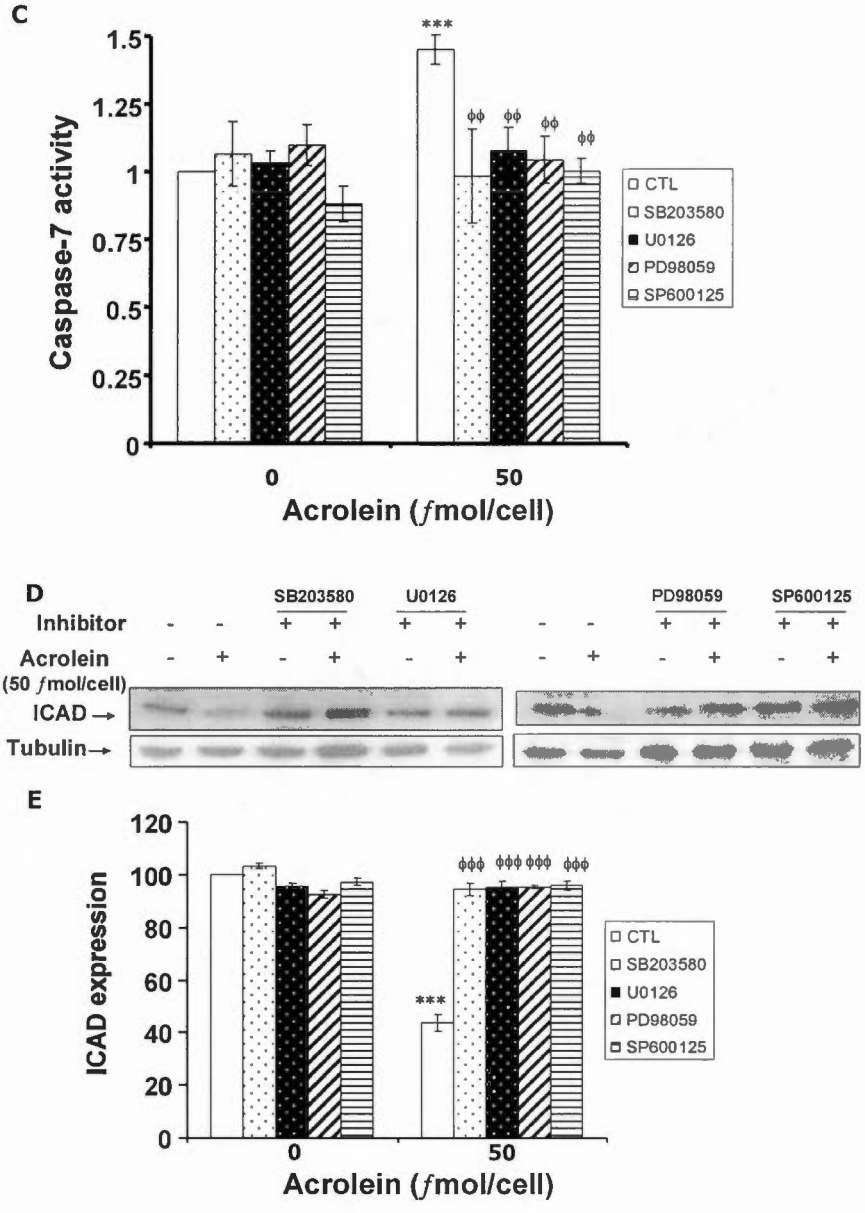

C
Caspase-7 activity
1.5
1.25
1
0.75
0.5
0.25
0
***
ϕϕ
ϕϕ
ϕϕ
ϕϕ
CTL
SB203580
U0126
PD98059
SP600125
0
50
Acrolein (fmol/cell)
D
Inhibitor
SB203580
U0126
PD98059
SP600125
Acrolein
(50 fmol/cell)
ICAD
Tubulin
E
ICAD expression
120
100
80
60
40
20
0
***
ϕϕϕ
ϕϕϕ
ϕϕϕ
ϕϕϕ
CTL
SB203580
U0126
PD98059
SP600125
0
50
Acrolein (fmol/cell)

Figure 4: Acrolein induces activation of AKT

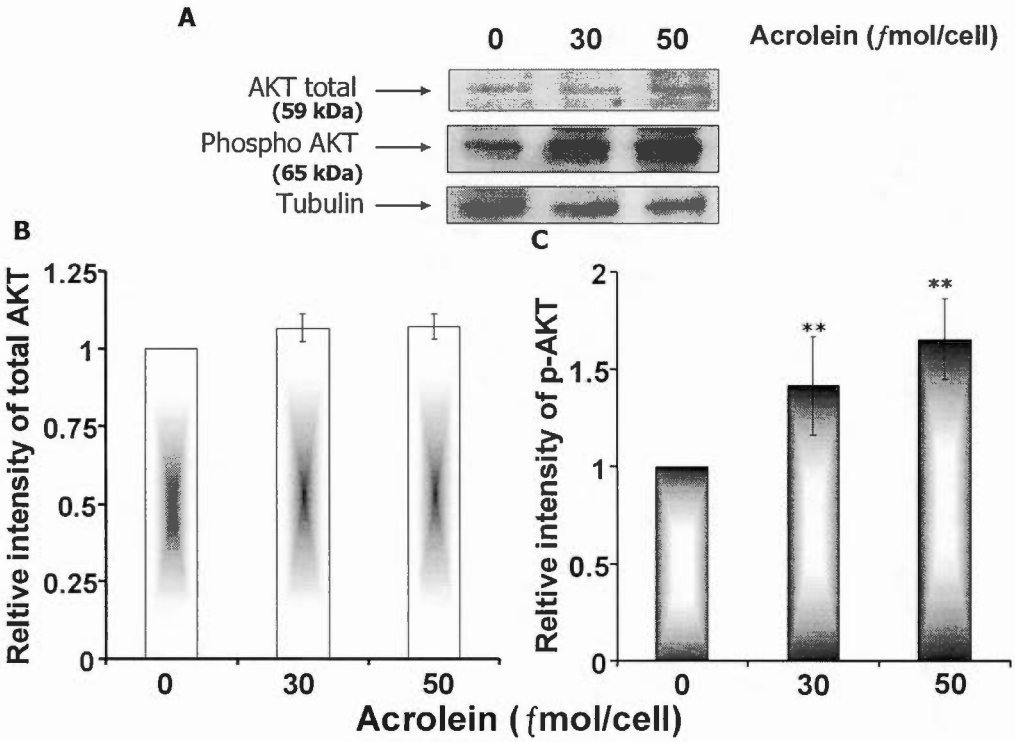
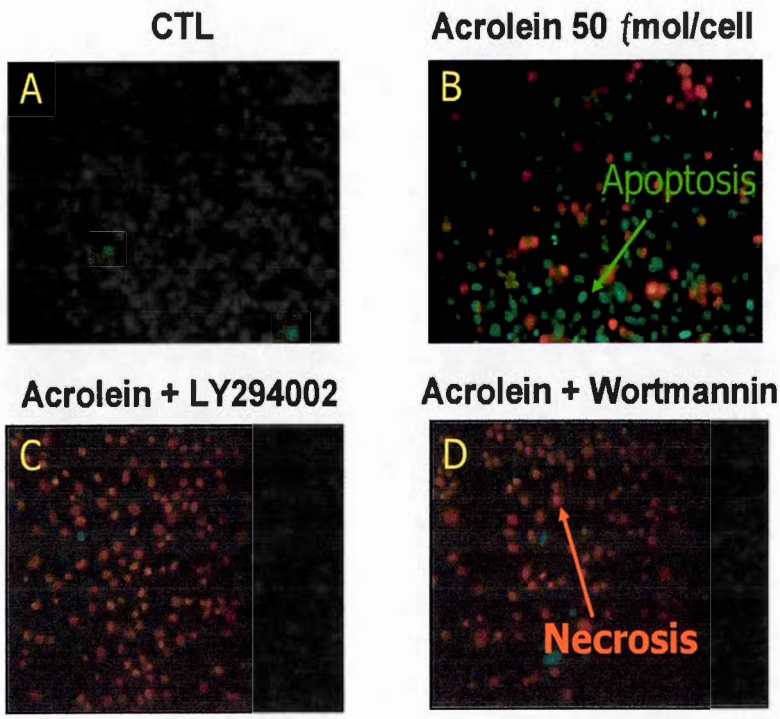


Figure 5: Inhibition of PI3K/AKT pathway by LY294002 or Wortmannin enhances necrosis induced by acrolein



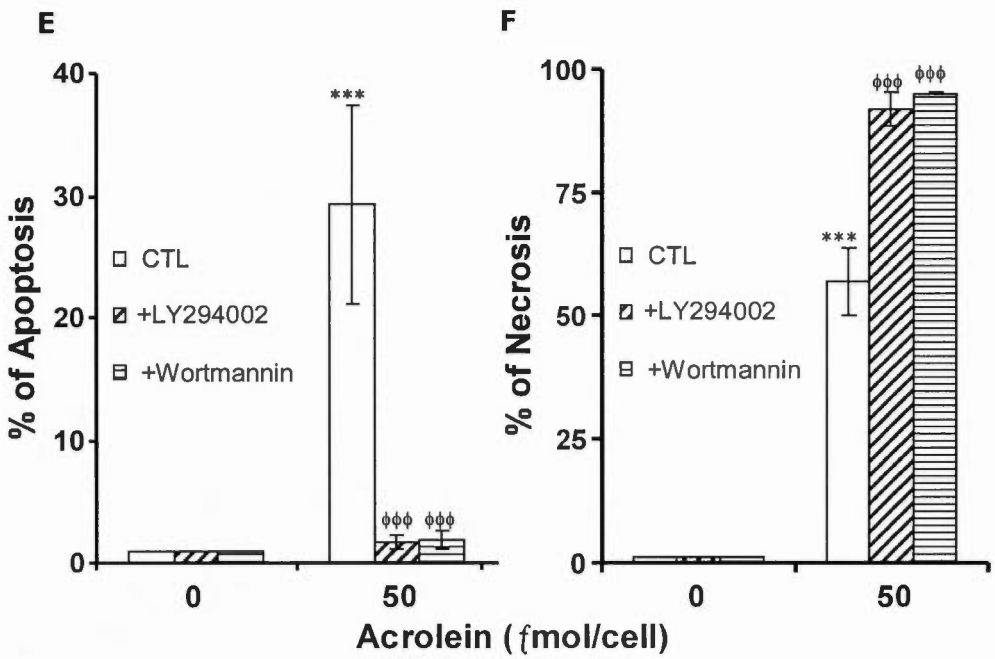
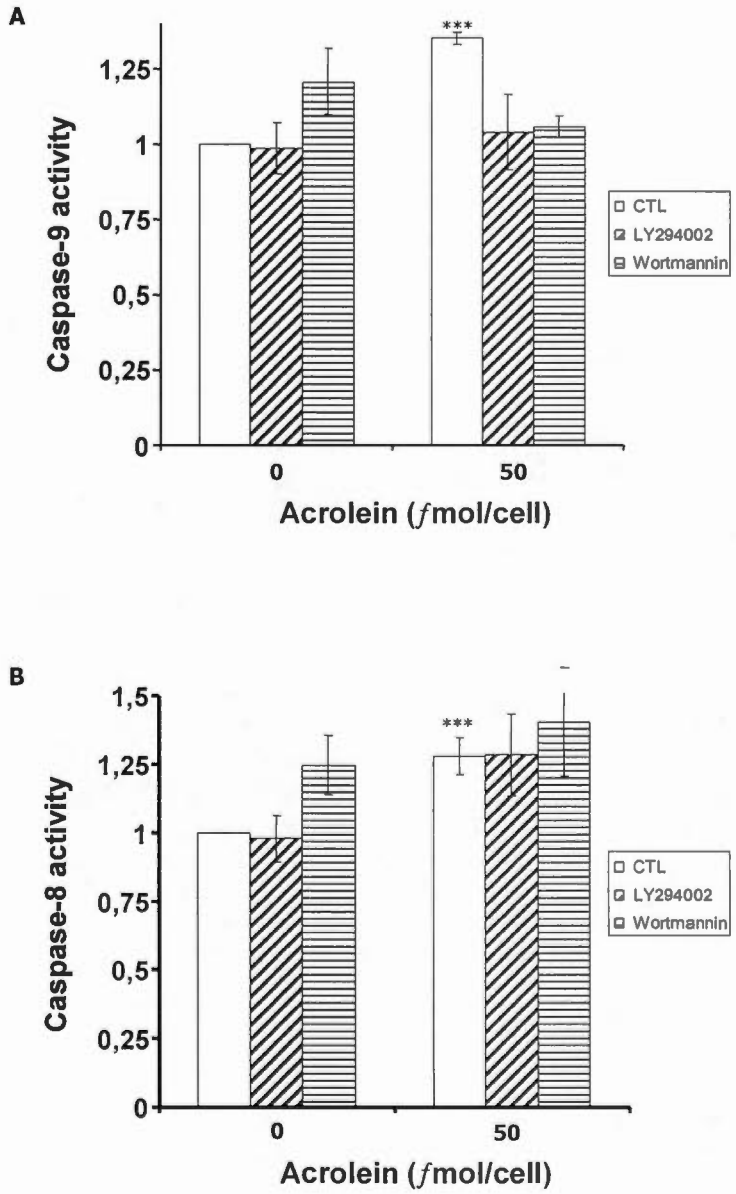

E
F
40
30
20
10
0
% of Apoptosis
CTL
+LY294002
+Wortmannin
***
ϕϕϕ
ϕϕϕ
100
75
50
25
0
% of Necrosis
CTL
+LY294002
+Wortmannin
***
ϕϕϕ
ϕϕϕ
0
50
0
50
Acrolein (fmol/cell)

Figure 6: Effects of AKT inhibitors on acrolein-induced activation of caspases-9, -8 and -7 and cleavage of ICAD



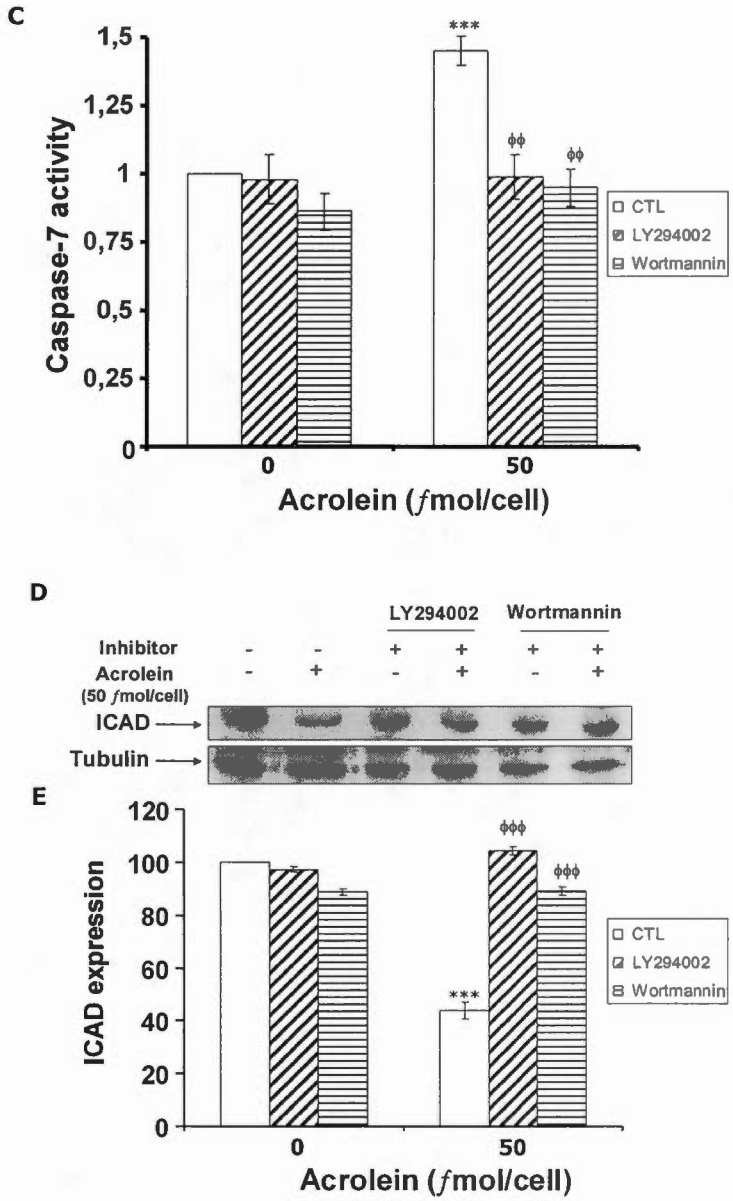

C
Caspase-7 activity
1,5
1,25
1
0,75
0,5
0,25
0
***
ϕϕ
ϕϕ
0
50
Acrolein (fmol/cell)
CTL
LY294002
Wortmannin
D
LY294002
Wortmannin
Inhibitor
Acrolein
(50 fmol/cell)
ICAD
Tubulin
E
ICAD expression
120
100
80
60
40
20
0
ϕϕϕ
ϕϕϕ
***
0
50
Acrolein (fmol/cell)
CTL
LY294002
Wortmannin

Figure 7: Acrolein induces phosphorylation of p53 at ser15 and ser46 as well as activation of ASK1

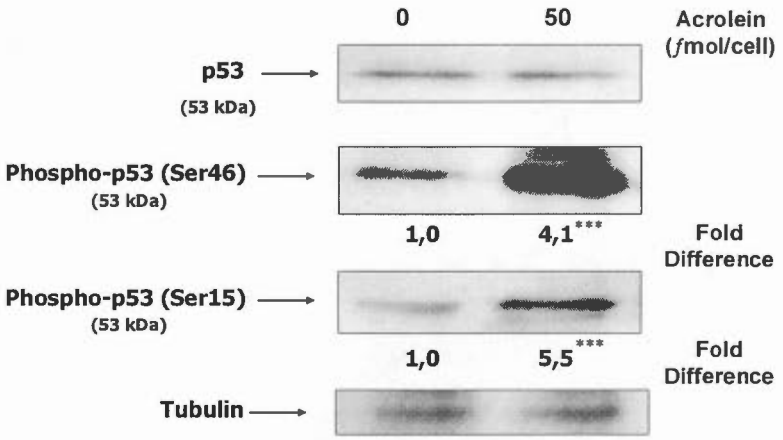


FIGURE LEGENDS

Fig. 1 Acrolein induces activation of p38 and ERK MAPKs, the JNK substrate c-jun, and ASK1. Cells (10^6/mL) were incubated with acrolein for 1 h and immunodetection of (A) p38 and p-p38, (D) ERK1/2 and p-ERK, (G) c-jun and p-c-jun and (K) ASK1 was carried out by Western blotting, using β-tubulin as a loading control. Representative blots are shown from five independent experiments. Densitometric analyses of the expression of (B) p38, (C) p-p38, (E) ERK1/2, (F) p-ERK, (H) c-jun, (I) p-c-jun and (K) ASK1 are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control.

Fig. 2. Inhibition of p38 and ERK MAPKs decreases acrolein-induced chromatin condensation. Confluent cells in monolayer were pre-incubated for 1 h with (C) 5 μM SB203580 (p38 MAPK inhibitor), (D) 10 μM U0126 (MEK1/2 activity inhibitor), (E) 50 μM PD98059 (inhibitor of MEK1/2 activation), or (F) 10 μM SP600125 (JNK inhibitor) before addition of acrolein. Cells were then incubated with (A) 0 (CTL) or (B-F) 50 *f*mol/cell of acrolein for 4 h. The fractions of (G) apoptotic and (H) necrotic cells are given relative to total cells. Apoptotic cells were visualized by fluorescence microscopy using Hoechst dye (blue fluorescence) and necrotic cells using propidium iodide (red fluorescence) (magnification 320x). Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.001$ ($\phi\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without inhibitor.

Fig. 3. Acrolein-induced activation of caspase-9 and caspase-7 and cleavage of ICAD are decreased by inhibitors of p38 and ERK MAPKs. (A, B, C, D) Pretreatment of cells with 5 μM SB203580 (p38 MAPK inhibitor), 10 μM U0126 (MEK1/2 activity inhibitor), 50 μM PD98059 (inhibitor of MEK1/2 activation), or 10 μM SP600125 (JNK inhibitor) was performed for 1h before addition of acrolein. CHO cells ($0.5x10^6$/mL) were then incubated with acrolein (50 *f*mol/cell) for (A, B) 1 h or (C) 2 h in α-MEM with 10% FBS. Activities of (A) caspase-9, (B) caspase-8 and (C) caspase-7 were measured in cell lysates using the fluorescent substrates Ac-LEHD-AFC, Z-IETD-AFC and MCA-VDQVDGWK(DNP)-NH_2, respectively. Caspase activity was expressed relative to the untreated control, designated as 1. Data represent means and SEM from eight independent experiments performed with multiple estimations per point. (D) Cells (10^6/mL) were incubated with acrolein for 2 h and immunodetection of ICAD was carried out by Western blotting, using β-tubulin as a loading control. A representative blot is shown from five independent experiments. Densitometric analysis of the expression of (E) ICAD is relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P<0.05$ (ϕ), $P<0.01$ (ϕϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without inhibitor.

Fig. 4 Acrolein induces activation of AKT. (A) Cells (10^6/mL) were incubated with acrolein for 1 h and immunodetection of AKT and p-AKT was carried out by Western blotting, using β-tubulin as a loading control. A representative blot is shown from five independent experiments. Densitometric analyses of the expression of (B) AKT and (C) p-AKT are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per

point. $P<0.01$ (**) indicates a statistically significant difference between treatment with acrolein and the control.

Fig. 5. Inhibition of PI3K/AKT pathway by LY294002 or wortmannin enhances necrosis induced by acrolein. Confluent cells in monolayer were pre-incubated for 1 h with (C) 50 μg/l Ly294002 (PI3-K inhibitor), or (D) 100 ηM Wortmannin (PI3-K inhibitor) before addition of acrolein. Cells were then incubated with (A) 0 (CTL) or (B-D) 50 *f*mol/cell of acrolein for 4 h. The fractions of (E) apoptotic and (F) necrotic cells are given relative to total cells. Apoptotic cells were visualized using Hoechst dye (blue fluorescence) and necrotic cells using propidium iodide (red fluorescence). Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P<0.001$ ($\phi\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without inhibitor.

Fig. 6. Effects of AKT inhibitors on acrolein-induced activation of caspases-9, -8 and -7 and cleavage of ICAD. (A, B, C) Pretreatment of cells with 50 μg/l Ly294002 (PI3-K inhibitor), or 100 ηM Wortmannin (PI3-K inhibitor) was performed for 1 h before addition of acrolein. CHO cells ($0.5x10^6$/mL) were then incubated with acrolein (50 *f*mol/cell) for (A, B) 1 h or (C) 2 h in α-MEM with 10% FBS. Activities of (A) caspase-9, (B) caspase-8 and (C) caspase-7 were measured in cell lysates and were expressed relative to the untreated control, designated as 1. Data represent means and SEM from eight independent experiments performed with multiple estimations per point. (D) Cells (10^6/mL) were incubated with acrolein for 2 h and immunodetection of ICAD was carried out by Western blotting, using β-tubulin as a loading control. A representative blot is shown from five independent experiments. Densitometric analysis of the expression of (E) ICAD is relative to the

untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.01$ ($\phi\phi$) or $P<0.001$ ($\phi\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without inhibitor.

Fig. 7. Acrolein induces phosphorylation of p53 at ser15 and ser46. Cells (10^6/mL) were incubated with acrolein for 1 h and immunodetection of p53, p-p53 (ser15) and p-p53 (ser46) was carried out by Western blotting, using β-tubulin as a loading control. Representative blots are shown from five independent experiments. Densitometric analyses of the expression of p53, p-p53 (ser15) and p-p53 (ser46) are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

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2.5. ARTICLE IV

INHIBITION OF ACROLEIN-INDUCED APOPTOSIS BY THE ANTIOXIDANT N-ACETYLCYSTEINE

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Abbreviations: AFC: amino trifluorocoumarin; AIF: apoptosis inducing factor; Apaf-1 : apoptotic protease activating factor; CAD: caspase activated DNase; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CHO: Chinese hamster ovary; FBS: fetal bovine serum; FCCP: *p*-trifluoromethoxy-phenyl-hydrazone; GSH: reduced glutathione; ICAD: inhibitor of caspase activated DNase; MEM: minimum essential medium; MMP: mitochondrial membrane potential; MOPS: 3-(N-morpholino)-propane sulfonic acid; NAC: N-acetylcysteine; PARP: polyADP-ribose polymerase; PBS: phosphate-buffered saline; PI: propidium iodide; PMSF: phenylmethylsufonyl fluoride; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PTP: permeability transition pore; γ-GCS: γ-glutamyl cysteine synthetase; Z-IETD-AFC : N-CBZ-L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic acid amide; Ac-LEHD-AFC: Ac-LEHD-AFC:Acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin .

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RÉSUMÉ

L'acroléine est un aldéhyde α,β-insaturé auquel les humains sont exposés dans de multiples situations. C'est un polluant environnemental qui cause plusieurs maladies respiratoires et neurodégénératives dont l'Alzheimer. La présente étude examine l'hypothèse que le N-acétylcystéine (NAC), un précurseur du glutathion, peut protéger contre la toxicité cellulaire engendrée par l'acroléine. L'exposition des cellules CHO à une concentration non-cytotoxique d'acroléine (4 *f*mol/cell), a diminué le glutathion intracellulaire de 45% de son taux initial. Le NAC a augmenté le niveau de glutathion intracellulaire et offert une protection contre la cytotoxicité et l'apoptose induite par l'acroléine en inhibant la voie mitochondriale. Le NAC a inhibé la translocation de Bad à la mitochondrie et la baisse du Bcl-2 mitochondriale induites par l'acroléine et ceci est démontré par immunobuvardage de type Western. Par contre, le NAC n'a pas d'effet sur la libération du cytochrome-c et la translocation de Bax induites par l'acroléine. Cependant, le NAC a inhibé la baisse du potentiel membranaire révélée par cytométrie de flux, le clivage de la procaspase-9, l'activation des caspases-9, -7 et -8 ainsi que le clivage de PARP. L'inhibition par NAC de l'apoptose induite par l'acroléine a été confirmée morphologiquement par la baisse de la condensation de la chromatine nucléaire. Ces résultats suggèrent que NAC pourrait être envisagé comme un antidote pour traiter les gens exposés à l'acroléine.

ABSTRACT

Acrolein is a highly electrophilic α,β-unsaturated aldehyde to which humans are exposed in many situations. It is an environmental pollutant that is responsible for multiple respiratory diseases and has been implicated in neurodegenerative diseases such as Alzheimer's disease. The hypothesis of the study is that the antioxidant N-acetylcysteine (NAC), a precursor of glutathione, could protect cells against acrolein-induced apoptosis. Exposure of CHO cells to a non-cytotoxic dose of acrolein (4 *f*mol/cell) depleted intracellular glutathione to 45% of initial levels. NAC, which increased intracellular glutathione levels by 30%, afforded protection against acrolein-induced cytotoxicity (loss of cell proliferation) and apoptosis. NAC protected against apoptosis by diminishing acrolein-induced activation of the mitochondrial death pathway. NAC inhibited acrolein-induced Bad translocation from the cytosol to the mitochondria, as well as Bcl-2 translocation from mitochondria to the cytosol, evaluated by Western blotting. However, NAC had no effect on acrolein-induced Bax translocation to mitochondria and cytochrome-c liberation into the cytosol. Meanwhile, NAC inhibited depolarisation of mitochondrial membrane potential, evaluated by rhodamine fluorescence using flow cytometry. NAC also inhibited procaspase-9 processing, activation of enzymatic activity of caspases -9, -7 and -8, as well as PARP cleavage induced by acrolein. Inhibition of acrolein-induced apoptosis using NAC was confirmed morphologically by diminished condensation of nuclear chromatin, evaluated by fluorescence microscopy. These findings suggest that NAC could be potentially useful as a protective agent for people exposed to acrolein.

INTRODUCTION

Acrolein is a highly reactive, α,β-unsaturated aldehyde pollutant to which humans are exposed in multiple situations (Kehrer and Biswal, 2000). It is a major component of smoke from cigarettes, forest and house fires, and is a constituent of automotive exhaust. It has been implicated in the development of atherosclerosis and various lung diseases including chronic obstructive pulmonary disease (Borchers et al., 1999). In addition, acrolein is found in the vapours of overheated cooking oil where severe human toxic exposures have occurred (Beauchamp et al., 1985). As one of the toxic products of endogenous lipid peroxidation (Adams and Klaidman, 1993), acrolein has been implicated in chronic neurodegenerative disorders such as Alzheimer's disease (Lovell et al., 2002; Pugazhenthi et al., 2006). Acrolein is a metabolic product of the anticancer drug cyclophosphamide, where it may be involved in its therapeutic effect as well as its toxic side effects (Schwartz and Waxman, 2001).

Acrolein toxicity has been demonstrated in many different cellular systems. It is known to induce apoptosis in several cell types such as keratinocytes (Takeuchi et al., 2001), neutrophils (Finkelstein et al., 2005), cultured neurons (Pugazhenthi et al., 2006), and Chinese hamster ovary (CHO) cells (Tanel and Averill-Bates, 2005), while necrosis occurs in others (Luo et al., 2005; Liu-Snyder et al., 2006).

Apoptosis is a physiological cell death process which plays an important role during development and maintenance of tissue homeostasis (Chandra et al., 2000). The mitochondrial pathway of apoptosis is triggered by different stresses such as growth factor deprivation, ionizing radiation, cortico-steroids and anticancer drugs. Anti-apoptotic (Bcl-2 and Bcl-X_L) and pro-apoptotic (Bax, Bad) members of the Bcl-2 family are thought to control apoptosis by modulating release of pro-apoptotic molecules such as cytochrome-c and apoptosis-inducing factor (AIF) from mitochondria. Bcl-2-Bax interactions at the outer mitochondrial membrane are

known to prevent induction of the mitochondrial apoptotic pathway. Translocation of Bad to mitochondria is necessary for promoting formation of pores consisting of Bax-Bax dimers on the outer mitochondrial membrane, which may be responsible for the release of pro-apoptotic factors (Orrenius et al., 2003). Bad translocation to the mitochondria removes the inhibitory effect of Bcl-2 on Bax, by interacting with Bcl-2 itself. The Bcl-2 family has two types of pro-apoptotic members: the Bax and Bak subfamily and the BH3-only members such as Bid, Bad, Bim/Bod and PUMA (Huang and Strasser, 2000). Studies with gene-targeted cells indicated that the presence of Bax or Bak is required for many forms of apoptosis (Lindsten et al., 2000), and each type of cell needs at least one of the anti-apoptotic Bcl-2 family members to survive (Cory and Adams, 2002; Wei et al., 2000). The BH3-only proteins are activated in response to various apoptotic stimuli and are able to induce apoptosis through interacting directly with Bax and Bak (Wei et al., 2000) or binding to the hydrophobic groove of anti-apoptotic members such as Bcl-2 or Bcl-xL, thus removing their inhibitory effect on pro-apoptotic molecules such as Bax and Bak.

Interaction of cytochrome-c and dATP with apoptosis protease activating factor (Apaf-1) in the cytosol leads to conversion of pro-caspase-9 to active caspase-9 through auto-proteolytic cleavage (Chandra et al., 2000). Caspase-9 subsequently activates effector caspases such as caspases-3, 6 and 7 (Salvesen and Dixit, 1997). Effector caspases then cleave cellular protein substrates such as polyADP ribose polymerase (PARP), lamins, gelsolins, fodrins and inhibitor of caspase activator DNase (ICAD). The cell then exhibits the characteristic morphological features of apoptosis such as chromatin condensation, cytoskeletal changes, nuclear membrane breakage, cell blebbing and formation of apoptotic bodies, which are then phagocytosed by macrophages. Elimination of these dying cells avoids an inflammatory response in surrounding tissues, such as occurs during necrosis.

Acrolein will rapidly bind to and deplete cellular nucleophiles such as glutathione (Heck, 1997). The antioxidant glutathione appears to have an important role in the detoxification of acrolein (Finkelstein et al., 2001). Cellular cytotoxic

responses to acrolein are likely to depend on intracellular glutathione levels. It is likely that increased glutathione levels could attenuate toxicity of acrolein. To increase glutathione levels, the thiol-containing compound, N-acetyl-L-cysteine (NAC) can be used as a glutathione precursor, since glutathione itself does not easily penetrate cells (Roberfroid and Calderon, 1995). NAC also increases cysteine pools inside cells and acts as a thiol-containing reducing agent (Cotgreave, 1997). Thiol compounds such as NAC could be potentially useful for protecting tissues and cells from acrolein-induced toxicity. Therefore, the objective of this study is to investigate whether NAC can protect cells against acrolein-induced cytotoxicity and activation of the mitochondrial pathway of apoptosis.

MATERIALS AND METHODS

Cell culture

CHO cells (AuxBl) were grown in monolayer in minimum essential medium-Alpha (α-MEM) (Gibco Canada, Burlington, ON) plus 10% fetal bovine serum (FBS) (Gibco Canada) and 1% penicillin (50 units/mL)-streptomycin (50 μg/mL) (Flow Laboratories, Mississauga, ON), in tissue culture flasks (Sarstedt, St Laurent, QC), in a humidified atmosphere of 5% CO_2 in a water jacketed incubator at 37°C (Lord-Fontaine and Averill, 1999). The cells were grown to near confluence and were then incubated for 24 h with fresh culture medium. Confluent cells were then harvested using PBS-citrate, washed by centrifugation (1000g, 3 min) and resuspended in α-MEM for experimental studies.

Pretreatment with NAC

Pretreatment of cells with 1 mM NAC (Sigma Chemical Co., St. Louis, MO) was performed for 24 h on confluent cells in monolayer (Lord-Fontaine and Averill, 1999). Cells were subsequently washed three times to remove NAC and then harvested. Intact cells were tested for cell survival, total glutathione levels and analysis of apoptosis. Treatment with NAC did not cause any loss of cell viability, determined by the trypan blue exclusion assay. Plating efficiency did not decrease relative to control cells.

Dosage of glutathione

Total glutathione was determined as previously described (Lord-Fontaine and Averill, 1999). The rate of formation of 5-thio-2-nitrobenzoic acid, which is proportional to total glutathione concentration, was followed at 412 nm by an enzymatic assay using glutathione reductase. Sample values were calculated from a standard curve of nmol glutathione versus rate and expressed as nmol/10^6 cells.

Clonogenic cytotoxicity assay

Cytotoxicity was evaluated as the ability of cells to proliferate to form macroscopic colonies after a toxic insult with acrolein, using a clonogenic cell survival assay (Lord-Fontaine and Averill, 1999). Cells (10^5/mL) were incubated with acrolein (Aldrich Chemical Co, Milwaukee, WI), in α-MEM containing 10% FBS for 1 h at 37 °C. Cytotoxicity was expressed as the mean number of macroscopic colonies (>50 cells) obtained relative to the mean number of colonies obtained in the control.

Morphological analysis of apoptosis

To visualize nuclear morphology and chromatin condensation, cells were incubated with acrolein for 4 h and then apoptotic cells were stained with Hoechst (33258) (0.06 mg/mL) (Tanel and Averill-Bates, 2005). Propidium iodide (PI) (50 μg/mL) was added to stain necrotic cells. Observations were made by fluorescence microscopy (Carl Zeiss Ltd, Montreal, QC) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc, Mississauga, ON). Images were analysed by Northern Eclipse software. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). A minimum of 600 cells was counted per dish.

Determination of caspase activity by fluorescence spectroscopy

Freshly harvested cells (0.5×10^6) were incubated with acrolein in α-MEM at 37^0C. Cells were lysed by freezing at -20^0C for 20 minutes and reaction buffer (20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) was added (Stennicke and Salvesen, 1997). The kinetic reaction was started after addition of the appropriate caspase substrate at 37°C using a spectrofluorimeter (Spectra Max

Gemini, Molecular Devices, Sunnyvale, CA) (Tanel and Averill-Bates, 2005). Caspase-7 activity was measured by cleavage of the fluorogenic substrate I MCA-VDQVDGWK(DNP)-NH_2 (Calbiochem, La Jolla, CA) with λmax excitation at 325 nm and λmax emission at 395 nm. Caspase-8 activity was measured by cleavage of the substrate Z-IETD-AFC to produce trifluorocoumarin (AFC) with λmax excitation at 415 nm and λmax emission at 490 nm. Caspase-9 activity was measured by cleavage of Ac-LEHD-AFC to produce AFC.

Subcellular fractionation and immunodetection of Bad, Bax, Bcl-2, cytochrome-c, caspase-9 and PARP

Following treatment with acrolein, cells were washed in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After 30 min incubation on ice, lysates were homogenised using a hand potter (Kontes glass CO, Duall 22, Fisher, QC). Unbroken cells and nuclei were pelleted by centifugation at 2500 *g* for 10 min. The supernatant was centrifuged at 15000 g for 20 min and the resultant pellet was designated as the mitochondrial fraction. A further centrifugation of the supernatant fraction at 100 000 *g* for 1 h resulted in a pellet fraction designated as the microsomal fraction, whereas the supernatant was designated as the cytosolic fraction (Samali et al., 1999). For immunodetection of caspase-9 and PARP, whole cell lysates were used.

Proteins (30 μg) were quantified according to Bradford (Bradford, 1976), separated using a 15% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane, as previously described (Tanel and Averill-Bates, 2005). The blots were probed with the following primary antibodies: anti-Bad, anti-Bax, anti-Bcl-2, anti-caspase-9, anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-cytochrome-c (BD Biosciences Canada, Mississauga, ON). Secondary antibodies (1:1000) consisted of horseradish peroxidase (HRP)-conjugated

goat anti-mouse, anti-rabbit and anti-goat IgG (Biosource, Camarillo, CA). Proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA) and protein expression quantified using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA). Purity of mitochondrial and cytosolic fractions was verified using antibodies to cytochrome-c oxidase (Santa Cruz Biotechnology) and GSTπ_1 (Calbiochem), respectively. Caspase-9 and PARP expression in whole cell lysates was quantified using IPGEL software, relative to β-tubulin loading controls.

Flow cytometry analysis of mitochondrial membrane potential (Δψm)

Freshly harvested cells (10^6) were incubated with acrolein, or the positive control *p*-trifluoromethoxy-phenyl-hydrazone (FCCP), in α-MEM for 1 h at 37 °C. To measure *Δψm*, cells were incubated with rhodamine 123 (800 ng/mL) for 5 min (Tanel and Averill-Bates, 2005). PI was added to identify dead cells. Cells were analysed with a FACScan flow cytometer equipped with an argon laser emitting at 488 nm (Becton Dickinson, Oxford, UK). Data was acquired and analysed using Lysis II software (Becton Dickinson). The mean fluorescence intensity of 20,000 cells was calculated for each sample. Rhodamine 123 fluorescence was detected on the FL1 detector and PI fluorescence on the FL2 detector. For each sample, only live cells were selected, and their mean fluorescence in FL1 was analysed. Apoptotic cells, which undergo a decrease in mitochondrial membrane potential, incorporate less of the rhodamine 123 dye, therefore emitting less fluorescence on the FL1 detector. Control measurements of mitochondrial mass were carried using the fluorescent probe MitoTracker Green (Molecular Probes).

Statistical analysis

Statistical differences between control and treated groups without NAC for figures 1, 2, 3I, 3J, 7D, 9A, 10A and 10B were determined using a one-way ANOVA

with a Bonferroni-Holm (a stepwise method) post-test correction for multiple comparisons. An adjustment was made to limit the familywise error rate (FWE) to 5% by calculating an adjusted *p*-value which is a simulated based *p*-value obtained from the multivariate *t* distribution (number of simulations = 1000 000) (Westfall et al., 1999). Statistical differences between control and treated groups with or without NAC for figures 1, 2, 3I, 3J, 7D, 9A, 10A and 10B were determined using a two-tailed unpaired Student's *t* test. Data for figures 4B, 4C, 5B, 5C, 6B, 6C, 8B, 8C, 9C, 9D, 11B and 11C were analysed using a bilateral *t* test. Values are expressed as means ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

NAC inhibits acrolein-induced cytotoxicity and apoptosis

The antioxidant glutathione appears to have an important role in the detoxification of acrolein (Finkelstein et al., 2001). Nevertheless, the possible protective effect of antioxidants against acrolein-induced apoptosis is not known. Therefore, we investigate whether NAC, a precursor of glutathione, can protect cells against acrolein-induced cytotoxicity and apoptosis. Induction of cytotoxicity (loss of cellular proliferation) occurred after a 1 h exposure to acrolein concentrations of 50 *f*mol/cell and higher (Fig. 1). Exposure to 350 *f*mol/cell induced 5 logarithms (10^5) of cell killing. NAC afforded significant protection against cytotoxicity of acrolein. The protective effect was only partial, as acrolein cytotoxicity was not completely prevented.

On the basis of the known reactivity of α,β-unsaturated aldehydes such as acrolein with thiols (Heck, 1997), it is likely that acrolein could mediate its toxic effects primarily by depleting cellular glutathione and/or by modifying protein cysteine residues. Indeed, exposure of cells to acrolein caused rapid and severe depletion of GSH (Fig. 2). GSH levels were reduced to 36 % of initial levels, from 2.28 ± 0.08 to 0.82 ± 0.13 nmol/10^6 cells, by treatment with 4 *f*mol/cell of acrolein during 30 min. We determined whether NAC could prevent the loss of glutathione following exposure to acrolein, by increasing glutathione levels. Pretreatment of cells with 1mM NAC for 24 h effectively increased total intracellular glutathione levels in untreated control cells by about 30%, from 2.28 ± 0.08 nmol/10^6 cells to 3.22 ± 0.17 (Fig. 2). However, NAC partially decreased the loss of intracellular glutathione in cells exposed to a low dose of acrolein (4 *f*mol/cell), but not at higher concentrations (≥ 10 *f*mol/cell) (Fig. 2). Even though initial levels of glutathione were elevated, acrolein still caused a severe loss of glutathione in cells pretreated with NAC (Fig. 2).

Given that NAC protected cells against acrolein-induced cytotoxicity (Fig. 1), it is possible that NAC could trigger a change in the type of cell death from necrosis to apoptosis. Morphological analysis demonstrates that acrolein induces both apoptosis and necrosis in CHO cells (Fig. 3). Apoptosis was revealed by condensation of chromatin with the fluorescent probe Hoescht (blue) whereas necrosis was revealed by the fluorescent probe PI (red). Acrolein (50 *f*mol/cell) induced apoptosis and necrosis in 21% and 3.5% of cells, respectively (Fig. 3C, 3I, 3J). A higher concentration of acrolein (100 *f*mol/cell) switched the mode of cell death from apoptosis (8.5%) to necrosis (20%) (Fig. 3D, 3I, 3J). We evaluated whether the NAC-induced increase in levels of intracellular glutathione could attenuate cell death induced by acrolein. When NAC-pretreated cells were exposed to 50 *f*mol/cell of acrolein (Fig. 3G, 3I), the fraction of apoptotic cells decreased from 21% to 2%. NAC also diminished necrosis in cells exposed to 50 and 100 *f*mol/cell of acrolein (Fig. 3G, 3H, 3J). Therefore, NAC increased the threshold level of acrolein for induction of apoptosis from 50 to 100 *f*mol/cell, relative to cells with native levels of glutathione (Fig. 3C, 3G, 3H, 3I). Treatment with NAC alone did not affect control cells (Fig. 3E versus 3A). Taken together, these results suggest that NAC is an effective compound to inhibit acrolein-induced cytotoxicity as well as acrolein-induced apoptosis.

NAC inhibits acrolein-induced translocation of Bad and Bcl-2

We previously reported that acrolein can induce apoptosis by the mitochondrial pathway in CHO cells (Tanel and Averill-Bates, 2005). However, the effect of acrolein on proteins in the Bcl-2 family is not known. Acrolein could alter the balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins at the mitochondrial membrane. Therefore, we examined whether acrolein could induce the translocation of Bad, Bax and Bcl-2 between the cytosolic and mitochondrial subcellular compartments. Exposure to acrolein (20 *f*mol/cell) for 1 h resulted in

translocation of the pro-apoptotic protein Bad from the cytosol to the mitochondria (Fig. 4). Translocation of Bad to mitochondria was partially inhibited by NAC (Fig. 4A, 4B). Acrolein also induced translocation of the pro-apoptotic protein Bax from the cytosol to the mitochondria (Fig. 5). However, NAC did not inhibit Bax translocation, but rather NAC alone promoted Bax translocation to mitochondria from the cytosol (Fig. 5). In addition, acrolein induced translocation of the anti-apoptotic protein Bcl-2 from mitochondria (Fig. 6A, 6B) to the cytosol (Fig. 6A, 6C). This was inhibited by NAC. Together, these findings indicate that acrolein has a pro-apoptotic effect at the mitochondrial membrane. NAC played an anti-apoptotic role at the mitochondrial level by inhibiting acrolein-induced translocation of Bad to mitochondria and by maintaining Bcl-2 levels at the mitochondrial membrane.

NAC inhibits acrolein-induced depolarisation of mitochondrial membrane potential

The balance between Bcl-2 proteins at the mitochondrial membrane is often a determinant in the opening of the permeability transition pore (PTP) and loss of mitochondrial membrane potential (MMP) under apoptotic stimuli. Since NAC can exert protective, anti-apoptotic effects at the level of the mitochondrial membrane, we examined whether it could prevent mitochondrial membrane depolarisation in acrolein-treated cells. Acrolein (10-20 *f*mol/cell) caused depolarisation of MMP (Fig. 7C, 7D). FCCP was used as a positive control for MMP depolarisation (Fig. 7A, 7D). However, pretreatment with NAC afforded a significant level of protection against acrolein-induced mitochondrial depolarisation (Fig. 7C, 7D). NAC alone caused a small decrease in MMP (Fig. 7B). To verify that changes in rhodamine fluorescence signals were not due to changes in the total mitochondrial mass, the effect of acrolein (20 *f*mol/cell), with and without NAC treatment, was determined on the fluorescence uptake of the redox-insensitive mitochondrial dye MitoTracker Green (Fig. 7E). There was no evidence for a shift in fluorescence intensity in cells treated with

acrolein alone, or acrolein and NAC, confirming that changes in rhodamine fluorescence were indeed due to changes in MMP, thus ruling out the possibility that acrolein and/or NAC affected the mitochondrial mass.

A decrease in MMP often leads to the release of cytochrome-c from mitochondria. Indeed, acrolein (20 *f*mol/cell) caused release of cytochrome-c into the cytosol (Fig. 8). However, NAC did not appear to inhibit this effect, but instead induced a small increase in cytochrome-c levels in both the mitochondrial and cytosolic fractions.

NAC inhibits acrolein-induced activation of caspase-9

Since NAC inhibited acrolein-induced translocation of Bad and Bcl-2, as well as mitochondrial membrane depolarisation, it is likely that NAC could inhibit post-mitochondrial events such as caspase-9 activation. Here we show that exposure to acrolein (4-50 *f*mol/cell) for 1 h activated the initiator caspase-9 (Fig. 9A), the first downstream caspase induced via the mitochondrial death pathway. Caspase-9 activation by acrolein was totally prevented by pretreatment with NAC (Fig. 9A). In addition, pretreatment with NAC inhibited the cleavage of procaspase-9 (Fig. 9B, 9C, 9D) and the generation of the p35 active cleavage fragment induced by acrolein (Fig. 9D). These findings show that NAC can inhibit acrolein-induced apoptosis at the post-mitochondrial level.

NAC inhibits acrolein-induced activation of caspase-7 and caspase-8, as well as PARP cleavage

We next determined whether NAC could inhibit other events in the apoptotic cascade. Acrolein induced activation of the effector caspase-7 (Fig. 10A) and initiator caspase-8 (Fig. 10B), and these events were significantly inhibited in cells that had been pretreated with NAC. Since caspase-7 was inhibited by NAC, we investigated whether NAC could prevent the cleavage of PARP, which is a downstream substrate

of caspase-7. PARP is a key nuclear enzyme involved in DNA repair and has a complex role in cell death. Acrolein (20 *f*mol/cell) caused cleavage of PARP protein, from the full-length 116 kDa form to generate the characteristic apoptosis-related 85-kDa cleavage fragment (Fig. 11A, 11B, 11C). Indeed, NAC inhibited the cleavage of PARP induced by acrolein (Fig. 11A, 11B, 11C).

DISCUSSION

The important new finding of this study is that NAC, a precursor of the major intracellular antioxidant glutathione, is capable of inhibiting acrolein-induced cytotoxicity and apoptosis in proliferating CHO cells. We provide new information on how NAC can inhibit activation of the mitochondrial pathway of apoptosis by acrolein.

Recently, we reported that acrolein induces apoptosis via the mitochondrial pathway by decreasing MMP, liberation of cytochrome-c, and activation of caspases-9 and -7 (Tanel and Averill-Bates, 2005). This study provides new information on the implication of the mitochondrial pathway in acrolein-induced apoptosis, where Bcl-2 family proteins are critical regulators. This is the first study to show that acrolein stimulates translocation of the pro-apoptotic Bcl-2 proteins, Bax and Bad, from the cytosol to mitochondria, as well as the translocation of anti-apoptotic protein Bcl-2 from mitochondria to the cytosol. The translocation of pro-apoptotic proteins such as Bax and Bad to mitochondria is an important step in removing the inhibitory effect of Bcl-2 on apoptosis at the mitochondrial level, thus promoting release of cytochrome c.

Acrolein induced a decrease in MMP as well as liberation of cytochrome-c from mitochondria into the cytosol. The implication of the PTP in acrolein-induced apoptosis was already demonstrated through inhibition of nuclear changes by cyclosporine A (Tanel and Averill-Bates, 2005), a blocker of opening of the PTP. Although the mechanisms are not completely understood, it is considered that cytochrome *c* can be released from mitochondria by several mechanisms, including the PTP, as well as through channels on the membrane involving Bax-Bax interactions (Orrenius et al., 2003). At the post-mitochondrial level, liberation of cytochrome-c leads to activation of caspase-9 and -7. Caspase-7 is an effector caspase that cleaves many substrates such as PARP. Several studies have reported that

acrolein can inhibit caspase-3 activity (Tanel and Averill-Bates, 2005; Finkelstein et al., 2001), which could occur by direct alkylation of its cysteine residue by acrolein.

The cellular effects of acrolein are believed to be due primarily to its ability to react rapidly with reduced thiols such as GSH (Heck, 1997). GSH plays a key role in cellular reductive processes and detoxification of harmful oxidative species and various xenobiotics (Meister and Anderson, 1983). GSH redox status is critical for a variety of biological process including transcriptional activation of various genes, regulation of cell proliferation, inflammation and apoptosis. An important consequence of severe glutathione depletion caused by toxic compounds such as acrolein is the resulting perturbation of the cellular redox balance between pro-oxidants and antioxidants in favor of a pro-oxidant state. This would leave the cell vulnerable to damage, and possibly death, induced by normal levels of cellular oxidants ($O_2^{\cdot-}$, H_2O_2) or mild exposure to other toxic compounds (Luo et al., 2005).

The present study demonstrates that acrolein causes glutathione depletion at much lower concentrations (4 *f*mol/cell) than those that induce cytotoxicity and apoptosis (30 to 50 *f*mol/cell). This indicates that glutathione depletion is an earlier event than cytotoxicity, evaluated by loss of cellular proliferation. There was also a correlation between acrolein-induced changes in GSH levels and inhibition of cell proliferation, in A549 lung carcinoma cells (Horton et al., 1997).

This is the first study that links GSH to induction of apoptosis by acrolein, since pretreatment with NAC, a glutathione precursor, inhibited acrolein-induced apoptosis. Acrolein concentrations (20 *f*mol/cell) that completely depleted GSH levels coincided with those required to induce early apoptotic events, such as translocation of Bax and Bad, mitochondrial membrane depolarisation, liberation of cytochrome-c and activation of caspase-9. Furthermore, this is the first study to report protection against acrolein-induced apoptosis with a thiol antioxidant. However, it was reported that sulfur compounds (thiamine, NAC, ascorbic acid) (Sprince, 1985), α-tocopherol and ascorbic acid (Nardini et al., 2002) as well as the nucleophilic drug

hydralazine (Kaminskas et al., 2004) could afford protection against acrolein-induced cytotoxicity. Indeed, NAC inhibited acrolein-induced apoptosis, at both the mitochondrial and post-mitochondrial levels. Caspase-9 activation, which is considered as an important indicator for activation of the mitochondrial pathway of apoptosis, was totally prevented by NAC. NAC inhibited acrolein-induced activation of caspase-7, PARP cleavage and chromatin condensation, which are downstream events from both caspase-9 and -8. Total inhibition of caspase-9 activation and partial inhibition of caspase-8 activation suggests that the mitochondrial pathway is primarily involved in protective mechanisms involving NAC. Caspase-8 activation mediates apoptotic signalling via death receptor pathways (Saikumar et al., 1999).

At the mitochondrial level, NAC inhibited the translocation of Bad to mitochondria as well as translocation of Bcl-2 to cytosol, stimulated by acrolein exposure. NAC maintained anti-apoptotic Bcl-2 protein at the mitochondrial membrane. Therefore, NAC changed the balance between pro- and anti-apoptotic Bcl-2 proteins at the mitochondrial membrane in favor of an anti-apoptotic state, confirmed by protection against the acrolein-induced decrease in MMP. Surprisingly, NAC did not appear to protect cells against acrolein-induced Bax translocation to the mitochondria. However, it is likely that the anti-apoptotic effect of NAC may predominate by increasing Bcl-2. Even though NAC does not prevent Bax translocation to mitochondria, NAC ensures increased levels of Bcl-2 at the mitochondrial membrane. Bcl-2 binds to Bax in order to prevent its pro-apoptotic effects.

Surprisingly, NAC did not protect cells against acrolein-induced cytochrome-c release from mitochondria to the cytosol. However, it was reported that the liberation of cytochrome-c persists in neutrophils exposed to acrolein even though apoptotic features such as caspase-9 and -8 activation, and externalisation of phosphatidylserine were inhibited by acrolein (Finkelstein et al., 2005). This suggests that cytochrome-c release is not necessarily a hallmark in acrolein-induced apoptosis. Moreover, glutathione depletion caused cytochrome-c release even in the absence of

apoptosis (Ghibelli et al., 1999). This again suggests that cytochrome-c release is not necessarily a terminal event leading to apoptosis, but could be the consequence of a redox disequilibrium arising from increased mitochondrial generation of reactive oxygen species that, under some circumstances, may be associated with apoptosis (Ghibelli et al., 1999). The mechanisms involved in cytochrome-c release during apoptosis are not completely understood and appear to involve multiple and distinct pathways, involving Bax-Bax dimers, t-Bid, the MPT and oxidation of cardiolipin (Orrenius et al, 2006). Further work is required to characterize the mechanisms involved in the mitochondrial regulation of apoptosis.

Depletion of GSH and other cellular thiols has been correlated with apoptosis in a number of cell types (Aoshiba et al., 1999; Sato et al., 1995). It was suggested that the effects of acrolein might be the result of redox changes of critical protein cysteine residues secondary to GSH depletion or to direct oxidation/alkylation of protein thiol residues that may occur along with or subsequent to the loss of GSH (Finkelstein et al., 2001). However, the relationship between GSH depletion and apoptosis is not clear and GSH depletion itself may not be sufficient to trigger apoptosis. In fact, it has been proposed that apoptosis could involve efflux of reduced GSH from the cell (Ghibelli et al., 1995). Thus, depletion of cellular GSH may be a consequence rather than a cause of apoptosis.

During recent years, many compounds have been tested as precursors of GSH or as inducers of the enzymes related to its synthesis (Anderson, 1997). Increasing GSH levels could be of great interest in designing new therapeutic strategies against environmentally toxic compounds, such as acrolein, and against the side effects of many therapeutic agents. NAC has proven to be a useful compound for increasing GSH synthesis. The protective role of NAC against acrolein-induced cytotoxicity and apoptosis could be explained by its ability to stimulate synthesis of glutathione, to increase cellular thiol pools or to act as a thiol-containing reducing agent. Besides increasing cellular thiols, NAC can act as an antioxidant by directly reducing reactive oxygen species such as $OH^{\cdot}$, H_2O_2 and HOCl. NAC reduced protein carbonyls

induced by acrolein, markers for lipid peroxidation, in rats developing acute hepatic injury (Kitamura et al., 2005). An advantage of using NAC as a protective agent is that it already has approval for clinical use. In fact, NAC is used clinically to treat overdose of the hepatotoxic drug acetaminophen (Perry and Shannon, 1998). Administering the cysteine delivery compound NAC to human subjects appears to be safer than administering cysteine itself, since cysteine has been reported to have toxic effects on the central nervous system (Dizdar et al., 2000).

On the basis of these results, we conclude that NAC appears to have a beneficial effect in protecting cells against acrolein-induced apoptosis and inhibition of cell proliferation. These findings also suggest that intracellular GSH levels may play an important role in cellular toxicity of acrolein, and in particular, the induction of apoptosis and cytotoxicity. These findings may also be relevant to our understanding of the toxicity of environmental exposures to low doses of acrolein as well as the normal tissue toxicity of cyclophosphamide, which generates acrolein as one of its metabolites.

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FIGURE LEGENDS

Fig. 1: NAC protects cells against acrolein cytotoxicity. CHO cells (10^5/mL), with or without pretreatment with NAC, were exposed to acrolein during 1 h at 37^0C in 1 mL of α-MEM containing 10% FBS. Acrolein was then removed, and cells were incubated to allow the formation of macroscopic colonies. The control value represents 10^5 cells and this was normalized to represent 100% cell survival. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P<0.05$ (ϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC. When not shown, error bars lie within the symbols.

Fig. 2: Acrolein causes severe depletion of intracellular glutathione levels: effect of NAC. CHO cells ($5x10^6$ /mL), with or without pretreatment with NAC, were incubated for 30 min with acrolein in α-MEM containing 10% FBS. Levels of glutathione were measured in cellular extracts. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P<0.05$ (ϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 3: Morphological analysis of apoptosis and necrosis in cells following exposure to acrolein: protective effect of NAC. Cells (0.3×10^6) were seeded and cultured for 2 days to near confluence in tissue culture dishes containing α-MEM and 10% FBS at 37°C. Cells, that were pretreated with (E, F, G, H) or without (A, B, C, D) NAC, were incubated with acrolein: (A, E) 0, (B, F) 30, (C, G) 50 and (D, H) 100 *f*mol/cell, for 4 h. The fractions of (I) apoptotic (Hoechst) and (J) necrotic (PI) cells

are given relative to total cells (magnification 320×). Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P<0.05$ (ϕ), $P<0.01$ (ϕϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 4: Acrolein-induced translocation of Bad to the mitochondria is inhibited by NAC. CHO cells (10^6 /mL), with or without pretreatment with NAC, were incubated for 1 h with acrolein (20 *f*mol/cell). For immunodetection of (A) mitochondrial and cytosolic Bad (25 kDa), anti-cytochrome oxidase and anti-GSTπ1 were used as a loading controls for cytosolic and mitochondrial fractions, respectively. A representative gel is shown from five independent experiments. Densitometric analyses of expression of (B) mitochondrial and (C) cytosolic Bad are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.05$ (*) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 5: Acrolein promotes translocation of Bax to mitochondria. CHO cells (10^6 /mL), with or without pretreatment with NAC, were incubated for 1 h with acrolein (20 *f*mol/cell). For immunodetection of (A) mitochondrial and cytosolic Bax (23 kDa), anti-GSTπ1 and anti-cytochrome oxidase were used as loading controls for cytosolic and mitochondrial fractions, respectively. A representative gel is shown from five independent experiments. Densitometric analyses of expression of (B) mitochondrial and (C) cytosolic Bax are relative to the untreated control. Data represent means and SEM from five independent experiments performed with

multiple estimations per point. $P<0.05$ (*) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ) or $P<0.01$ ($\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 6: NAC inhibits acrolein-induced translocation of Bcl-2 to the cytosol. CHO cells (10^6 /mL), with or without pretreatment with NAC, were incubated for 1 h with acrolein (20 *f*mol/cell). For immunodetection of (A) mitochondrial and cytosolic Bcl-2 (26 kDa), anti-GSTπ1 and anti-cytochrome oxidase were used as loading controls for cytosolic and mitochondrial fractions, respectively. A representative gel is shown from five independent experiments. Densitometric analyses of expression of (B) mitochondrial and (C) cytosolic Bcl-2 are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.01$ (**) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig.7: NAC inhibits acrolein-induced depolarization of the mitochondrial membrane. CHO cells (10^6/mL) were treated with (A) 5 μM FCCP for 1 h, or (B) NAC (24 h) alone for 24 h, or (C) acrolein (20 *f*mol/cell) for 1 h (with or without NAC pretreatment), relative to untreated control cells (red). Cells were then analysed by flow cytometry for rhodamine 123 fluorescence in channel FL1. (D) Data represent means and SEM of relative fluorescence intensity of rhodamine from eight independent experiments. The absolute data value for the untreated control cells from 8 experiments was 38±2 (mean ± S.E.M) relative fluorescence units. The control value was designated as 100%, and data for treated cells were normalized to this value. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference

between treatment with acrolein or FCCP and the untreated control. $P<0.05$ (ϕ) or $P<0.01$ ($\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC. (E) Controls for mitochondrial mass were stained with the redox-insensitive living dye MitoTracker Green and analyzed in the FL-1 channel of a flow cytometer. Control (red), acrolein-treated (black), NAC-treated (green) and acrolein-NAC-pretreated (blue) cells were compared in a histogram overlay.

Fig. 8: Acrolein-induced liberation of cytochrome-c to the cytosol is not affected by NAC. CHO cells (10^6 /mL), with or without pretreatment with NAC, were incubated for 1 h with acrolein (20 *f*mol/cell). For immunodetection of (A) mitochondrial and cytosolic cytochrome-c (15 kDa), anti-GSTπ1 and anti-cytochrome oxidase were used as loading controls for cytosolic and mitochondrial fractions, respectively. A representative gel is shown from five independent experiments. Densitometric analyses of expression of (B) mitochondrial and (C) cytosolic cytochrome-c are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 9: Activation of initiator caspase-9 by acrolein is inhibited by NAC. (A) CHO cells (0.5×10^6/mL), with or without pretreatment with NAC, were incubated with acrolein for 1 h. Caspase-9 activity in cell lysates was expressed relative to the untreated control, designated as 1. Data represent means and SEM. from eight independent experiments performed with multiple estimations per point. (B) Cells (10^6/mL), with or without pre-treatment with NAC, were incubated with acrolein (20

*f*mol/cell) for 1 h. For immunodetection of procaspase-9 and its cleavage fragment (35 kDa), β-tubulin was used as a loading control. Densitometric analyses of expression of (C) procaspase-9 and (D) the cleavage fragment are relative to the untreated control. Data represent means and SEM from four independent experiments performed with multiple estimations per point. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.01$ (ϕϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 10: NAC inhibits activation of effector caspase-7 and initiator caspase-8 by acrolein. (A) CHO cells ($0.5x10^6$/mL), with or without pretreatment with NAC, were incubated with acrolein for (A) 2 h (caspase-7) or (B) 1h (caspase-8) in α-MEM with 10% FBS. Activities of caspases were expressed relative to the untreated controls, designated as 1. Data represent means and SEM from seven (caspase-7) or nine (caspase-8) independent experiments performed with multiple estimations per point. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control. $P<0.05$ (ϕ), $P<0.01$ (ϕϕ) or $P<0.001$ (ϕϕϕ) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 11: NAC inhibits acrolein-induced cleavage of PARP. (A) Cells (10^6/mL), with or without pretreatment with NAC, were incubated with acrolein (20 *f*mol/cell) for 1 h. For immunodetection of PARP (116 kDa) and its cleavage fragment (85 kDa), β-tubulin was used as a loading control. Densitometric analyses of expression of (B) PARP (116 kDa) and (C) the cleavage fragment (85 kDa) are relative to the untreated control. Data represent means and SEM from five independent experiments performed with multiple estimations per point. $P<0.05$ (*) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the control.

$P<0.05$ (ϕ) or $P<0.001$ ($\phi\phi\phi$) indicates a statistically significant difference between cells exposed to acrolein, with and without NAC.

Fig. 12: Proposed schema for NAC-induced inhibition of acrolein-induced apoptosis. NAC, which increased intracellular glutathione levels, afforded protection against acrolein-induced apoptosis. NAC diminished apoptosis by inhibiting acrolein-induced activation of the mitochondrial death pathway. NAC inhibited acrolein-induced Bad translocation to the mitochondria, as well as Bcl-2 translocation from mitochondria to the cytosol. NAC had no effect on Bax translocation to the mitochondria and cytochrome-c liberation to the cytosol. NAC inhibited depolarisation of mitochondrial membrane potential, procaspase-9 processing, caspase-9, -7 and -8 activation, as well as PARP cleavage induced by acrolein. Acrolein causes procaspase-3 cleavage, but caspase-3 activity is immediately inhibited and caspase-7 is responsible for downstream cleavage events. Inhibition of acrolein-induced apoptosis using NAC was confirmed morphologically by condensation of nuclear chromatin. Apaf-1: apoptosis protease activating factor-1; t-bid: truncated bid, CAD: caspase activated DNase; Cyt-c: cytochrome-c; FADD: Fas associated death domain; Fas: fibroblast-associated; ICAD: inhibitor of caspase activated DNase; NAC: N-acetylcysteine; PARP: poly ADP-ribose polymerase.

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Figure 1: NAC protects cells against acrolein cytotoxicity

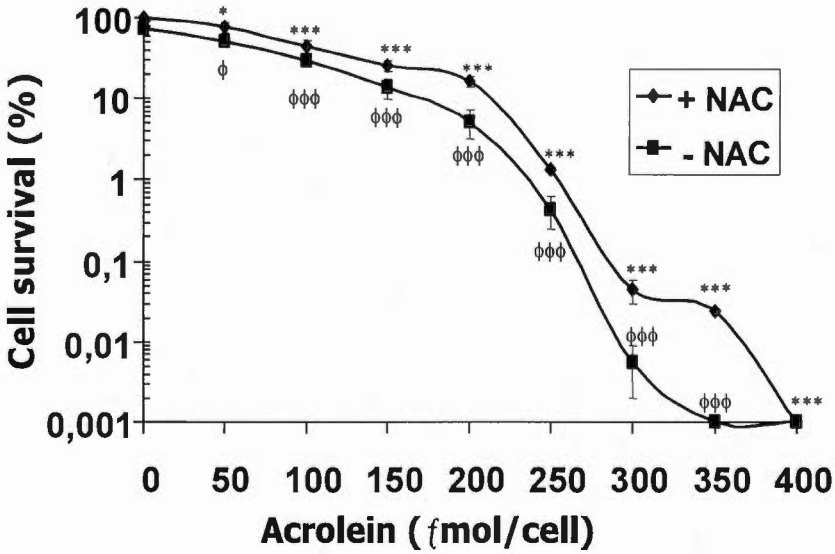


Figure 2: Acrolein causes severe depletion of intracellular glutathione levels: effect of NAC

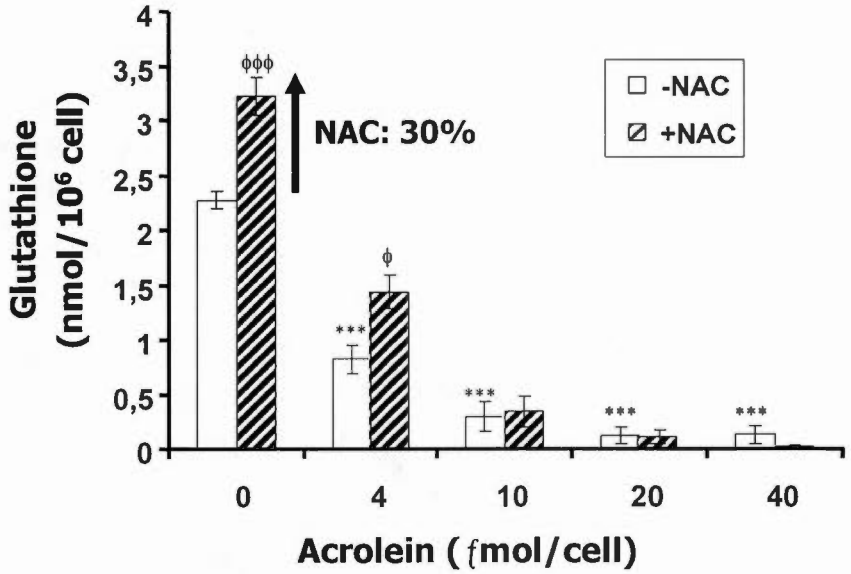
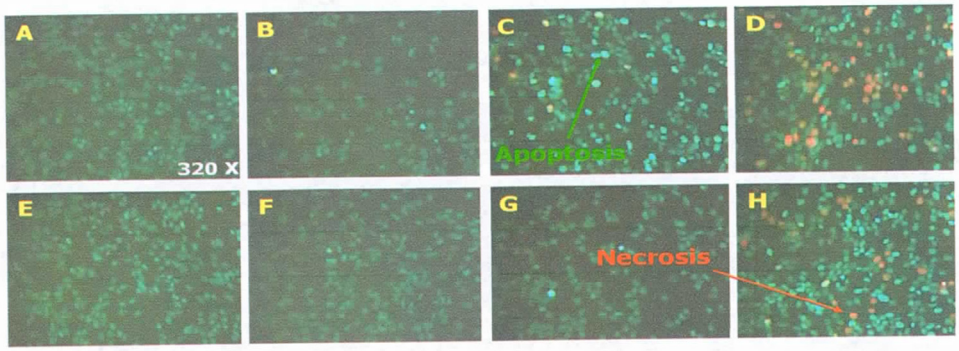


Figure 3: Morphological analysis of apoptosis and necrosis in cells following exposure to acrolein: protective effect of NAC



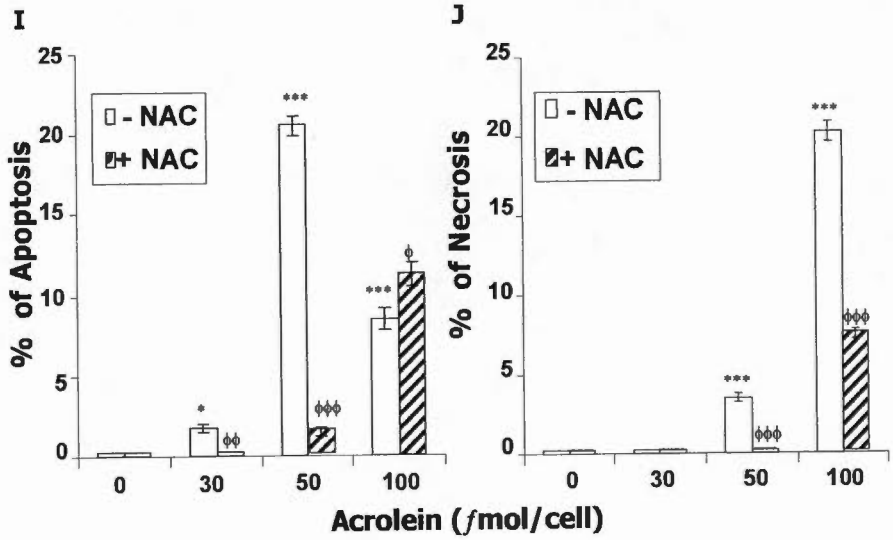


Figure 4: Acrolein-induced translocation of Bad to the mitochondria is inhibited by NAC

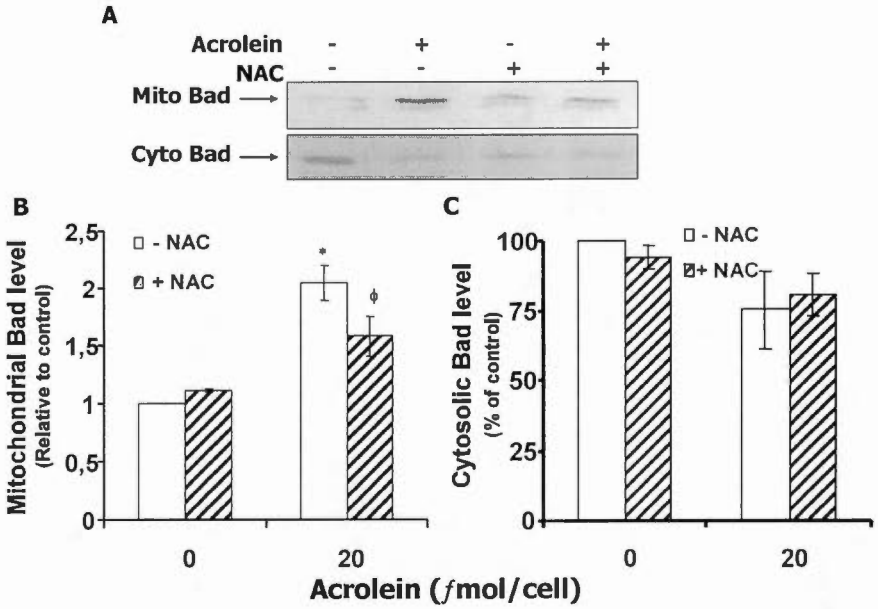


Figure 5: Acrolein promotes translocation of Bax to mitochondria

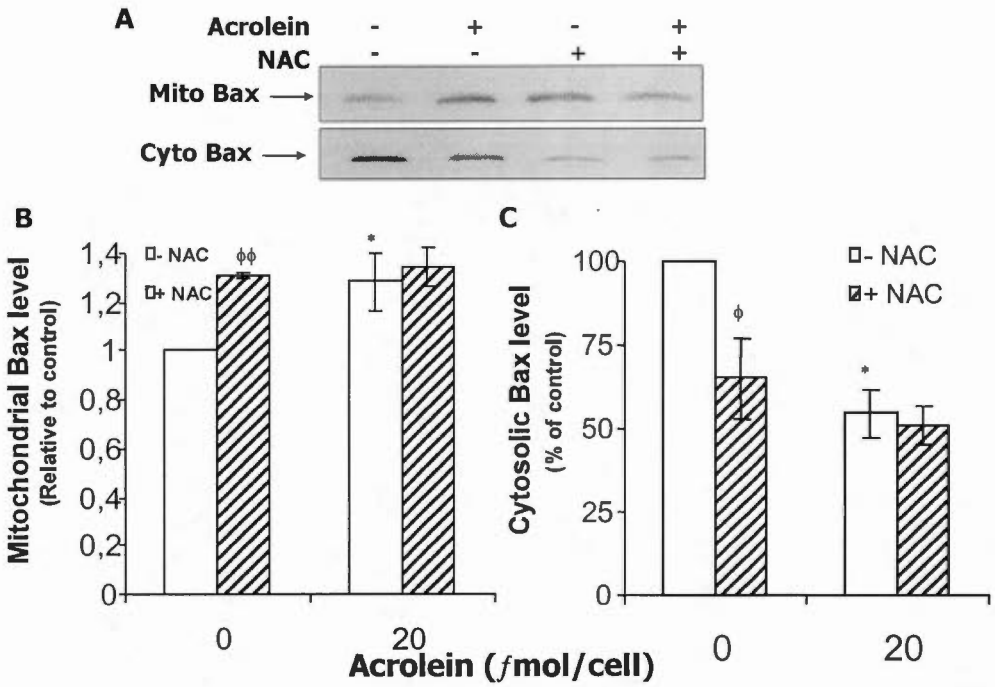


Figure 6: NAC inhibits acrolein-induced translocation of Bcl-2 to the cytosol

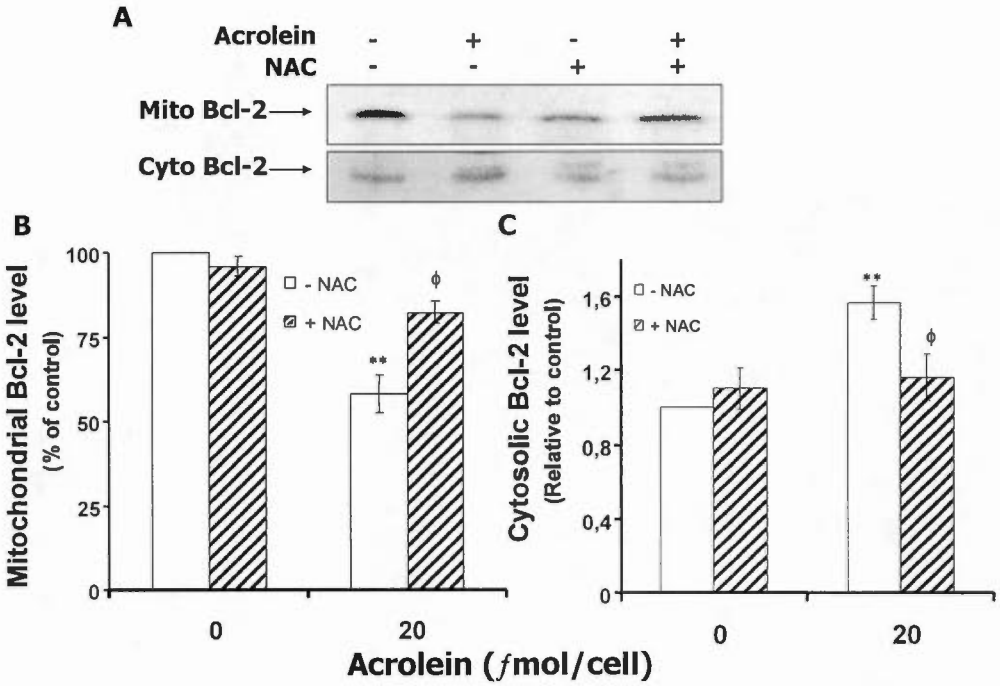


Figure 7: NAC inhibits acrolein-induced depolarization of the mitochondrial membrane

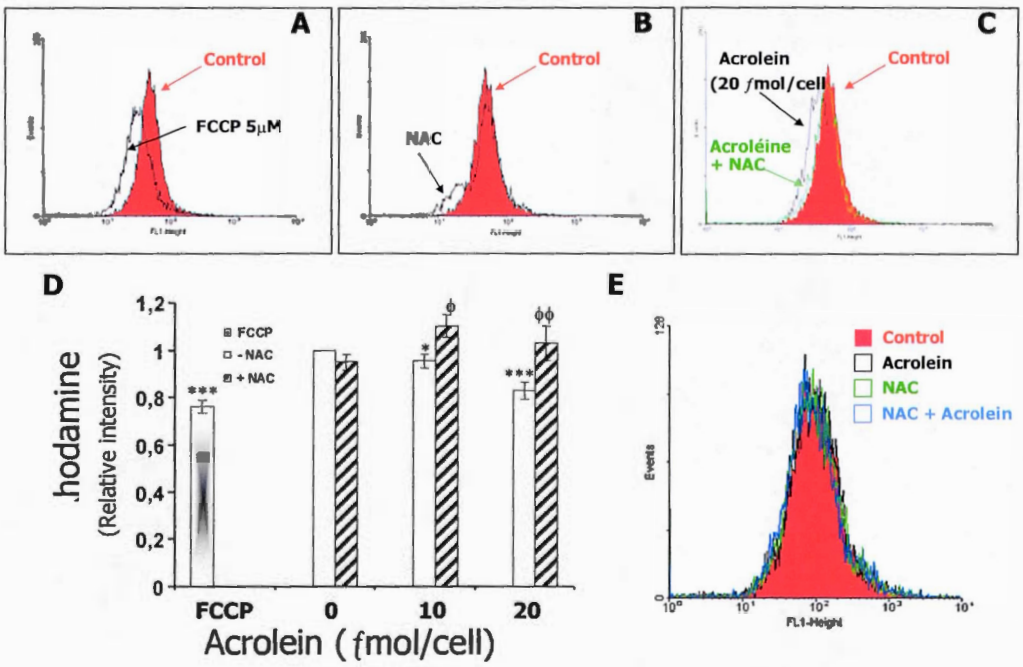


Figure 8. Acrolein-induced liberation of cytochrome-c to the cytosol is not affected by NAC

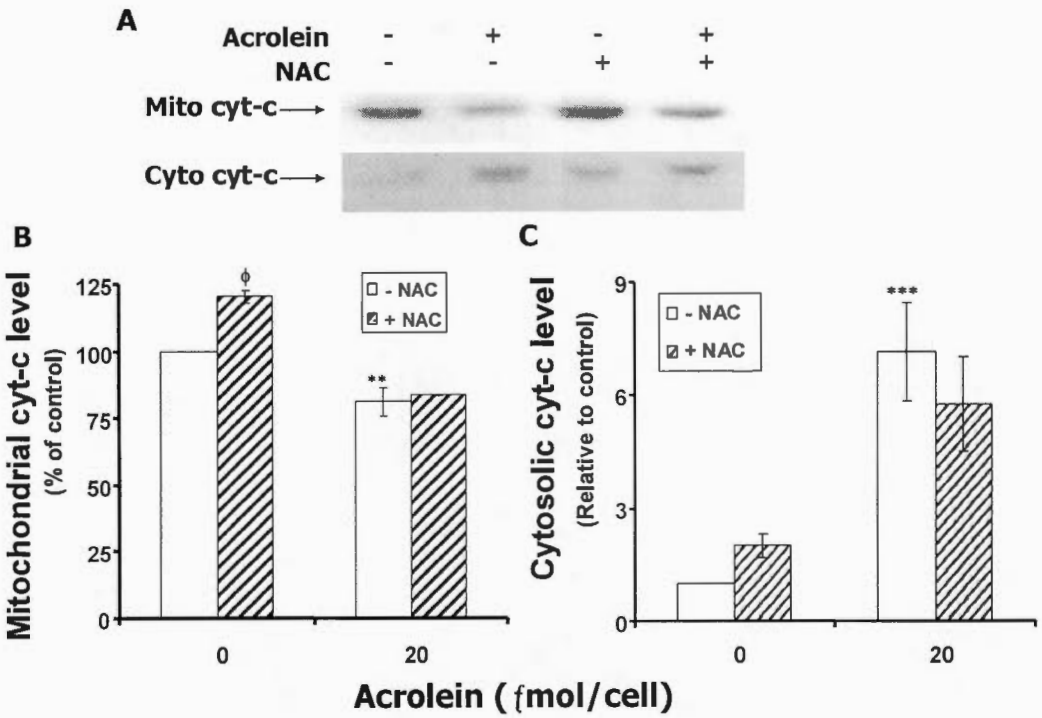


Figure 9: Activation of initiator caspase-9 by acrolein is inhibited by NAC

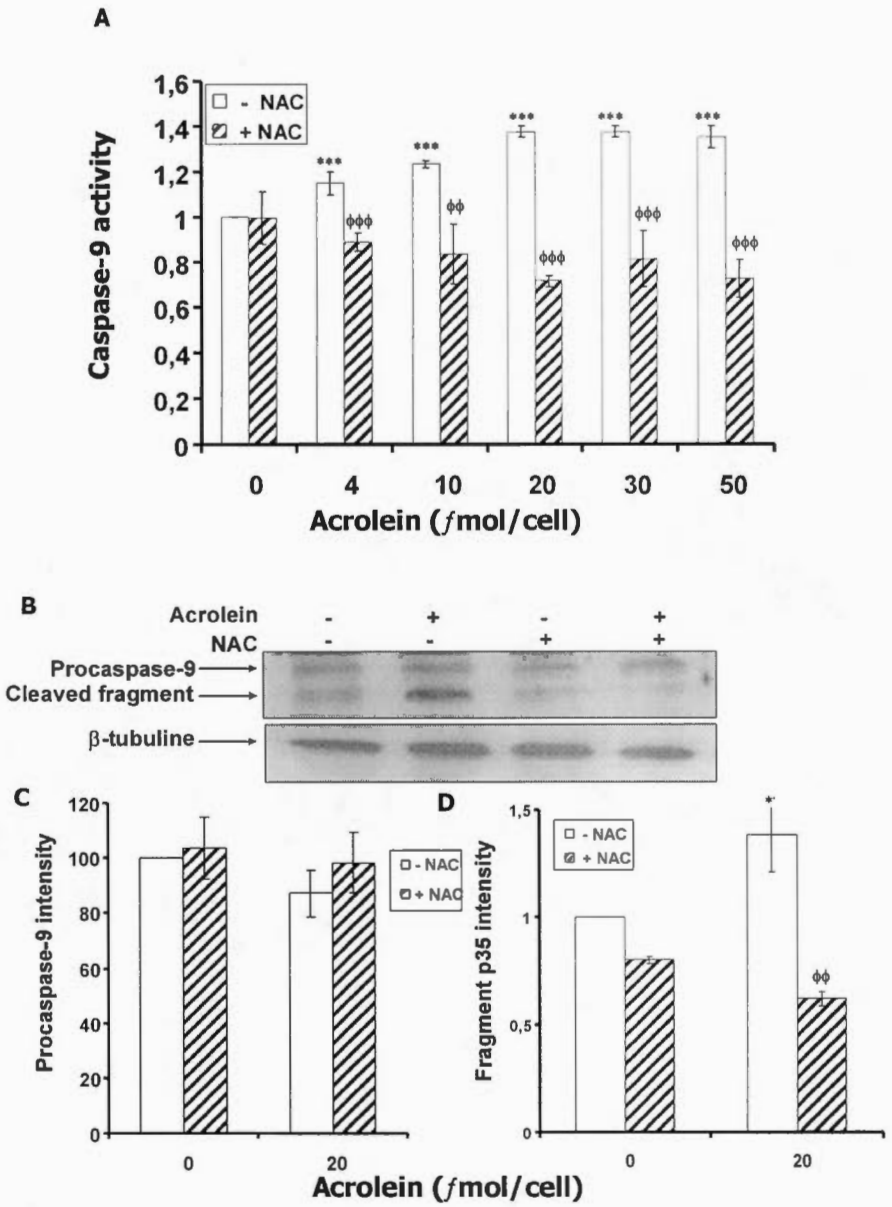


Figure 10: NAC inhibits activation of effector caspase-7 and initiator caspase-8 by acrolein

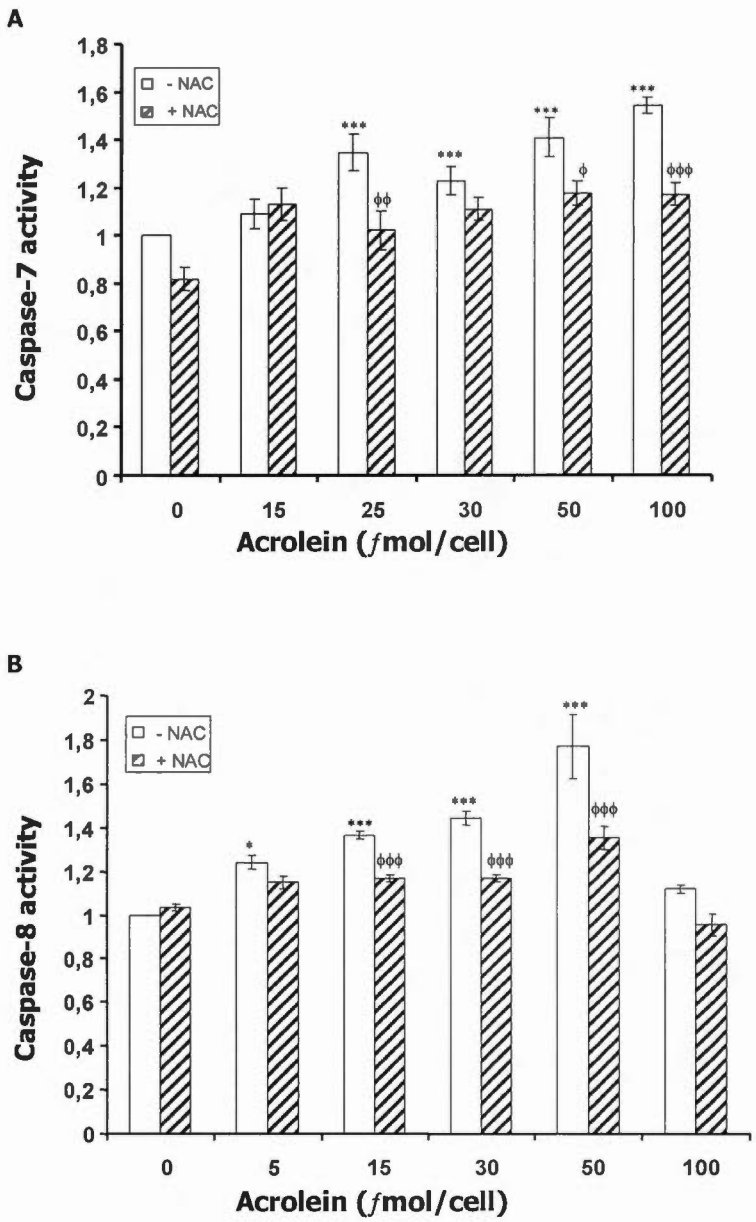


Figure 11: NAC inhibits acrolein-induced cleavage of PARP

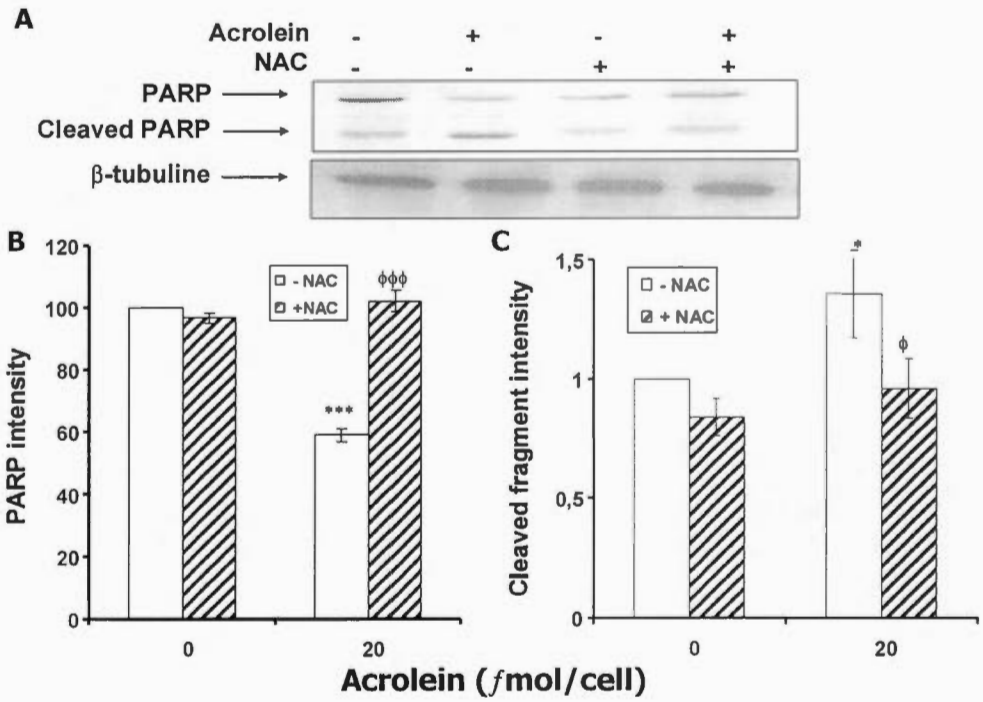
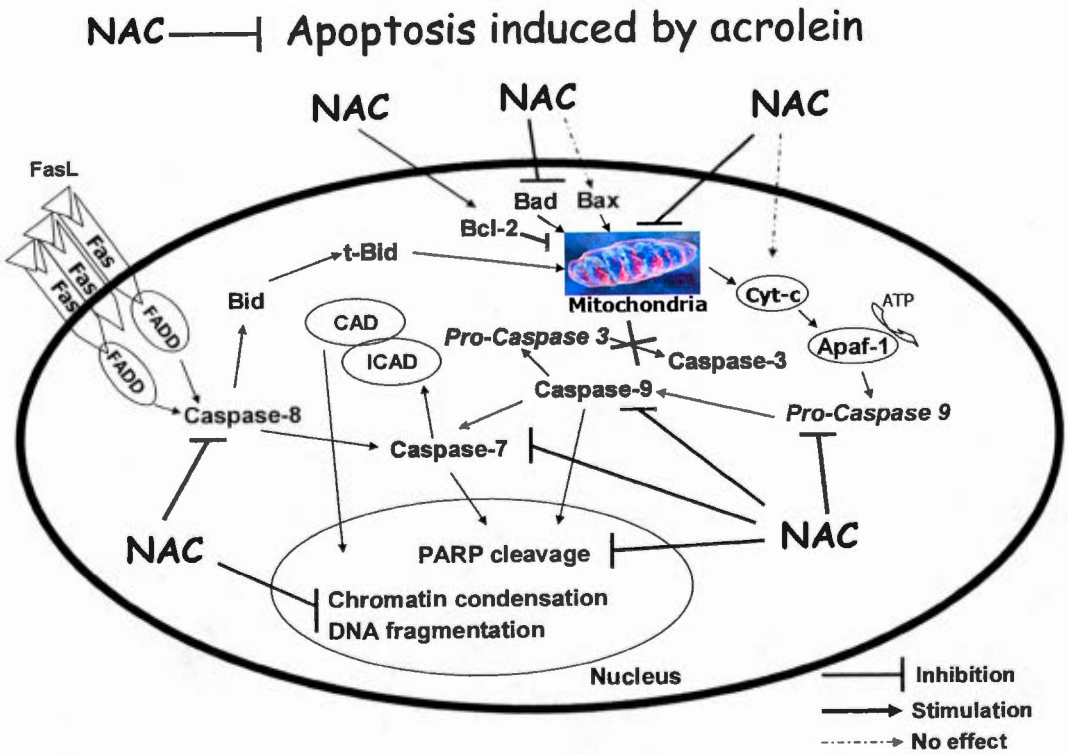


Figure 12: Proposed schema for NAC-induced inhibition of acrolein-induced apoptosis



CHAPITRE III

CONCLUSION

L'acroléine est la plus électrophile des aldéhydes α,β-insaturés, extrêmement toxique et elle est très présente dans l'environnement. Elle est utilisée comme herbicide et générée par la fumée de cigare tte, la cuisson d'huile, le métabolisme de certains agents anticancéreux, lors de la peroxidation des lipides et après exposition aux rayons UV. L'acroléine est impliquée dans différentes maladies respiratoires chroniques (bronchite, asthme) (Hogg 2001) et neurodégénératives (Alzheimer) (Arlt *et al.*, 2002) ainsi que l'athérosclérose (Biswal *et al.*, 2002). La peau est aussi une des cibles de l'acroléine, puisque la molécule est formée de façon endogène suite aux irradiations des lipides par les rayons ultraviolets du soleil et la quantité d'acroléine formée peut être mesurée par immunohistochimie. Dans la peau, l'acroléine peut causer une hypersensibilité non-immunologique, une inflammation, le développement de cancer, le vieillissement, une cytotoxicité et une génotoxicité.

Cette étude montre pour la première fois que l'induction de l'apoptose par l'acroléine passe par la voie mitochondriale et par la voie du récepteur de mort Fas et que cette induction est MAPK-dépendante et caspase-dépendante.

3.1. Induction de l'apoptose par l'acroléine selon la littérature

La majorité des données de la littérature concernant la toxicité de l'acroléine porte sur sa nature électrophilique et son effet toxique *in vivo* et *in vitro*. En ce qui concerne les mécanismes d'activation de l'apoptose par l'acroléine, peu de recherches ont été effectuées. Ces études ont montré plutôt que l'acroléine induit l'apoptose par détection des évènements tardifs (fragmentation de l'ADN), sans déterminer les mécanismes par lesquels l'acroléine induit l'apoptose. L'étude des mécanismes d'activation de la cascade apoptotique constitue la contribution importante et originale de cette étude. Dans la littérature, on rapporte une induction de l'apoptose

par l'acroléine et ceci est démontré par la fragmentation de l'ADN au niveau des macrophages humains (Li *et al.*, 1997 et 1999) et des kératinocytes humains (Takeuchi *et al.*, 2001), par l'externalisation des phosphatidylsérines (PS) chez les cellules épithéliales bronchiales humaines HBE1 (Nardini *et al.*, 2002). En plus, l'apoptose était révélée par la coloration nucléaire et le clivage de la caspase-3 chez les cellules endothéliales HUVEC (Misonou *et al.*, 2005) et par l'activité de la caspase-3 chez les cellules tubulaires proximales humains (Schwerdt *et al*, 2005). Cependant, d'autres études démontrent que l'acroléine inhibe l'apoptose en faveur de la nécrose et ceci en abaissant l'activité enzymatique des caspases-3, -8 et -9 chez les lymphocytes B provenant de souris (Kern et Kehrer, 2002). De plus, dans les cellules neuronales PC12, l'acroléine inhibe l'activité de la caspase-3 et la fragmentation de l'ADN, et rend les cellules perméables à l'iodure de propidium (Liu-Snyder *et al.*, 2006; Luo *et al.*, 2005). En plus, l'acroléine inhibe l'apoptose chez les neutrophiles humains en supprimant l'activité de la caspase-3, la fragmentation de l'ADN et l'externalisation des PS (Finkelstein *et al.*, 2001). Mais, une récente étude par la même équipe a démontré que l'acroléine induit l'apoptose à faible concentration chez les neutrophiles humains (<10μM) et celle-ci est révélée par l'induction de l'activité des caspases-3, -8 et -9, l'externalisation des PS et la libération du cytochrome-c. Mais à plus forte dose (>10μM) l'acroléine a induit la nécrose, une baisse de l'activité des caspases -8, -9 et -3 ainsi qu'une diminution de l'externalisation des PS. Pourtant la libération du cytochrome-c est restée forte à ces deux conditions (Finkelstein *et al.*, 2005). Ces résultats variables de l'activation ou l'inhibition de l'apoptose par l'acroléine peuvent être dus aux différences dans les processus biochimiques impliqués dans l'induction de la mort cellulaire dans ces différents types cellulaires, qui incluent des cellules primaires non-prolifératives, des cellules immunitaires et des lignées cancéreuses, ainsi que le temps et la dose d'acroléine administrée. En plus, différentes méthodologies ont été utilisées dans ces études, comme des temps d'exposition à l'acroléine beaucoup plus long, de 24 h à 48 h,

comparativement à des courtes expositions de l'ordre de 30 min à 4h. Dans quelques études incluant la présente, les cellules étaient incubées dans un milieu contenant du sérum (Hamilton *et al.*, 1997); pourtant dans d'autres études, l'acroléine était incubée dans un milieu sans sérum (Takeuchi *et al.*, 2001; Nardini *et al.*, 2002; Finkelstein *et al.*, 2001; Kern et Kehrer, 2002). L'acroléine réagit avec les protéines du sérum diminuant ainsi sa concentration libre disponible. Ceci peut ralentir ou modifier le processus toxique induisant l'apoptose. La nécrose semble la forme de mort cellulaire prédominante quand les cellules sont exposées à l'acroléine dans un milieu sans sérum (Kern et Kehrer, 2002). Dans cette étude, l'acroléine induisait la nécrose mais à plus forte dose d'acroléine.

Alors, on remarque que les deux principales voies de l'activation de l'apoptose, la voie mitochondriale et la voie des récepteurs, n'ont pas été investiguées en profondeur jusqu'à présent ainsi que le rôle de la voie de signalisation des MAPK dans l'activation de la cascade apoptotique par l'acroléine.

Cependant, de récentes études ont démontré que d'autres aldéhydes peuvent induire l'apoptose. En fait, le 4-hydroxynonénal, un marqueur de la maladie d'Alzheimer, induit l'activation des caspases -2, -3, -8 et -9, le clivage de PARP et la libération du cytochrome-c (Ji et al., 2001). En plus, le 4-hydroxynonénal stimule la voie de signalisation des MAPKs et ceci par la phosphorylation de c-jun (Pugazhenthi et al., 2006; Bruckner et Estus, 2002) et par l'activation de JNK (Castello et al., 2005). Cependant, une nouvelle étude sur des fibroblastes a démontré que l'apoptose induite par l'aldéhyde 4-hydroxynonénal est JNK-dépendante (Kutuk et al., 2006). D'autre part, la p53 qui joue un rôle majeur dans l'apoptose est surexprimée suivant une exposition à l'hydroxynonénal chez une lignée de neuroblastomes (Laurora et al., 2005). Enfin, l'induction de l'apoptose par la voie mitochondriale par le cinnamaldéhyde est p38 et JNK-dépendantes (Wu et al., 2006).

3.2. Les mécanismes d'action de l'apoptose induite par l'acroléine

3.2.1. Induction de la voie mitochondriale de l'apoptose par l'acroléine

Lors de la présente étude, des tests de cytotoxicité ont démontré que l'acroléine est un composé très toxique pour les cellules CHO puisqu'une concentration de 200 *f*mole/cellule éliminait 95% des cellules après une heure d'exposition. On a observé que l'acroléine induit l'apoptose (condensation de la chromatine après quatre heures d'exposition) et la nécrose à petite dose (30 *f*mole/cellule), mais une élevation de la concentration à 100 *f*mole/cellule entraîne la nécrose plutôt que l'apoptose. Notre investigation sur les mécanismes d'activation de l'apoptose a montré que l'acroléine stimule la voie mitochondriale en causant la baisse du potentiel membranaire mitochondrial, la libération du cytochrome-c dans le cytosol, la translocation des protéines pro-apoptotiques Bax et Bad vers la membrane mitochondriale, la translocation de la protéine anti-apoptotique Bcl-2 vers le cytosol, ainsi qu'une activation de la caspase-9 initiatrice (Figure 3.1). Cette cascade enzymatique a entraîné le clivage de différents substrats des caspases dont l'inhibiteur ICAD et la protéine PARP, l'externalisation des phosphatidylsérines et l'apparition de la condensation de la chromatine (Figure 3.1). Les caspases -3 et -6 n'étaient pas activées mais plutôt inhibées et ceci pourrait probablement résulter d'une liaison directe au site actif par l'addition de Michael puisque les caspases sont des cystéines protéases qui sont régulées suite à un stress oxydatif par modification de leur thiol du site actif (Chandra *et al.*, 2000; Mannick *et al.*, 1999). Il était rapporté que les cellules du carcinome du sein MCF7, qui sont dépourvues de la caspase-3, peuvent subir l'apoptose par l'intermédiaire de la caspase-7 effectrice (Degterev *et al.*, 2003). En effet, l'apoptose induite par l'acroléine chez les cellules CHO semble être médiée par la caspase-7 effectrice (Figure 3.1). En plus de la caspase-3, la caspase-9 est capable de cliver directement la caspase-7 et entraîner son activation (Slee *et al.*, 1999). Dans cette étude, le clivage de l'ICAD semble être médié par la caspase-7 (Wolf *et al.*,

1999; Houde *et al.*, 2004). C'est souvent difficile de distinguer entre l'implication de la caspase-3 ou -7 dans l'apoptose puisque les deux partagent plusieurs substrats en commun (PARP, ICAD) et le peptide Ac-DEVD-AMC (Cohen, 1997).

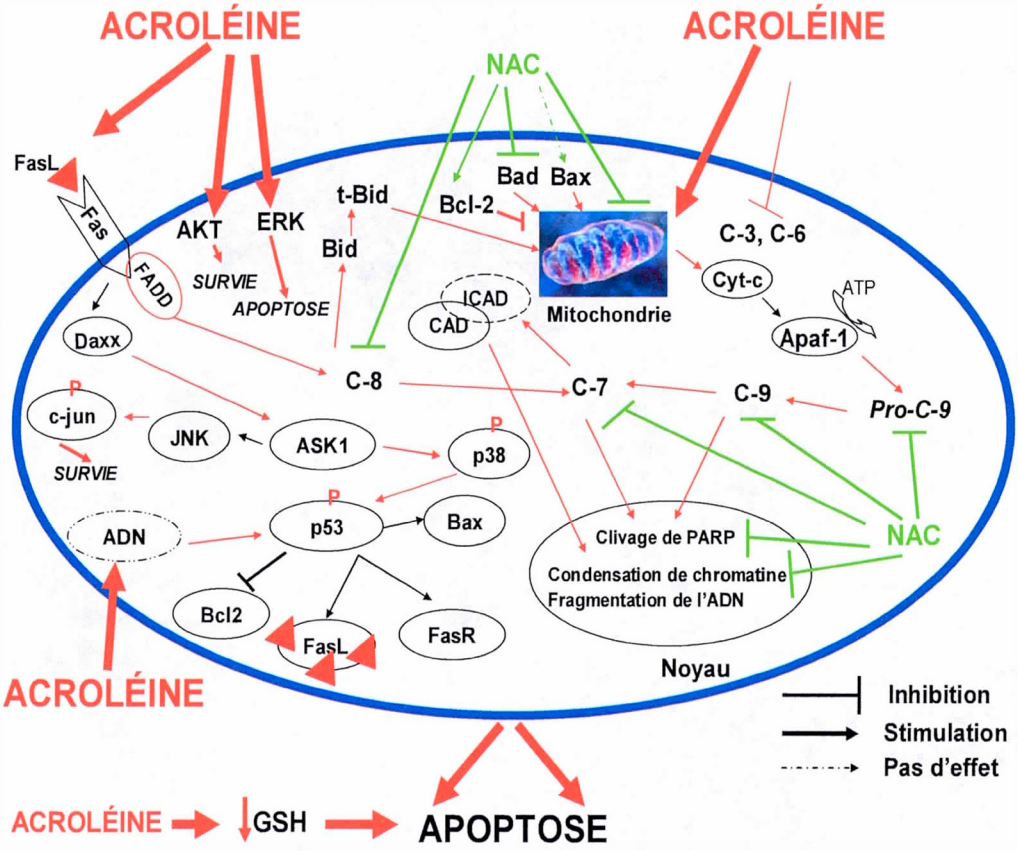


Figure 3.1 : Schéma représentant les voies de signalisation dans l'induction de l'apoptose par l'acroléine : inhibition par le NAC.

Nous avons observé que l'acroléine clive la procaspase-3 (2-50 *f*mole/cellule) mais inhibe très rapidement (< 5 min) son activité enzymatique. Le clivage de la procaspase-3 résulte probablement de l'action enzymatique directe de la caspase-9 active (Lawen, 2003). En plus, l'utilisation d'un inhibiteur spécifique de la caspase-3 n'a pas emmené une baisse de l'apoptose induite par l'acroléine. L'inhibition enzymatique peut être due à une alkylation directe du site actif par l'acroléine, ce qui est en accord avec d'autres études (Finkelstein *et al.*, 2001). La baisse de l'activité de la caspase-3 se produit chez les neutrophiles après 2 à 8 h d'exposition à 10 μM d'acroléine (Finkelstein *et al.*, 2001). De même, les activités des caspases -3, -8 et -9 étaient inhibées après 30 min d'exposition à l'acroléine (5 to 40 μM) chez les lymphocytes murins (Kern et Kehrer, 2002). Les caspases -7 et -9 sont aussi clivées et activées par l'acroléine dans les cellules CHO mais il semble qu'elles sont moins susceptibles que la caspase-3 à l'inhibition par l'acroléine. L'inhibition des caspases-7 et -9 se produit également, mais seulement après 3 h et 2 h d'exposition, respectivement, puisqu'elles possèdent toutes les deux un résidu cystéine nucléophilique dans leur site actif qui peut être alkylé par l'acroléine (Finkelstein *et al.*, 2001). L'activation des caspases par l'acroléine semble être le résultat d'un net équilibre entre deux processus, soit l'activation et l'inhibition des enzymes. Bien que ces caspases puissent être inhibées, il reste une activité caspase nette mais minimale pour induire le clivage de l'ICAD et la condensation de la chromatine (Figure 3.1). La différence dans l'inhibition de ces caspases peut s'expliquer aussi par le fait que les caspases ne se ressemblent pas toutes au niveau du site actif mais plutôt au reste de la structure, ce qui expliquerait la limitation de l'accès et les différences des substrats (Degterev *et al.*, 2003).

Récemment, on a rapporté que le clivage protéolytique n'est pas suffisant pour l'activation de la caspase-9 initiatrice (Boatright et Salvesen, 2003). La dimérisation plutôt que le clivage semble le plus important dans la formation du site actif et l'activation de la caspase-9. Des changements conformationnels identiques pour

former le site actif ont été observés pour les caspases -9 et -7, même si elles sont activées par des mécanismes distincts. D'autre part, un clivage interdomaine par les caspases initiatrices est nécessaire pour l'activation des caspases effectrices -3 et -7 (Boatright et Salvesen, 2003).

L'activation des caspases -9 et -7 était confirmée par le clivage de leurs formes pro-enzymes et la génération des fragments appropriés, mais surtout par l'augmentation de l'activité enzymatique. De plus, le rôle de la caspase-9 dans l'induction de l'apoptose par l'acroléine était confirmé en utilisant un inhibiteur de son activité. Jusqu'à présent, il n'y a pas d'inhibiteur spécifique disponible pour la caspase-7.

L'implication du pore de transition de perméabilité (PTP) et du relargage du cytochrome-c dans l'apoptose induite par l'acroléine étaient confirmés en utilisant la cyclosporine A, un inhibiteur du pore PTP. Bien que le mécanisme du relargage du cytochrome-c n'est pas complètement connu, il est considéré que le cytochrome-c est libéré via les pores PTP et les canaux formés par les interactions des protéines pro-apoptotiques Bax (Orrenius *et al.*, 2003). L'inhibition de l'apoptose par la cyclosporine A supporte un rôle pour les pores PTP et la libération du cytochrome-c dans l'apoptose induite par l'acroléine. L'inhibition partielle par la cyclosporine A suggère que l'acroléine peut induire l'apoptose par des mécanismes alternatifs à la voie mitochondriale et que le cytochrome-c peut être relargué par plusieurs mécanismes.

3.2.2. Induction de la voie des récepteurs de mort par l'acroléine

Une autre nouvelle contribution de cette étude est que l'exposition des cellules prolifératives CHO à l'acroléine stimule la voie du récepteur de mort Fas (Figure 3.1). Cette affirmation est confirmée par l'augmentation de l'expression du ligand Fas, la translocation de l'adapteur de domaine de mort de Fas (FADD) à la membrane plasmique et l'activation de la caspase-8 initiatrice. Le Kp7-6, un antagoniste du

récepteur Fas et / ou Z-IETD-FMK, un inhibiteur de la caspase-8, bloquaient les évènements apoptotiques en aval de la caspase-8, comme l'activation de la caspase-7 exécutrice et la condensation de la chromatine nucléaire. L'acroléine induit une transduction croisée entre la voie de signalisation des récepteurs de mort et celle de la mitochondrie en clivant la protéine Bid en sa forme tronquée t-bid qui se transloque à la membrane mitochondriale pour stimuler cette voie. L'inhibition du récepteur Fas ou de la caspase-8 inhibait partiellement l'activation de la caspase-9, un évènement post-mitochondrial, par l'acroléine. Ces résultats démontrent que l'acroléine active la voie du récepteur Fas en amont de la voie mitochondriale. La caspase-9 demeurait partiellement active malgré l'inhibition du récepteur Fas et de la caspase-8, suggérant que l'acroléine peut induire la voie mitochondriale indépendamment de la voie des récepteurs. En plus, le Z-LEHD-FMK, un inhibiteur de la caspase-9, inhibait partiellement la condensation de la chromatine induite par l'acroléine. Ces résultats suggèrent que les deux voies sont activées indépendamment et que la voie des récepteurs peut stimuler la voie mitochondriale par l'intermédiaire de t-bid.

Chez les neutrophiles, de faibles doses d'acroléine activaient la caspase-8 et au contraire, de fortes doses inhibaient son activité (Finkelstein *et al.*, 2005). En plus, l'acroléine inhibait l'activité de la caspase-8 chez les lymphocytes B murins (Kern et Kehrer, 2002). Dans ces études, l'exposition à l'acroléine était courte mais la détection de l'activité se faisait de 6 à 24 h après l'exposition. Après une longue incubation, il est possible que le type de mort cellulaire change de l'apoptose en nécrose. Il a été rapporté que les composés toxiques induisent l'apoptose à des faibles doses et la nécrose à des conditions plus sévères et que le type de mort dépend du type cellulaire (Orrenius et Zhivotovsky, 2006).

3.2.3. Implication de la voie de signalisation des MAPK dans l'apoptose induite par l'acroléine

La voie de récepteur de mort Fas converge aussi à l'activation de la MAPKKK ASK1 par l'intermédiaire de l'adapteur Daxx. Ensuite, ASK1 active les voies MAPKs, JNK et p38 qui sont impliquées dans la transcription des gènes des protéines apoptotiques dont Bax, Bim, le ligand Fas et le récepteur Fas.

L'acroléine induit l'apoptose par l'activation du récepteur du facteur de croissance épidermale (EGFR) qui active la voie des MAPKs chez les kératinocytes humains (Takeuchi *et al.*, 2001). Mentionnons ici que l'activation des MAPKs peut prendre différentes voies de signalisation et ainsi emmener l'apoptose dans le cas des kératinocytes humains (Takeuchi *et al.*, 2001) et l'inhibition de l'apoptose chez les neutrophiles humains (Finkelstein *et al.*, 2001). Toutefois le lien entre l'apoptose et l'activation des MAPK n'était pas défini dans ces deux études. La présente étude montre pour la première fois que l'apoptose induite par l'acroléine est MAPK-dépendante. On a démontré par des inhibiteurs pharmacologiques, que l'acroléine active les voies de signalisation ASK1/p38 et ERK, induisant plusieurs caractéristiques de l'apoptose dont l'activation des caspases-9 et -7, le clivage de l'ICAD et la condensation de la chromatine.

Trois différentes MAP kinases, p38, JNK et ERK sont impliquées dans l'apoptose. Bien qu'elles jouent un rôle important dans la signalisation de l'apoptose, leurs rôles dans l'induction de l'apoptose par l'acroléine demeurent inconnus. Cette étude démontre que l'acroléine active la c-jun (Figure 3.1), un substrat de JNK, et l'inhibition de l'activité de JNK par le SP600125, change le mode de mort par apoptose en une mort totale par nécrose. Ceci confirme que la JNK est impliquée dans une voie de survie contre l'agression par acroléine. En plus, le SP600125 inhibait l'activation des caspases -7 et -9, et le clivage de l'ICAD. Ces observations sont des résultats attendus puisque l'activation des caspases n'est pas une caractéristique de la nécrose. Il était rapporté que JNK est impliquée dans l'apoptose

des cellules du carcinome pulmonaire (Chuang *et al.*, 2000) et celle des macrophages murins (Kim et Sharma, 2004) mais elle n'était pas impliquée dans l'apoptose des cellules neuronales HT4 (Rockwell *et al.*, 2004) où p38 jouait le rôle majeur. Cependant, JNK est importante dans le développement et la survie des macrophages (Himes *et al.*, 2006). Ainsi, la fonction de JNK dans l'apoptose est contradictoire.

Par contre, la p38 MAPK est impliquée dans l'induction de l'apoptose par l'acroléine. L'incubation des cellules CHO avec l'acroléine induisait la phosphorylation de la p38 (Figure 3.1) et un prétraitement avec le SB203580, un inhibiteur de la p38, inhibait l'apoptose induite par l'acroléine. L'inhibiteur SB203580 inhibait l'activation des caspases-7 et -9, le clivage de l'ICAD et la condensation de la chromatine induites par l'acroléine. Bien que les stimuli de stress activent la p38 et induisent l'apoptose dans des modèles de dommages cellulaires, ce n'est pas le cas chez tous ces modèles. L'activation de la p38 était requise pour l'apoptose induite par le cadmium (Galan *et al.*, 2000), la soustraction des facteurs trophiques (Kummer *et al.*, 1997), et l'ischémie (Mackay et Mochly-Rosen, 1999), pourtant l'incapacité de SB203580 à prévenir l'apoptose induite par les UV (Franklin *et al.*, 1998) et la S-nitrosoglutathion (Callsen et Brune, 1999) prouve la faible corrélation entre p38 et l'apoptose. D'autres résultats contradictoires sont obtenus chez des cellules neuronales. Dans une étude récente dans les cellules neuronales HT4, l'inhibition de la p38, et non de JNK, bloquait l'apoptose induite par le cadmium (Rockwell *et al.*, 2004), des résultats qui concordent avec notre étude où une inhibition de la p38 bloquait l'apoptose induite par l'acroléine. Une telle anomalie suggère l'existence d'une grande différence dans la régulation de la réponse face au stress dépendamment du type cellulaire et de l'agent administré.

En plus des facteurs de croissance, des cytokines, et des mitogènes, ERK est aussi activé par des agents cytotoxiques. Dans notre étude, ERK semble être impliquée dans l'apoptose induite par l'acroléine. Acroléine induit la phosphorylation de ERK, et l'inhibition de son activité par le U0126 inhibait l'activation des caspases-7 et -9, le clivage de l'ICAD et la condensation de la chromatine par l'acroléine. En

plus, un autre inhibiteur de ERK, le PD98059, inhibait l'activation des caspases-7 et -9, et le clivage de l'ICAD sans toutefois avoir un effet sur la condensation de la chromatine, une des étapes finales dans l'induction de l'apoptose. PD98059 et U0126 sont toutes les deux des inhibiteurs de MEK qui sont utiles pour étudier les mécanismes biologiques et surtout ceux impliqués dans l'activation des MAPKs. Ils sont des inhibiteurs non-compétitifs des substrats de MEK, l'ATP et ERK respectivement (Favata *et al.*,1998). Elles ont des mécanismes d'action différents (Dudley *et al.* 1995; Favata *et al.* 1998), U0126 est in inhibiteur spécifique de l'activité de MEK1 et 2 tandis que le PD98059 est un inhibiteur de l'activation de MEK1. Ainsi le U0126 est un plus puissant inhibiteur que le PD98059 (Alessi *et al.*, 1995; Dudley *et al.*, 1995; Favata *et al.*, 1998). L'activation de la MAPK ERK peut inhiber l'apoptose (Chuang *et al.*, 2000), l'activer (Lee *et al.*, 2006; Ruffels *et al.*, 2004; Yang G. *et al.*, 2004) ou même n'avoir aucun effet (Galan *et al.*, 2000). En plus, le rôle de ERK est différent d'un agent toxique à l'autre dans le même type cellulaire. Par exemple, chez les cellules de neuroblastomes humains SH-SY5Y, l'apoptose induite par le peroxyded'hydrogène était inhibée par un inhibiteur de ERK (Ruffels *et al.*, 2004) qui n'avait pas d'effet sur l'apoptose induite par le cadmium (Kim *et al.*, 2005). Ceci suggère que les agresseurs n'induisent pas tous les mêmes voies de signalisation.

3.2.4. Induction de la voie de survie AKT par l'acroléine

Afin de vérifier le lien entre l'acroléine et les voies de survie cellulaire, on a entrepris des expériences sur l'AKT (protéine kinase B ou PKB) qui est une protéine activée en aval des récepteurs de facteurs de croissance. AKT est la plus caractérisée des kinases qui sont connues de promouvoir la survie cellulaire et de jouer un rôle majeur dans le métabolisme du glucose. AKT est activée en réponse à plusieurs facteurs de croissance, à des hormones ainsi qu'à des agresseurs exogènes (Datta *et al.*, 1999; Lawlor et Alessi, 2001; Talapatra et Thompson, 2001; Downward, 1998;

Brazil et Hemmings, 2001). Les facteurs de croissance et les hormones qui activent la voie AKT incluent le facteur de croissance ressemblant à l'acroléine (IGF), le facteur de croissance épidermale (EGF), le facteur de croissance des fibroblastes (bFGF), l'insuline et l'interleukine 6 (Datta *et al.*, 1999; Talapatra et Thompson, 2001; Downward, 1998). Dans nos cellules, l'AKT joue un rôle dans la survie cellulaire contre l'agression par l'acroléine. En effet, l'acroléine phosphoryle l'AKT et le prétraitement avec des inhibiteurs pharmacologiques de l'AKT, qui sont le LY294002 et le Wortmannin, changeait la mort cellulaire de l'apoptose en nécrose totale. Ces deux inhibiteurs agissent sur la voie de survie cellulaire de la phosphoinositide 3-kinase (PI3-K) et ils sont très utiles dans l'étude des mécanismes biologiques impliquant la voie PI3-K (Stein et Waterfield, 2000; Fruman, 1998). Le LY294002 et le Wortmannin diminuaient significativement l'activation des caspases-7 et -9 et le clivage de l'ICAD par l'acroléine, des résultats attendus dans des conditions nécrotiques.

Parmi les protéines kinases kinases kinases activées par des mitogènes (MAPKKK), la kinase régulatrice du signal d'apoptose 1 (ASK1) est activée en réponse à des stresseurs cytotoxiques tels que les EOR, le FasL, le H_2O_2 et le TNF-α, et induit l'activation de JNK et p38, et l'apoptose (Tobiume *et al.*, 2001). Le mécanisme de toxicité de l'acroléine implique la génération du stress oxydatif. L'acroléine génère des EORs (Luo et Shi, 2004 et 2005) et des radicaux libres, et des antioxydants comme le glutathione et l'hydralazine réduisent sa toxicité (Nunoshiba et Yamamoto, 1999; Burcham *et al.*, 2000). Comme attendu, nos résultats démontrent que l'acroléine entraîne l'augmentation de l'expression de ASK1 (Figure 3.1) et la phosphorylation de la p38 et du substrat de JNK, c-jun, à des concentrations apoptotiques.

3.2.5. Phosphorylation de la p53 par l'acroléine

Un nouvel aspect de cette étude démontre la phosphorylation de la p53 aux sérines 15 et 46 par l'acroléine (Figure 3.1) qui est un agent endommageant de l'ADN (Esterbauer *et al.*, 1991). Les sérines 15 et 46 de la p53 sont phosphorylées en réponse aux dommages à l'ADN (Cnman *et al.*, 1998; Bulavin *et al.*, 1999). La phophorylation de la sérine 15 réduit la liaison de l'oncogène mdm2 à la p53 *in vitro* (Shieh *et al.*, 1997) pour inhiber sa dégradation par la mdm2 (Haupt *et al.*, 1997). Effectivement, l'acroléine induit la phosphorylation de la p53 à la sérine 15, et comme attendu celle de la sérine 46 qui est la cible de la p38 (Bulavin *et al.*, 1999) qu'on a démontré son activation. Parmi les évènements médiés par la p53, il y a l'apoptose et l'arrêt du cycle cellulaire. La p53, qui est un facteur de transcription induit la transcription des protéines proapoptotiques comme Bax et le ligand Fas, et supprime celle des antiapoptotiques comme le Bcl-2 (Bras *et al.*, 2005). D'ailleurs, nous avons démontré que l'acroléine induit une augmentation de Bax et une baisse de Bcl-2 à la membrane mitochondriale ainsi que l'augmentation de l'expression du ligand Fas, tous des changements régulés par la p53. Ainsi, la présente étude démontre que p38 et ERK sont des molécules clés dans l'induction de l'apoptose par l'acroléine et que AKT et JNK sont des voies de survie empruntée par la cellule pour contrer sa toxicité cellulaire.

3.2.6. L'antioxydant N-acétylcystéine inhibe l'apoptose induite par l'acroléine

Puisque l'acroléine est un toxique omniprésent dans notre environnement, on a étudié l'effet protecteur des antioxydants sur les cellules afin de pouvoir traiter les gens qui y sont exposés. On a démontré que le glutathion qui est parmi les plus importants antioxydants intracellulaires, joue un rôle important dans la détoxification de l'acroléine. Ainsi, le NAC, un précurseur du glutathion, a inhibé la toxicité induite par l'acroléine dans les cellules prolifératives CHO. L'acroléine se lie rapidement aux

molécules nucléophiliques comme l'antioxydant glutathion, qui est impliqué dans la défense cellulaire, et emmène une diminution de sa concentration intracellulaire (Heck, 1997; Kehrer et Biswal, 2000). De plus, elle inactive l'enzyme glutathione réductase qui regénère la forme réduite du glutathione (GSH) par le cycle rédox du glutathione. La baisse de la forme réduite du GSH rend la cellule plus vulnérable aux dommages causés par le stress oxydatif. Nos résultats montrent une baisse accrue du GSH intracellulaire avec des faibles concentrations de l'ordre de 10 *f*mole/cellule. Une exposition des cellules CHO à une concentration non-léthale d'acroléine (4 *f*mole/cellule) diminue le glutathione intracellulaire de 55% dans 30 min. Cette baisse est plus rapide et dramatique par rapport à celle observée chez les cellules CHO exposées au H_2O_2 pendant 1 heure (25%) (Lord-Fontaine et Averill-Bates, 2002). Puisque les concentrations d'acroléine qui abaissent le glutathion intracellulaire sont beaucoup plus faibles que celles qui induisent la cytotoxicité, cela suggère que la baisse du glutathion est une étape importante qui précède les conditions cytotoxiques. Dans les cellules du carcinome pulmonaire A549, il y avait une forte corrélation entre la baisse du glutathion et l'inhibition de la prolifération cellulaire (Horton *et al.*, 1997).

Le NAC, qui augmente le niveau intracellulaire de glutathion, offre une protection contre la cytotoxicité, la nécrose et l'apoptose (Figure 3.1) engendrées par l'acroléine. Le GSH joue un rôle important dans la détoxification cellulaire des espèces oxydatives tels que le H_2O_2 et les hydroperoxydes lipidiques (Meister et Anderson, 1983) et la protection des cellules épithéliales pulmonaires de l'oxydation et de l'inflammation. La balance oxydo-réductrice du glutathion est critique pour plusieurs fonctions cellulaires et des changements induits par l'acroléine semblent affecter plusieurs voies de signalisation. Ceci inclut l'activation de plusieurs gènes de transcription, la régulation de la prolifération cellulaire et l'apoptose (Horton *et al.*, 1997, 1999; Ramu *et al.*, 1996; Rahman et MacNee, 1999, Rahman *et al.*, 1999; Arrigo, 1999; Bojes *et al.*, 1999).

En plus, nous avons apporté de nouvelles informations sur les mécanismes de protection par le NAC contre l'apoptose induite par l'acroléine. Les concentrations d'acroléine qui abaissent le glutathion coincident avec celles qui induisent les évènements précoces de l'apoptose comme la translocation du Bax et Bad, la dépolarisation de la membrane mitochondriale, la libération du cytochrome-c et l'activation de la caspase-9 (Figure 3.1). Aucune autre étude n'a rapporté une protection contre l'apoptose induite par l'acroléine par n'importe quel antioxydant. La protection de l'apoptose par le NAC a été confirmée morphologiquement par l'inhibition de la condensation de la chromatine, un évènement tardif de l'apoptose, induite par l'acroléine. Un prétraitement avec NAC inhibait l'induction de la voie mitochondriale par l'acroléine puisque le clivage de la caspase-9 et son activation ont été totalement inhibés. L'activation de la caspase-9 est considérée comme un indicateur de la voie mitochondriale (Chandra *et al.*, 2000). NAC inhibait la translocation du Bad vers la mitochondrie ainsi que la translocation du Bcl-2 au cytosol induite par l'acroléine. NAC inhibait l'activation de la caspase-7 effectrice et celle de la caspase-8 initiatrice ainsi que le clivage de PARP par l'acroléine.

L'inhibition totale de la caspase-9 et l'inhibition partielle de la caspase-8 suggèrent que la voie mitochondriale est la première cible du mécanisme du protection par le NAC puisque l'activation de la caspase-8 médie l'apoptose par la voie du récepteur Fas (Saikumar *et al.*, 1999). Ainsi, nos résultats suggèrent que le GSH joue un rôle important dans la toxicité et la modulation de l'apoptose induite par l'acroléine.

La baisse du glutathion et d'autres thiols cellulaires corrèlent avec l'apoptose dans plusieurs types cellulaires (Aoshiba *et al.*, 1999; Sato *et al.*, 1995). En effet, l'apoptose induite par l'acroléine chez les neutrophiles coincide avec la baisse du glutathion intracellulaire (Finkelstein *et al.*, 2001). Il était suggéré que les effets de l'acroléine sont probablement dues aux changements d'oxydoréduction dans des molécules connexes au GSH (Finkelstein *et al.*, 2001). Cependant, la relation entre la baisse du glutathion et l'apoptose n'est pas encore claire et la baisse du GSH toute

seule peut ne pas être suffisante pour induire l'apoptose. En effet, il était proposé que l'apoptose impliquerait une sortie du GSH intracellulaire (Ghibelli *et al.*, 1995; Van den Dobbelsteen *et al.*, 1996). Ainsi, la diminution du GSH est plutôt une conséquence et non une cause de l'apoptose.

Un rôle pour le stress oxydatif dans l'apoptose intrigue de plus en plus de chercheurs. Pour plusieurs années, on a pensé qu'une exposition directe des cellules au peroxyded'hydrogène ou à des quinones induit seulement la nécrose, mais de plus récentes études démontrent que ces agents à des plus faibles doses peuvent induire l'apoptose (Hampton et Orrenius, 1997). En plus, de plus en plus de chercheurs suggèrent que la production des espèces réactives de l'oxygène (EOR) fait partie intégrante de l'apoptose et que ces espèces sont déterminantes dans la toxicité associée à la radiation ionisante et aux médicaments chimiothérapeutiques (Zamzami *et al.*, 1995; Macho *et al.*, 1997). Puisque l'acroléine génère un stress oxydatif en augmentant la production des EORs et que le NAC inhibe l'induction de l'apoptose par le NAC, on pense que l'apoptose induite par l'acroléine est médié par le stress oxydatif. La capacité de plusieurs antioxydants cellulaires comme la catalase et le NAC à bloquer l'apoptose induite par divers agents autres que des oxydants aussi confirme le rôle central du stress oxydatif dans l'apoptose (Buttke et Sandstrom, 1994). Réciproquement, on a assigné une fonction antioxydante à la famille anti-apoptotique Bcl-2 et à la protéine du baculovirus p35 (Jacobson, 1996; Sah, 1999), démontrant que la génération des EOR est probablement une condition prérequise pour démarrer l'apoptose. Cependant, d'autres études ont démontré ques les radicaux libres peuvent atténuer l'apoptose (Hampton et Orrenius, 1998).

Durant les récentes années, plusieurs précurseurs du GSH ou inducteurs de ses enzymes de synthèse ont été développés (Anderson, 1997). L'augmentation du GSH est d'un grand intérêt dans le développement de nouvelles stratégies thérapeutiques contre les composés toxiques de l'environemment, comme l'acroléine, et les effets secondaires de plusieurs médicaments tel que l'hépatotoxicité de l'acétaminophène. Le NAC diminue les protéines conjuguées à l'acroléine, un marqueur de la

peroxidation lipidique, chez les rats développant des dommages hépatiques chroniques (Kitamura *et al.*, 2005). En plus, le NAC supprimait l'apoptose induite par des agresseurs toxiques ou par le facteur de nécrose tumorale (TNF-α) (Cotgreave, 1997). Le NAC est utilisé pour ses propriétés chimioprotectrices contre les effets secondaires de plusieurs médicaments (Mclellan *et al.*, 1995; Gillissen *et al.*, 1997). Le NAC est utilisé cliniquement pour traiter les overdoses de l'acétaminophène (Perry et Shannon, 1998). En outre, le NAC peut agir directement comme un antioxydant en réduisant les espèces réactives de l'oxygène comme le radical hydroxy ($OH^{\cdot}$), le péroxyde d'hydrogène (H_2O_2) et l'acide hypochloreux (HOC1). Administrant le précurseur de la cystéine, le NAC, est plus sûr pour les humains que la cystéine elle-même qui a montré des effets toxiques pour le système nerveux central (Dizdar *et al.*, 2000). En plus, le GSH ne peut pas être administré car il n'entrera pas dans la cellule à cause de son volume.

3.2.7. Pertinence de l'acroléine dans le traitement des cancers

De nos jours la thérapie anticancéreuse par induction d'apoptose prend de plus en plus d'intérêt. Cette étude vise à comprendre le mécanisme de mort cellulaire induit par l'acroléine ainsi que l'impact de la modulation du glutathion intracellulaire sur la toxicité de cet aldéhyde. Nous sommes intéressés à étudier l'acroléine parce qu'elle est produite par l'oxydation des polyamines (Agostinelli *et al.*, 1996). Il est déjà connu que les tumeurs contiennent des concentrations élevées en polyamines (Bachrach et Heimer, 1989), qui sont des molécules nécessaires à la prolifération cellulaire (Heby et Persson, 2003). Ainsi, en administrant une enzyme de la famille des amines oxydases, l'amine oxydase du sérum du bovin (BSAO, EC 1.4.3.6), les polyamines seront oxydés pour produire de l'acroléine et du peroxyded'hydrogène (Alarcon, 1970; Tabor et Tabor, 1984). Ce faisant, la taille de la tumeur diminuera puisqu'on la prive des polyamines et on la met en présence de produits apoptogènes (Lord-Fontaine *et al.*, 2001), qui sont le peroxyded'hydrogène et l'acroléine. La

BSAO a été immobilizée avec succès dans des hydrogels de polyéthylène glycol biocompatibles, ce qui peut être utile pour la stabilité et la livraison de l'enzyme dans des tumeurs *in vivo* (Demers *et al.*, 2001). La BSAO immobilizée était injectée *in vivo* dans des tumeurs de mélanome chez la souris et elle a diminué la croissance tumorale (Averill-Bates *et al.*, 2005). Nous avous caractérisé le type de mort cellulaire, qui était l'apoptose, révélée par la condensation de la chromatine, chez les cellules tumorales (test de Hoescht) (Averill-Bates *et al.*, 2005). En ce qui me concerne, j'ai participé à cette étude *in vivo* en confirmant l'apoptose à l'aide du test de l'annexine V / propidium iodide et ceci par cytométrie de flux (Averill-Bates *et al.*, 2005). Les résultats de cette étude suggèrent que l'enzyme immobilisée possède un potentiel anticancéreux en clinique, puisque les produits générés sont relachés graduellement ce qui stimule l'apoptose des cellules plutôt que la nécrose. Ainsi, les résultats de mon projet de doctorat pourront aider au développement de cette nouvelle thérapie anticancéreuse dont les mécanismes sont investigués en détails afin d'optimiser le traitement.

En conclusion, nos résultats ont permis une meilleure compréhension de la toxicité de l'acroléine, un des produits majeurs de l'oxydation des polyamines impliqués dans la régulation de la prolifération cellulaire et la croissance des tumeurs. En plus, cette étude a permis une explication possible de l'activité pharmacologique du cyclophosphamide, un agent anticancéreux, qui est métabolisé en acroléine et en un mélange de phosphoramides, ce qui ouvre la voie au développement de l'enzymothérapie par la BSAO pour lutter contre les cancers. D'autre part, les résultats de ce projet envisagent la possibilité d'utiliser les composés qui augmentent le niveau de GSH dont le NAC dans des cas de toxicité à l'acroléine, molécule qui prend de l'ampleur dans la recherche sur la maladie d'Alzheimer.

En perspective, il sera important et original de démontrer si l'acroléine peut induire l'apoptose *in vivo* et ceci sur des tissus pulmonaires des rats Sprague-Dawley suite à une exposition par inhalation. L'étude se fera par voie respiratoire puisque normalement les gens sont exposés à l'acroléine par l'air comme la cuisson d'huile,

l'émission des moteurs et les feux de forêt. Différentes études *in vivo* se sont intéressées au métabolisme de l'acroléine et son effet toxique sans avoir étudier la mort cellulaire par apoptose (Morris *et al.*, 2003; Penn *et al.*, 2001; Borchers *et al.*, 1999; Linhart *et al.*, 1996). Les rats seront exposés à différentes concentrations en acroléine suivie des étapes de prélèvement de poumons et du lavage bonchoalvéolaires. Afin d'investiguer l'apoptose induite *in vivo* par inhalation de l'acroléine, on utilisera un homogénat de poumons. Les analyses à utiliser seront : la microscopie de fluorescence pour confirmer la présence des noyaux apoptotiques à l'aide du colorant Hoescht, le test du TUNEL pour démontrer la fragmentation de l'ADN (Barazzone *et al.*, 1998), la fluorimétrie et l'immunobuvardage pour étudier l'activité et le clivage des caspases -7, -8 et -9. Ensuite, par HPLC on effectuera le dosage de l'hydroxyproline qui démontre le dépôt de collagène qui est un indicateur de fibrose (Hagimoto *et al.*, 1997). La fibrose se caractérise par une surpopulation de fibroblastes au niveau des poumons en cas de toxicité pulmonaire (Hagimoto *et al.*, 1997). Enfin, la microscopie de fluorescence pour étudier l'activité de la phagocytose qui est un mécanisme de défense majeure des poumons et ceci sur les différents types cellulaires du système immunitaire obtenus par lavage bonchoalvéolaire (Kuronuma *et al.*, 2004). Au niveau des poumons les macrophages sont responsables d'attaquer et de digérer les bactéries et microorganismes qui entrent par la voie respiratoire. Ainsi, une activité phagocytose dérégulée représente un danger pour la santé humaine.

Enfin, nos résultats suggèrent que l'acroléine induit l'apoptose par la voie de récepteur de mort Fas et par la voie mitochondriale et que ces mécanismes sont MAPK- et caspases-dépendant. En plus, l'antioxydant NAC peut être utilisé dans le traitement d'une toxicité à l'acroléine et pour abaisser les effets secondaires indésirables de l'agent anticancéreux, le cyclophosphamide.

ANNEXE

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Anti-tumoral effect of native and immobilized bovine serum amine oxidase in a mouse melanoma model

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Abstract

Bovine serum amine oxidase (BSAO) oxidatively deaminates polyamines containing primary amine groups, spermidine and spermine, to form the cytotoxic products hydrogen peroxide and aldehyde(s). Polyamines are present at elevated levels in many tumor tissues. The aims of the study were to evaluate the anti-tumoral activities of native and immobilized BSAO in mouse melanoma and also to determine the mechanism of tumor cell death. C57BL mice received a subcutaneous injection of B16 melanoma cells to induce formation of tumors, prior to antitumor treatments with native and immobilized BSAO. The enzyme was immobilized in a polyethylene glycol) (PEG) biocompatible matrix. Antitumor treatments consisted of a single injection of enzyme into the tumor. When immobilized BSAO (2.5 mU) was injected into the tumor, there was a marked decrease of 70% of the tumor growth. This was compared with a decrease of only 32% of tumor size when the same amount of native BSAO was administered. The type of cell death was analysed in tumors that were treated with native or immobilized BSAO. When tumors were treated with immobilized BSAO, there was induction of a high level of apoptosis (around 70%), compared to less than 10% with the native enzyme. Apoptotic cell death was assessed by nuclear chromatin condensation using Hoechst staining and labelling of externalized phosphatidylserine using Annexin V. However, native BSAO, probably due to a burst of cytotoxic products, induced a high level of necrosis of about 40%, compared to less than 10% with immobilized BSAO. In conclusion, the advantage is that immobilized BSAO can act by allowing the slow release of cytotoxic products, which induces tumor cell death by apoptosis rather than necrosis.

Keywords: Amine oxidase; Apoptosis; Immobilized; Mouse melanoma; Necrosis; Polyamine

1. Introduction

The naturally occurring polyamines (spermine, spermidine and putrescine) play an essential role in cellular growth and differentiation [1]. Recently, they have also been implicated in apoptosis, which is a highly regulated process of cell death [2], and in cellular signal transduction [3]. The polyamine biosynthetic pathway is very active during the growth of various cancer cells. Polyamines are often present at increased concentrations in tumor cells and tissues, for example, breast and colon cancers [4]. Reasons for these increased levels include enhanced putrescine synthesis by ornithine decarboxylase and increased uptake of polyamines [5]. In addition, depletion of polyamines leads to inhibition of tumor growth [6].

Given the role of the natural polyamines in growth-related processes, enormous efforts have been made to synthesize inhibitors for the different enzymes involved in polyamine biosynthesis: spermidine and spermine synthase, ornithine decarboxylase and *S*-adenosyl-methionine decarboxylase [7]. In fact, new strategies for cancer treatment are currently under development [8] using (i) inhibitors of polyamine synthesis such as DFMO, a specific inhibitor of ornithine decarboxylase [9], and methylglyoxal-bis-guanidylhydrazone, an inhibitor of *S*-adenosyl-methionine decarboxylase,

Abbreviations: BSAO, bovine serum amine oxidase; DAH, 1,6-diaminohexane; DFMO, α-difluoromethylornithine; DMEM, Dulbecco's modified Eagle medium; GPx, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione *S*-transferase; IU, international unit; PEG, polyethylene glycol; PBS, phosphate buffered saline; PI, propidium iodide; SD, standard deviation; S.E.M., standard error of the mean

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(ii) analogues of polyamines [10,11], which can deplete polyamine content and interfere with polyamine metabolism and/or function, (iii) polyamine transport inhibitors which can prevent uptake of exogenous polyamines by blocking membrane transporters [12] and (iv) the use of polyamine-degrading enzymes such as BSAO [13–18]. A recent development is that the polyamine oxidase inactivator, N^1,N^3-bis (2,3-butadienyl)-1,4-butanediamine (MDL 72527), can improve the anticancer effect of DFMO [7]. Currently, DMFO is undergoing clinical evaluation as a chemoprevention agent [19].

BSAO (EC 1.4.3.6) is a copper enzyme, Mw 170 kDa, which oxidatively deaminates polyamines containing primary amine groups (putrescine, spermidine and spermine) in the presence of oxygen and water [20]. The reaction products are hydrogen peroxide (H_2O_2), the corresponding aldehydes and ammonia [21]. Acrolein is formed by the spontaneous β-elimination of the dialdehyde, an unstable intermediate oxidation product of spermine [22,23]. Fig. 1 shows the reaction scheme for the enzymatic action of BSAO on spermine and the formation of cytotoxic products. Products of polyamine oxidation have been implicated in programmed cell death [24] and inhibition of DNA and protein synthesis [25].

We previously reported that spermine oxidation products could inhibit mammalian cell proliferation and that both H_2O_2 and aldehyde(s) were involved [18,26]. Furthermore, an important finding is that the BSAO/spermine enzymatic system was able to eliminate multidrug-resistant cells with overexpression of P-glycoprotein [17,27]. These findings suggest that BSAO could prove to be useful in cancer treatment. To take advantage of the higher levels of polyamines in tumor versus normal tissues [4], toxic

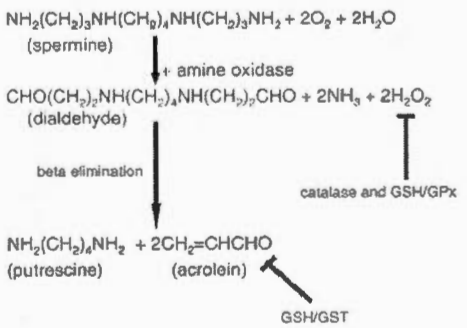

Fig. 1. Reaction scheme for the enzymatic oxidation of the polyamine spermine by BSAO. Spermine undergoes oxidative deamination in the presence of oxygen and water to form hydrogen peroxide (H_2O_2), ammonia and the unstable aminoaldehyde intermediate [N,N' bis(3 propionaldehyde) 1,4 butanediamine]. The aminoaldehyde undergoes spontaneous β elimination to form acrolein and putrescine. H_2O_2 is detoxified by two cellular defence systems: (i) by catalase and (ii) by reacting with reduced glutathione (GSH), catalyzed by glutathione peroxidase (GPx). Acrolein is detoxified by conjugation with GSH, catalyzed by glutathione *S*-transferase (GST).

products such as H_2O_2 and aldehyde(s) could be generated in situ by delivering amine oxidases into tumors to induce cytotoxicity [18,27]. Furthermore, BSAO could also act by depleting tissue levels of polyamines, necessary for tumor growth.

The aim of this study is to evaluate in vivo, using a mouse melanoma model, whether BSAO, when injected directly into the tumor, is able to induce tumoricidal activity by converting polyamines to toxic products in situ. It has been well established that immobilization of enzymes such as asparaginases into polymeric matrices such as PEG increases their structural stability and functional activities in vitro and in vivo [28]. Immobilization of BSAO into a biocompatible matrix made of bovine serum albumin and PEG was reported recently [29] and the enzyme showed a high operational stability relative to its native form. Therefore, both native and immobilized BSAO will be compared in vivo in terms of their antitumor efficacy against mice melanomas.

For cancer therapies, it is also important to establish the mechanism(s) by which cytotoxic agents cause tumor cell death. Apoptosis is a highly regulated form of cell death involving many different genes and proteins [30]. During apoptosis, caspase enzymes are activated, chromatin condensation and internucleosomal degradation of DNA occur in the nucleus and blebs appear on the surface of the cell membrane. An early morphological event in apoptosis is the loss of plasma membrane asymmetry, resulting in exposure of phosphatidylserine at the outer membrane leaflet. Cells are subsequently dismantled in an orderly manner into apoptotic bodies, which are removed by phagocytosis. This process avoids liberation of cellular contents into surrounding tissue and induction of inflammation, as occurs when cells lose membrane integrity and die by necrosis. Therefore, native and immobilized BSAO will be compared in vivo in terms of their respective abilities to induce tumor cell death by either apoptosis or necrosis.

2. Materials and methods

2.1. Materials

Benzylamine, Hoechst (# 33258), Annexin V-FITC, propidium iodide, bovine serum albumin, epidermal growth factor, hydrocortisone, insulin, polyethylene glycol of 3.5 kDa and transferrin were purchased from Sigma Chemical Co. Triethylamine, perchloric acid (70%) and DAH were purchased from J.T. Baker. DMEM, foetal bovine serum, penicillin, streptomycin and trypan blue were from Gibco Canada. Mouse melanoma cells B16-F0 (CRL-6322) were purchased from the American type culture collection. Female C57BL mice, aged 6–8 weeks and weighing 16–20 g, were purchased from Charles River Inc.

2.2. Methods

2.2.1. Tissue culture

Melanoma cells (B16-F0) were grown in monolayer in DMEM supplemented with 10% foetal bovine serum, 4 mM L-glutamine, 1% penicillin (50 units/ml)-streptomycin (50 μg/ml), 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml epidermal growth factor and 1.4 μM hydrocortisone, in an atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C. Confluent cells were harvested with 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution and centrifuged at 1000 × *g* for 3 min. The cell pellet was resuspended in sterile PBS. Cell viability was estimated by the trypan blue exclusion test [27].

2.2.2. Purification of BSAO

Bovine blood was withdrawn at a slaughterhouse and mixed with 3.8% sodium citrate solution (an anticoagulant) and then treated according to Turini et al. [31] to purify the enzyme amine oxidase. Some modifications were added to the method:

(a) CM-Cellulose column, equilibrated with phosphate buffer (0.01 M) at pH 5.8, to remove haemoglobin, followed by (b) an AE-Agarose column, in phosphate buffer (0.01 M) at pH 7.2 to eliminate ceruloplasmin and then, as the last steps were added two ionic exchange chromatographies performed using a Q-Sepharose column, in phosphate buffer (0.025 M) at pH 6.8 and a Q-Sepharose column, in phosphate buffer (0.02 M) at pH 8.0, according to Janes et al. [32]. The enzyme was eluted highly purified with a NaCl gradient. All purification steps were carried out in a cold room, at 4 °C.

2.2.3. Measurement of soluble and immobilized BSAO activities

The benzylamine oxidase activity of native BSAO, diluted at 4 μg of protein/ml, was measured at 25 °C in the presence of 5 mM benzylamine in 100 mM phosphate buffer, pH 7.2 [33]. After 5 min of incubation at room temperature, the quantities of benzaldehyde formed were monitored at 250 nm (ε = 12 500 M^{-1} cm^{-1}). The native enzyme had a specific activity of 0.24 IU/mg, where 1 IU was the amount of enzyme required to catalyze the oxidation of 1 μmol of substrate per min.

Immobilization of BSAO was performed into a film of hydrogel made from rat serum albumin crosslinked by di-1 nitrophenylcarbonate PEG [29]. Microbeads were obtained by crunching the film through a series of mesh filter sheets to obtain a microbead size which is injectable into the tumor [34]. The hydrogel slab was first crunched through a 100 μm polyethylene Spectra/Mesh filter sheet (Spectrum Laboratories) and then after the particles were crunched again through a 10 μm filter sheet. The microbeads looked like elongated spheres and their final size was greater than the sieve used, due to swelling during the washing procedure. The exact size of the microbeads was not evaluated, but their size would be between 10 and 15 μm. The size of the microbeads obtained was small enough to pass through a 21 g needle mounted on a syringe, for tumor injection. The activity of immobilized BSAO was evaluated by incubating 100 mg of wet microbeads for 5 min at room temperature in the presence of 5 mM benzylamine in 100 mM phosphate buffer, pH 7.2, as previously described [29]. After centrifugation (16,000 × *g*, 2 min) (Biofuge Pico, Heraeus), the absorbance of the supernatant was measured at 250 nm following the formation of benzaldehyde during 5 min.

2.2.4. Analysis of plasma polyamines

The analysis of polyamines in blood samples was carried out according to De Vera and co-workers [35]. Briefly, blood was collected in heparinized microvettes CB 300 (Sarstedt) from the saphen vein of the mice. Polyamines were extracted and derivatized with dansyl chloride (5 mg/ml) solution. The dansyl-polyamines (putrescine, cadaverine, spermidine and spermine) were analyzed by HPLC equipped with a Phenomenex Synergi Hydro-RP reverse-phase column (150 × 4.60 mm, particle size 4 μm). 1.0 mM DAH was added as internal standard. The column eluate was monitored using a Shimadzu RF-551 fluorescence detector at λ excitation (λ_{ex}) = 350 nm and λ emission (λ_{em}) = 520 nm. A gradient of solution A (75:25, methanol: 1.2 mM Na_2HPO_4/12 mM NaCl buffer solution, pH 7.0) and solution B (methanol) was used. At initial time, the composition of the eluate was 100:0, A:B and at t = 4.5 min the eluate composition was 0:100, A:B. The retention times of the polyamines, putrescine, cadaverine, DAH, spermidine and spermine, were 3.85 ± 0.4, 4.38 ± 0.7, 4.77 ± 0.4, 5.97 ± 0.4 and 6.67 ± 0.4 min, respectively. Amounts of polyamines were determined using the internal standard method.

The concentrations of the different polyamines were evaluated in the sera of the various experimental groups of mice to ensure that the enzyme substrates, spermine and spermidine, were present in sufficient quantities. The quantification of polyamines showed a mean plasma concentration of 250 pmol/ml for spermine, 1700 pmol/ml for spermidine, 388 pmol/ml for putrescine and 163 pmol/ml for cadaverine. A chromatogram representing the polyamines in mice plasma is shown in Fig. 2. The quantities of polyamines in the mouse chow (n = 3) were 6.7 ± 0.5 μmol/g of dry food for spermine, 29.9 ± 2.1 μmol/g for spermidine, 55.2 ± 3.3 μmol/g for putrescine and 10.7 ± 1.8 μmol/g for cadaverine.

2.2.5. Treatment of mice tumors

Animal experiments were carried out according to the guidelines of the Canadian Council for Animal Care and with approval by the institutional animal care committee. All mice, with the exception of the negative control groups, were injected subcutaneously in the dorsal position with 1×10^6 B16-F0 cells suspended in 120 μl of sterile PBS.

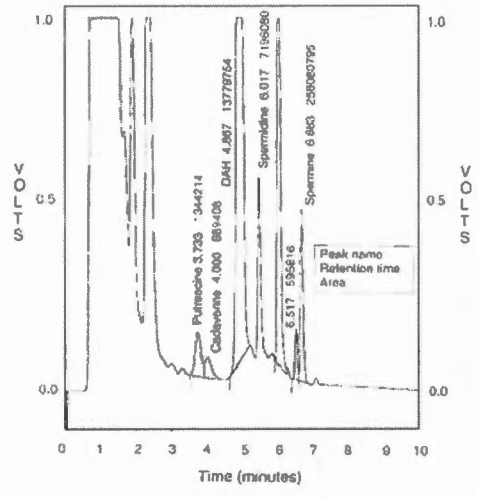

Fig. 2. Analysis of polyamine content in plasma of mice by reversed-phase HPLC. The separation of different polyamines was carried out by reversed phase HPLC using dansyl precolumn derivatives as described in Section 2. Putrescine, cadaverine, DAH (internal standard), spermidine and spermine had retention times of 3.7, 4.0, 4.87, 6.0 and 6.5 min, respectively. A representative chromatogram is shown from at least four experiments.

Negative control groups were injected with 120 μl of PBS alone.

A series of three experiments (4–5 mice per treatment) was carried out to determine the optimal parameters such as (1) enzyme dosage and (2) tumor size before starting the treatment, as well as (3) the effect of adding exogenous spermine to the drinking water. Doses of enzyme tested were 2.5, 5 and 15 mU. Treatment of mice with enzyme was started at tumor sizes of 0.02 g, 0.05 g or 0.08 g. Spermine was added in the drinking water at a concentration of 100 mg/l, once BSAO was injected into the tumor, and remained in the water throughout the duration of the treatment. Once the optimal parameters were determined, four series of experiments were carried out using the optimal dose (2.5 mU) for native or immobilized BSAO and a tumor size of 0.02 g (e.g. 26.0 mm^3), before beginning the treatment. This size was reached approximately 6–8 days after inoculation with the tumor cells. For these subsequent four experiments, spermine was not added to the drinking water.

Tumor size was determined four times per week, until mice were sacrificed. Euthanasia of mice was performed using CO_2 inhalation when the size of the tumor exceeded 3.5 g. The length (l) and the width (w) were measured with a digimatic calliper (Mitutoyo). From these dimensions, the tumor volume (v) (mm^3) was calculated using the formula $(w/2)^2 l\pi$ [36].

To assess the effect of BSAO on tumor growth rate, four series of experiments were carried out in this study and 26 mice were used in each series. For each series, there were three control groups. For the negative control group, mice received an injection of only 120 μl of PBS alone, but were not inoculated with tumor cells. The mice in the positive control group were inoculated with tumor cells and received an injection of PBS as treatment. The third control group of mice was inoculated with tumor cells and received a naked hydrogel as treatment. The experimental groups were composed of two groups of mice carrying the tumor. The first group was treated with an intratumoral injection of native BSAO (2.5 mU), and the second group was treated with an intratumoral injection of BSAO immobilized in a hydrogel (2.5 mU). All groups were composed of 5–6 mice. The development of cutaneous wounds at the site of tumor injection was also evaluated under these conditions in 3–7 independent experiments with 5–6 mice per group.

2.2.6. Viability test and death mechanism of tumoral cells

In this study, four series of experiments were carried out. In each series, 18 mice were used and were subdivided into subgroups of 5–6 mice to determine the time course for kinetics of cell death. The tumors were excised from euthanized mice at specific times after the beginning of the treatment with either PBS alone, native BSAO or immobilized BSAO. The tumor mass was chopped finely into small pieces and incubated with 200 units of collagenase IV for 60 min under gentle agitation. After the incubation, cells were detached by gentle passage through a pipette. The cells obtained from the tumors were suspended in DMEM medium and then centrifuged at 400 × *g* for 30 s. The cell pellet was then washed twice with PBS. Then, 15 μl of Hoechst solution (1 mg/ml) was added to the cells, along with 500 μl of PBS. The cells were incubated under weak agitation at 37 °C for 15 min and then centrifuged at 120 × *g* for 30 s. The resulting cell pellet was then washed in PBS and centrifuged at 120 × *g* for 30 s. Then, 500 μL of DMEM medium with PI (50 μg/ml) were added to the tumor cells before observation under a fluorescent microscope (Carl Zeiss Canada Ltd.). Pictures were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc.). Images were analysed by Northern Eclipse software. Cells were classified using the following criteria: (a) live cells (normal nuclei, pale blue chromatin with organized structure); (b) membrane-intact apoptotic cells (bright blue condensed or fragmented chromatin); (c) necrotic cells (red, enlarged nuclei with smooth normal structure) [37]. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). At least 400 cells were counted per tumor.

2.2.7. Determination of cell death by Annexin V-FITC staining

Externalized phosphatidylserine on the outer surface of the cytoplasmic membrane becomes labelled by fluores-

cein-labelled Annexin V, which has a high affinity for phosphatidylserine-containing phospholipid bilayers [38]. To analyze apoptotic cell death by flow cytometry, B16-F0 cells (1×10^6/ml) were incubated for 4 h with empty PEG hydrogels, or with either immobilized or native BSAO (6.0 mU/ml) and spermine (50 μM). Cells were then washed twice with PBS and resuspended in 1 ml of binding buffer (10 mM Hepes/NaOH, pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$). Five hundred microliters of cell suspension were then incubated with 5 μl of Annexin V-FITC and 10 μl of PI for 10 min at room temperature in the dark. The populations of Annexin V-positive/PI-negative cells (early apoptosis) and Annexin V-positive/PI-positive cells (late apoptosis) were evaluated by flow cytometry [38]. Data were collected using a FACS scan equipped with an argon laser emitting at 488 nm and analyzed using Lysis II software (Becton Dickinson).

2.3. Statistical analysis

Data represent the mean value and S.E.M. The effects of the different treatments on the rate of tumor growth were compared to the growth of the tumor from the positive control group by the extra sum of squares (log% of tumor growth versus day post treatment). The percentages of apoptosis at various times of tumor excision for the following groups: untreated, treated with native or immobilized BSAO, were compared using the Bartlett's test for homogeneity of variance, followed by a Tukey multiple comparison test. For significant differences, $P < 0.05$.

3. Results

3.1. Effect of dietary supplementation of spermine on tumor growth rate in mice

It was first necessary to establish whether sufficient levels of polyamines were present in mice to allow the enzymatic reaction to occur, or whether dietary supplementation would be necessary. Following inoculation of mice with tumor cells, the effect of dietary addition of spermine on the rate of tumor growth was determined. One would expect that addition of spermine would allow an increased BSAO kinetic reaction rate, which should be reflected by a lower growth rate of the tumor, due to increased generation of toxic products. In fact, this was not the case. Instead, dietary addition of spermine caused marked acceleration of the rate of tumor growth (Fig. 3). Furthermore, in the presence of dietary spermine and immobilized BSAO (5 mU), a higher percentage of wound tissue was observed at the tumor site (group 7), when compared to mice bearing tumors but without addition of spermine in their water (group 1) (Fig. 4). Effectively, up to 80% of the mice having tumors and receiving dietary spermine and immobilized BSAO treatments developed

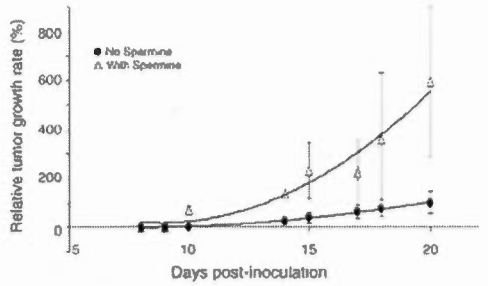

Fig. 3. Dietary supplementation with spermine accelerates rate of tumor growth. All of the animals were injected subcutaneously in the dorsal position with melanoma B16-F0 cells (1×10^6 cells) to induce formation of tumors. Mice received water either with (△), open triangle) or without (●), solid circle) addition of spermine (100 mg/l). Data represent means and S.D. from three to five mice and the experiment was repeated three times.

severe skin wounds at the site of tumor implantation (Fig. 4). The severity of the tissue wound was in many cases high enough to require euthanasia of mice before completing the experiment. This high percentage has to be compared with a level of 20–40% for all other groups of mice bearing tumors and drinking regular water (Fig. 4). These findings indicate that increasing polyamine substrate availability for the BSAO kinetic reaction by dietary addition of spermine would not provide an advantage

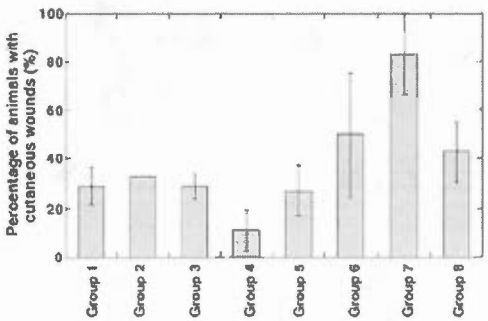

Fig. 4. Percentage of cutaneous wounds for the various groups of mice following different treatments. All of the animals were injected subcutaneously in the dorsal position with melanoma B16-F0 cells (1×10^6 cells) to induce formation of tumors. The various treatments began when tumors had grown to a weight of 0.02 g. The control groups 1 and 2 were treated with PBS, however, group 2 had spermine (100 mg/l) in its drinking water. Group 3 control received a naked hydrogel as treatment. Groups 4 and 5 received native BSAO at concentrations of 5 and 2.5 mU, respectively. Groups 6 and 7 received immobilized BSAO at a concentration of 5 mU, but group 7 had spermine (100 mg/l) in its drinking water. Group 8 received immobilized BSAO at a concentration of 2.5 mU. The data represent means and S.E.M. from at least 3 (groups 1, 3, 4, 6) to 7 (groups 2, 5, 8) independent experiments, each with 5–6 mice per group. For group 7, data are the mean and standard deviation of only one experiment (five mice).

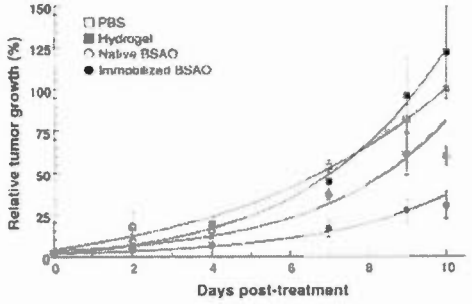

Fig. 5. Effect of different treatments with native or immobilized BSAO on tumor growth rate. All of the animals were injected with melanoma B16-F0 cells (1×10^6 cells) and groups received different treatments when their tumor reached 0.02 g. All measurements of tumor size were made from day 0 post treatment. Animals were sacrificed at day 10 after the beginning of the treatment. The data represent means and S.E.M. from four independent experiments, each with 4–6 mice per group. The positive control groups were injected with PBS ((□), open square) or with a naked hydrogel ((■), solid square). The two other groups were treated with a single injection of 2.5 mU of native BSAO ((○), open circle) or with immobilized BSAO ((●), closed circle), respectively.

for the anticancer strategy since it was, in fact, deleterious to health of the animals. Furthermore, spermine was previously reported to cause renal toxicity [39]. Therefore, further experiments were carried out without dietary addition of spermine.

3.2. Antitumor effect of native and immobilized BSAO in mice

The various treatments of the tumors consisted of a single injection (PBS, empty hydrogel, native BSAO or immobilized BSAO) in the center of the tumor. The day at which the treatment begins corresponds to day 0 post-treatment. When native BSAO (2.5 mU) was used for the treatment, the size of the tumor was 40–50% smaller than the size of tumor in the positive control group at day 10 (Fig. 5). A more striking reduction of tumor size was observed in the mice treated with immobilized BSAO (2.5 mU). Effectively, the tumor was 70% smaller than the tumor in the positive control group at day 10. The tumor growth curves for the groups that received native or immobilized BSAO as treatments were statistically compared to the tumor growth curve of the positive control group. A significant difference was observed in terms of growth rate between mice receiving native BSAO and the positive control group ($P = 0.0409$). However, a highly significant difference was obtained for a similar comparison with the group treated with immobilized BSAO and the positive control group ($P = 0.0001$). When a higher dose (15 mU) of immobilized or native BSAO was used, there was no variation in the rate of the tumor growth, relative to control groups (Table 1).

Table 1
Lack of effect on tumor growth by a higher concentration of immobilized BSAO

Days post-treatment	Relative tumor growth (%)	
	Control	Immobilized BSAO (15 mU)
0	0	0
1	0	8.30
3	4.53	5.43 ± 3.50
4	4.38 ± 2.85	11.70 ± 6.03
5	9.96 ± 3.55	19.25 ± 7.43
6	17.58 ± 5.71	20.75 ± 5.87
8	36.83 ± 6.46	28.15 ± 15.03
9	40.75 ± 10.50	48.83 ± 21.07
10	44.38 ± 9.70	46.64 ± 12.34
11	54.71 ± 10.09	49.74 ± 5.00
12	70.19 ± 14.06	67.17 ± 0.75
13	69.81 ± 9.48	79.47 ± 10.45
15	100.00 ± 12.17	78.49

Tumor growth was determined in melanoma bearing mice at different days post treatment with PBS (controls) or immobilized BSAO, which were both administered by subcutaneous injection. Data represent mean and S.D. from two experiments each with three mice per group.

3.3. Mechanism of tumor cell death following treatment with native and immobilized BSAO

Subsequently, the mechanism by which the tumor cells died was determined. The tumor mass was excised at different times following the treatment with both forms of BSAO from the various groups of mice. The cells obtained from the untreated tumors excised from the positive control group showed a constant viability of around 80% and few apoptotic (5%) or necrotic (15%) cells were observed at any time after the saline injection (Fig. 6A and D).

In contrast, cells isolated from tumors treated with 2.5 mU of native BSAO showed a gradual decrease in cell viability from around 80% to 55%, 48–72 h after the injection (Fig. 6B). At 96 h post-injection, the tumor cell viability increased gradually and returned to initial levels (approx. 80%) at 120 h after the administration of native BSAO. There was a corresponding gradual increase in necrotic cells (Fig. 6B and E) during the first 72 h after the treatment, to reach a maximum close to 40%. The percentage of cell death by apoptosis remained constant at low levels (Fig. 6B), which were similar to those observed in the control group (Fig. 6A).

Interestingly, Fig. 6C and F highlight a different mechanism of cell death in the group of mice treated with immobilized BSAO. Effectively, after the injection of immobilized BSAO, tumor cells underwent cell death by apoptosis. Levels of apoptotic cells increased to 35% at 4 h and reached a maximal value of about 70% at 120 h after the treatment (Fig. 6C). Subsequently, there was a gradual decrease to 30% apoptotic cells at 216 h. Relative to native BSAO, there was a more pronounced decrease in cell viability following treatment of mice with immobilized BSAO. A minimal viability of less than 20% was observed

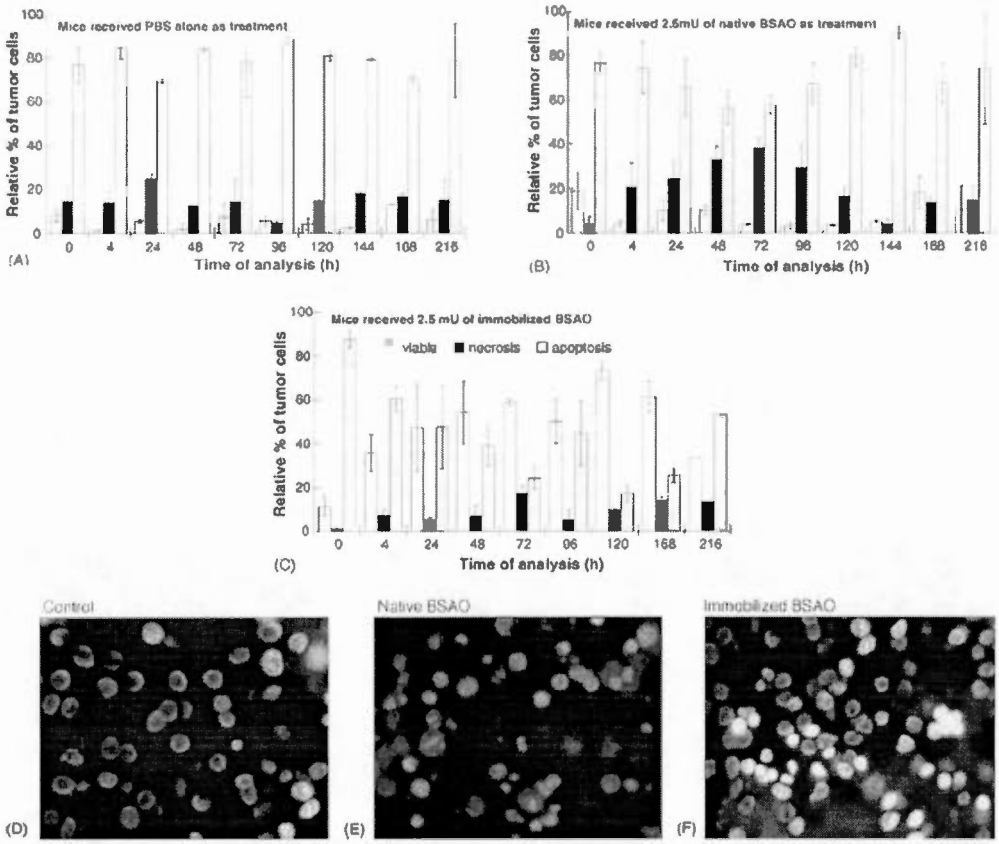

Fig. 6. Determination of cellular death pathway in tumors excised following a treatment with native or immobilized BSAO. Mice had received, by subcutaneous injection in the dorsal position, melanoma B16-F0 cells (1×10^6 cells) and tumors were allowed to grow to 0.02 g before beginning the various treatments. Tumor cells were obtained by excision of the tumor at various times following one of the treatments, with (A) PBS alone, (B) native BSAO or (C) immobilized BSAO. Apoptotic ((□), open square), necrotic ((■), solid square) and viable cells ((▩), grey) were determined by fluorescence microscopy and the total number of cells under bright field illumination. The fractions of apoptotic and necrotic cells are given relative to total cells. A minimum of 400 cells was counted per tumor. Representative images (320×) are shown of cells from tumors of mice treated with (D) PBS alone, or with either (E) native or (F) immobilized BSAO, after 72 h. The data represent the mean and S.E.M. from four independent experiments, each with 4–6 animals per group. The percentage of apoptosis showed significant differences ($P < 0.05$) between groups of mice treated with immobilized BSAO and groups treated with native BSAO or untreated controls at 4, 24, 48, 72, 96, 120 and 168 h after the beginning of the various treatments.

in tumor cells in this group of treated mice at 120 h (Fig. 6C). The percentage of necrotic cells remained low and was similar to that in the positive control group of mice. Statistical analysis of the percentage of apoptosis showed significant differences ($P < 0.05$) between groups of mice treated with immobilized BSAO (Fig. 6C) relative to groups treated with native BSAO (Fig. 6B) or untreated controls (Fig. 6A) at 4, 24, 48, 72, 96, 120 and 168 h after the beginning of the various treatments. The percentage of apoptotic cells in the group treated with native BSAO (Fig. 6B) was significantly different ($P < 0.05$) from that observed in the untreated group (Fig. 6A) at the experimental time of 24 h only. The level of apoptotic cells was similar for the three groups at the beginning ($T = 0$) and at the end of the experiment ($T = 216$ h) (Fig. 6A, B and C).

To further confirm that the BSAO/spermine enzymatic system can in fact cause cell death by apoptosis, Annexin V-FITC/PI fluorescence was analyzed by flow cytometry (Fig. 7). B16-F0 melanoma cells were exposed to either immobilized BSAO (Fig. 7C) or native (Fig. 7D) BSAO and spermine for 4 h, followed by Annexin V/PI staining. When cells were treated with immobilized or native BSAO,

exposed native BSAO, relative to immobilized BSAO. The naked PEG hydrogels (Fig. 7B) showed similar effects to untreated controls (Fig. 7A, Table 2).

4. Discussion

In this study, native and immobilized forms of the BSAO enzyme were used as anti-tumoral drugs on mice bearing melanomas and their efficiencies were evaluated in terms of inhibition of tumor growth. Also, the type of death pathway of tumor cells exposed in vivo to the enzyme was evaluated with the aim of determining whether cell death was related to an apoptotic or necrotic mechanism. BSAO could be useful as an antitumor agent, by causing inhibition of proliferation by exerting two separate mechanisms. Firstly, it is well known that BSAO is able to generate highly cytotoxic products, including hydrogen peroxide and aldehydes such as acrolein and aminoaldehydes, by deaminating polyamine substrates such as spermine and spermidine [6,22,26]. When BSAO is directly injected into the tumor, it is likely that polyamines may be secreted by membrane-leaky necrotic cells in the tumor core, thus rendering them available for the enzymatic reaction. Secondly, when injected directly into a solid tumor, the BSAO enzyme could modify polyamine concentrations in the tumor, leading to polyamine deprivation to a sufficient level that the proliferation of the tumor cells could be affected. In this case, a decrease in polyamine levels could lead to decreased tumor growth.

The polyamine biosynthetic pathway is very active during the growth of various cancer cell lines in vitro [4] and also in human cancers in vivo [40–42]. The addition of spermine in the drinking water of C57BL mice caused a marked increase in the size of untreated melanoma tumors, when compared to tumors of mice drinking regular water. This finding is consistent with the necessity of polyamines for tumor growth. Dietary supplementation of spermine (group 2) did not increase the frequency (35%) of skin wounds when compared to controls (group 1) (Fig. 4). However, marked increases in skin wound frequencies, up to 50% and 80%, occurred in mice treated with 5 mU of immobilized BSAO and either regular (group 6) or spermine-enriched water (group 7), respectively (Fig. 4). At lower doses of BSAO, 2.5 mU, there were no major differences in skin wound frequencies compared to controls. It appears that the higher concentration of immobilized BSAO (5 mU) produced cytotoxic products in concentrations high enough to diffuse to the normal skin tissue surrounding the melanoma and to induce severe wounds. This effect was more pronounced when spermine was added to the drinking water, in combination with the high dose of BSAO. Under these conditions, the velocity of the kinetic reaction would be more rapid, generating higher concentrations of cytotoxic products. Hydrogen peroxide is detoxified by two cellular defence systems: by the enzyme catalase and by reacting with reduced glutathione (GSH), catalyzed by glutathione peroxidase (Fig. 1). Acrolein is detoxified by a conjugation reaction with GSH, catalyzed by glutathione *S*-transferase (Fig. 1). These higher levels of cytotoxic products would overwhelm the detoxification ability of the cellular defences, therefore contributing to normal tissue toxicity. For these reasons, addition of exogenous spermine and high doses of BSAO were not studied in the subsequent series of experiments.

Treatment of mice bearing subcutaneous melanoma tumors with a low dose (2.5 mU) of native (group 5) or immobilized (group 8) BSAO, induced inhibition of tumor growth by 40% and 70%, respectively, during a period of 10 days after a single injection of the enzyme into the center of the tumor, compared to the positive control group (group 1) (Fig. 5). The PEG-albumin matrix used for enzyme immobilization had no significant effect on tumor growth. The difference in the decrease of tumor growth between the two forms of BSAO could be explained by several reasons. Firstly, the native enzyme is more susceptible to deactivation and degradation in the physiological environment than the immobilized form of the enzyme [43]. Secondly, it was previously reported that the immobilization of BSAO into a hydrogel matrix of PEG-albumin increased its operational stability in vitro from 1.5 h to 70 h [29]. Operational stability was defined as the half-life of the enzyme continuously exposed to a constant substrate concentration at 25 °C. Also, the apparent K_m (binding affinity between enzyme and substrate) of the immobilized BSAO was very similar to the K_m of the native BSAO, when benzylamine was the substrate. However, the V_{max} (maximum velocity of enzymatic reaction) of the immobilized BSAO was two times lower than the V_{max} of the native soluble enzyme. This was explained by the fact that the rate of catalysis was diffusion limited by the matrix, i.e. the polyamine substrates need to diffuse inside the PEG matrix in order to have access to the enzyme. Similar results of catalytic stability in vivo were obtained when immobilized asparaginase was injected into the rat peritoneal cavity to eliminate blood levels of asparagine [28]. The lower V_{max} of the immobilized form of BSAO would allow a prolonged, slow release of cytotoxic products compared to more rapid generation of higher levels of cytotoxic products with the native enzyme.

Several in vitro studies have shown that the cytotoxic products of BSAO and spermine, H_2O_2 and acrolein, can induce either apoptosis or necrosis, depending on their concentrations and the cell type [44–46]. However, there are few, if any, in vivo studies highlighting the mechanism of cell death caused by polyamine-mediated generation of these cytotoxic products. To determine the type of cell death induced by BSAO, tumors were excised at different times from the various groups of mice after the single injection of saline, 2.5 mU of native BSAO or 2.5 mU of immobilized BSAO. Tumors from the untreated mice showed a high level of cell viability and the few remaining

cells were necrotic or apoptotic. On the other hand, when mice were treated with BSAO, the viability of tumor cells decreased, but the response was more rapid and for a longer time (5 days) with immobilized rather than native BSAO (2–3 days). However, an important finding is that immobilized BSAO caused cell death mainly by apoptosis. These findings with immobilized BSAO could be beneficial since the ultimate aim of anticancer treatments is to induce tumor cell death by apoptosis, a process which avoids inflammatory damage to surrounding tissue.

The difference in the mechanisms of cell death observed when native or immobilized BSAO were used to treat the mice can be explained in terms of the kinetics of generation of cytotoxic products. As discussed previously, native BSAO has a higher V_{max} than immobilized BSAO. In addition, when using native BSAO, the reaction products do not have to cross a matrix to reach the tumor tissues, nor does the substrate have to enter the matrix to have access to the enzyme. It is also possible that the area of enzyme distribution covers a larger volume of the tumor than that for the immobilized one. Under these conditions, the native BSAO generates a burst of hydrogen peroxide and aldehyde(s) at such concentrations that necrosis is favoured. On the other hand, a lower V_{max} and diffusional constraints due to the matrix should allow a more gradual release of the cytotoxic products generated by the immobilized enzyme in the tumor. Effectively, it is well known that during the translocation of the substrate to the center of the bioreactor to reach the deeply embedded enzyme, the substrate is transformed into the product(s). So, under these conditions only a part of the enzyme is operational. These results are consistent with findings generally observed in vitro, where toxic compounds usually cause necrosis at higher doses and apoptosis at lower doses. Effectively, it was demonstrated that higher concentrations of acrolein favoured necrosis by inhibiting caspases in lymphocytes [46]. Also, high concentrations of H_2O_2 (1–10 mM) induced necrosis in lung fibroblasts, whereas lower amounts (10–100 μM) caused cell death by apoptosis [45].

Native BSAO caused tumor cell toxicity during a shorter period of time, compared to immobilized BSAO (Fig. 6B and C). Native BSAO caused toxicity by necrosis during 5 days, with a maximum level of toxicity after 2–3 days (Fig. 2B). There was a gradual recovery of viable cells starting from day 4, indicating that the native enzyme was no longer functionally active. Immobilized BSAO caused toxicity by apoptosis throughout the 9 days, with a maximum level of toxicity after 5 days (Fig. 6C). It was demonstrated that native BSAO can be inactivated by H_2O_2 formed during the enzymatic reaction [47]. Furthermore, aldehydes such as acrolein can react with proteins and generate a protein-linked carbonyl derivative [48], which could alter protein function. Thus, the initial burst release of the cytotoxic products could inactivate native BSAO, which could partly explain the shorter duration of tumor cell toxicity, compared to the immobilized form of BSAO. Furthermore, the tumor environment can contain high levels of proteases, which would be detrimental to the structural integrity of the enzyme. Immobilization into a matrix protects BSAO against such detrimental factors and this could result in the more sustained cytotoxic action of the immobilized enzyme, which was at least two times longer (minimum viability observed at day 5 compared to days 2–3 for native BSAO).

PEG is a non-toxic, non-immunogenic biocompatible polymer which has been approved for clinical use, conjugated to certain enzymes and drugs, by the Food and Drug Administration. PEG conjugation increases the circulating half-life of proteins and drugs and reduces their renal clearance, while maintaining their biological activity, compared to the parent compound. The immunogenicity of certain proteins can be reduced, by masking their antigenic sites by the polymeric chains of PEG [43]. In cancer treatment, PEG-asparaginase is useful in acute lymphocytic leukemia [49]. PEG is used to reduce the immunogenicity of liposome-encapsulated doxorubicin and pegfilgrastim is a pegylated form of granulocyte colony-stimulating factor filgrastim, used in the management of chemotherapy-induced neutropenia [50].

The ultimate aim of the current research is to develop a new type of cancer treatment for solid tumors, which takes advantage of the fact that levels of polyamines are higher in many solid tumor tissues, such as breast, colon, melanoma, brain and kidney [4]. The major objective of this paper is to demonstrate anti-tumor activity in vivo with the spermine/amine oxidase enzymatic system, following the promising results that were previously obtained in vitro in several tumor cell lines [15–18,26]. The microbeads used in this study were prepared with the objective of direct injection into solid tumors. This allows targeting of the toxic treatment to the solid tumor, which is not yet attainable by intravenous injection. Direct injection of a protein/enzyme into solid tumors is applicable to melanomas as well as to other inoperable solid tumors (e.g. brain), which are accessible by needle injection in some cases. This approach is also applicable to solid tumors which develop resistance following treatment with chemotherapy or radiotherapy. We also reported that multidrug resistant cells are sensitive to the toxic effects of the BSAO/spermine enzymatic system [17,27]. Multidrug resistance is a major reason for the failure of chemotherapy in tumors which are often initially responsive to treatment. If drug resistance could be overcome, the impact on cancer patient survival would be highly significant [51]. Targeting of solid tumors is an important objective for improved treatment as well as for decreasing undesirable side effects in the cancer clinic.

In conclusion, the treatment of melanomas in mice with BSAO immobilized in a matrix of PEG hydrogel represents an effective strategy to eliminate this type of cancer. The immobilization allows an improved performance of the enzyme by maintaining its activity for 9

days and represents a 'cleaner' treatment since the cells die by apoptosis and not by necrosis. Compared to the native form of the enzyme, the advantage with respect to anticancer treatment is that immobilized BSAO can act by allowing the slow release of cytotoxic products within the tumor.

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