UNIVERSITÉ DU QUÉBEC À MONTRÉAL

THE ROLE OF OXIDATIVE STRESS IN THE INDUCTION
OF APOPTOSIS BY HEAT SHOCK

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LE RÔLE DU STRESS OXYDATIF DANS L'INDUCTION DE L'APOPTOSE PAR LE CHOC THERMIQUE

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Two years of work finally comes to an end. When I look back at the very beginning of my research career, I feel it has just happened by accident. A casual e-mail asking for a place in the laboratory of Prof. Diana Averill led to a pleasant interview in which I received suggestions and encouragement from that delicate and devoted scientist. Two years of help and inspirations from colleagues André Tanel, Ahmed Bettaieb, Kamini Barot, Oliver Didur and Paulina Wrzal accompany me to the final stage. I am more grateful than they know.

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease activating factor-1
ARC	Apoptosis repressor with CARD
ATF6	Activating transcription factor 6
BAR	Bifunctional apoptosis regulator
BIR	Baculoviral IAP repeats
CAD	Caspase-activated DNase
CAR1	Cerevisiae arginase
CARD	Caspase recruitment domain
CAT	Catalase
c-FLIP	FADD-like ICE-inhibitory protein
CHO	Chinese hamster ovary
CHOP	C/EBP homologous protein
c-JNK	c-jun N-terminal kinase
DBD	DNA binding domain
DD	Death Domain
DDC	Diethyldithiocarbamate
DED	Death effector domain
DFF	DNA fragmentation factor
DIABLO	Direct IAP-binding protein with low pI
DR3	Death receptor 3
DR5	Death receptor 5
DRs	Death receptors
ER	Endoplasmic reticulum
FADD	Fas-associated with death domain

FAK	Focal adhesion kinase
FLICE	FADD-associated ICE
G6PDH	Glucose 6-phosphate dehydrogenase
GADD 153	Growth-arrest and DNA damage 153
GPx	Glutathione peroxidase
GR	GSH reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HILP	Hyperthermic isolated limb perfusion
HPP	Hyperthermic peritoneal perfusion
Hsf1	Heat shock factor
HSPs	Heat shock proteins
HtrA2	High temperature requirement protein A2
IAPs	Inhibitors of apoptosis proteins
IBMs	IAP binding motifs
ICAD	Inhibitor of caspase-activated DNase
IGF-BP3	Insulin-like growth factor binding protein 3
MAP4	Microtubule-associated protein 4
MDR	Multidrug resistant
MMP	Mitochondrial membrane permeabilization
MnTBAP	Manganese (III) tetrakis (4-benzoic acid)porphyrin
MRP1	Multidrug-resistance protein-1
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
PARP	Poly (ADP-ribose) polymerase
PERK/PEK	Phosphorylated extracellular signal-regulated protein kinase
PTP	Permeability transition pores

ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
TNFR1	Receptor of TNF
TRAF2	TNF receptor-associated factor 2
UCP-2	Uncoupling protein-2
VDAC	Voltage-dependent anion channel
WBH	Whole-body hyperthermia

R É SUM É

L'apoptose est une mort cellulaire qui, contrairement à la nécrose, représente un moyen actif d'élimination des cellules endommagées. Elle est impliquée dans plusieurs situations pathologiques chez les organismes pluricellulaires et est caractérisée par le rétrécissement cellulaire, la fragmentation de l'ADN et la condensation de la chromatine, le bourgeonnement membranaire conduisant à la formation des corps apoptotiques (vésicules membranaires). L'apoptose peut également survenir suite à une exposition des cellules à plusieurs types de stress, entre autres les radiations, le stress oxydatif, le choc thermique, certains polluants environnementaux et certains agents chimiothérapeutiques. Toutefois, le mécanisme de la mort cellulaire induite par le choc thermique est encore mal élucidé. Nous pensons que le choc thermique pourrait générer un stress oxydatif en causant un déséquilibre de la balance entre oxydants et anti-oxydants. Ceci survient lorsque le taux des espèces réactives de l'oxygène, telles que l'ion superoxyde et le peroxyde d'hydrogène, augmente et les défenses anti-oxydantes cellulaires sont inactivées. De tels changements, causés par le choc thermique, pourraient causer des dommages conduisant à la mort cellulaire.

Les objectifs de ce projet consistent premièrement, à étudier l'effet protecteur de l'anti-oxydant Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) contre l'induction de l'apoptose par le choc thermique chez les cellules ovariennes de hamster chinois (CHO). On s'est intéressé en second lieu, à la capacité de l'inhibiteur de la défense anti-oxydante diéthyldithiocarbamate (DCC) à potentialiser la mort cellulaire induite par le choc thermique. Les résultats montrent que le MnTBAP protège les cellules CHO contre l'apoptose induite par le choc thermique en inhibant la translocation des protéines pro-apoptotiques Bax et cytochrome c entre la mitochondrie et le cytosol et en inhibant l'activité de la caspase 9 et de la caspase 3. Le MnTBAP protège également contre le clivage des substrats des caspases tels que l'inhibiteur de la DNase activée par les caspases (ICAD) et la poly(ADP-ribose) polymérase 1 (PARP). De même, l'inhibition de la superoxyde dismutase, par le DCC, diminue l'activation de la voie mitochondriale de l'apoptose induite par le choc thermique sans pour autant protéger contre l'induction de la nécrose. En conclusion, l'antioxydant MnTBAP est en mesure de prévenir l'apoptose induite par le choc thermique via la voie mitochondriale. De son côté, l'inhibition de la superoxyde dismutase par le DCC augmente la mort cellulaire par nécrose plutôt que par apoptose. Ces résultats suggèrent que l'apoptose chez les cellules CHO, induite par le choc thermique survient suite à la génération d'un stress oxydatif.

Mots clés : CHO, Apoptose, Choc thermique, Stress oxydatif, Caspases

ABSTRACT

Apoptosis is a form of cell death that is distinct from necrosis. As a way to remove damaged cells, apoptosis occurs during severe pathological situations in multicellular organisms and is characterized by DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of apoptotic bodies (membrane enclosed vesicles). Apoptosis can also arise following exposure of cells to various types of stress, including radiation, oxidative stress, heat shock, environmental pollutants and chemotherapeutic agents. The mechanism of heat shock-induced cell death is not understood. It appears that heat shock could promote an increase in oxidative stress, thus creating a redox imbalance in favour of peroxidants. This could arise by increasing generation and reactivity of oxidants such as superoxide and H_2O_2 and by inactivating cellular antioxidant defences. As a consequence, heat is likely to induce oxidative changes in cells leading to cell damage and eventually cell death.

The objectives of this study are to determine 1) the ability of the antioxidant Mn(III)tetrakis(4–benzoic acid) porphyrin chloride (MnTBAP) to decrease heat shock-induced apoptosis, and 2) the ability of an inhibitor of the antioxidant defence system diethyldithiocarbamate (DDC) to potentiate heat-induced cell death in Chinese hamster ovary cells. The superoxide scavenger MnTBAP protected cells from heat shock-induced apoptosis by blocking the translocation of the pro-apoptotic proteins Bax and cytochrome c between the mitochondria and cytosol, and by inhibiting the activities of caspase-9 and caspase-3. MnTBAP also reduced the cleavage of the caspase substrates, inhibitor of caspase-activated DNase (ICAD) and poly (ADP-ribose) polymerase-1 (PARP). The inhibition of superoxide dismutase using DDC also decreased activation of the mitochondrial pathway of apoptosis by heat shock. Instead, DDC induced necrosis rather than apoptosis in Chinese hamster ovary cells. In conclusion, the antioxidant MnTBAP is able to prevent apoptosis through the mitochondrial pathway of apoptosis. On the other hand, the inhibition of superoxide dismutase by DDC increased cell death and shifted the type of cell death from apoptosis to necrosis. These findings indicate that heat shock-induced apoptosis is mediated by oxidative stress in Chinese hamster ovary cells.

Key words : CHO, Apoptosis, Heat shock, oxidative stress, Caspases

CHAPTER 1: INTRODUCTION

1.1. Programmed cell death

Multicellular animals often need to get rid of cells that are in excess and that are potentially dangerous (Hengartner, 2000). Three types of morphological and biochemical cell death have been identified. Type I is apoptotic cell death, type II is autophagic cell death and type III is necrotic cell death (Bursch *et al.*, 2000; Kim *et al*, 2005). Among these different types of cell death, apoptosis and necrosis have drawn a lot attention. They have very different morphological and biochemical features (Chandra *et al.,* 2000) (Figure 1.1). The term apoptosis comes from a Greek word, which describes the falling off of leaves from trees. It was first coined in 1972 by Kerr and coworkers (Kerr *et al.,* 1972) to describe an alternative type of cell death which is distinct from necrosis. Apoptosis is characterized by DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of apoptotic bodies (membrane enclosed vesicles). Apoptosis can be triggered by a number of factors, including ultraviolet or γ-irradiation growth factor withdrawal, chemotherapeutic drugs, or signaling by death receptors (DRs) (Ashkenazi and Dixit, 1998; Krammer *et al.,* 1998). In brief, apoptosis is a form of cell death that occurs during severe pathological situations in multicellular organisms and constitutes a common mechanism of cell replacement, tissue remodelling and removal of damaged cells (Delong, 1998). Necrosis appears to be the result of an acute cellular dysfunction in response to severe stress conditions after exposure to toxic agents and is a relatively passive process associated with rapid cellular ATP depletion (Chandra *et al.,* 2000). During necrosis cells swell and then rupture, releasing their contents and thereby eliciting an inflammatory response (Wyllie *et al.,* 1980).

Autophagic cell death, characterized by the formation of autophagic vacuoles in the cytoplasm of dying cells, occurs in many eukaryotic cell types. During

autophagic cell death, organelles and other cell components are sequestered into lysosomes, a cellular compartment enriched in hydrolases able to cleave proteins, lipids, nucleic acids and carbohydrates, and then the cell is degraded (Klionsky and Emr, 2000).

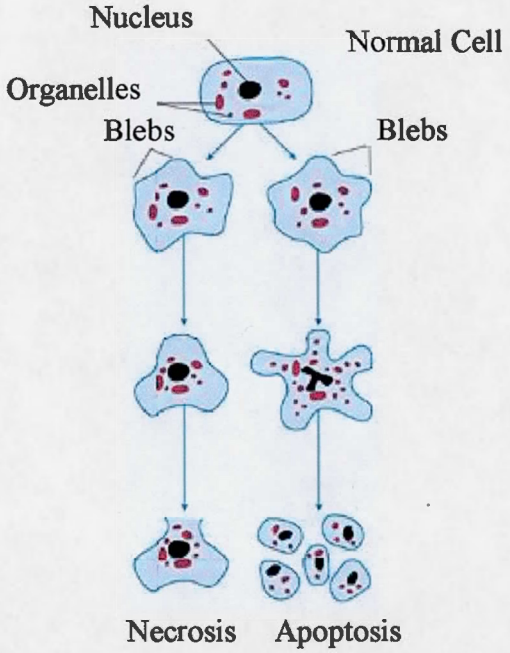

Figure 1.1: Structural changes of cells undergoing necrosis or apoptosis. Cells undergoing necrosis initially swell and the organelles break down. The cells eventually rupture and debris is spilled out that causes local inflammation. This inflammation can cause further death of the adjacent cells. Apoptosis affects only individual cells. When cells undergo apoptosis, the cell body shrinks, the DNA in the nucleus condenses and breaks apart into small fragments. However, other cellular organelles remain intact. The cell breaks up into several smaller bodies surrounded by a membrane. Scavenging cells such as macrophages engulf and destroy these "apoptotic bodies" (Adapted from Goodlett and Horn, 2001).

1.2. Components of the apoptotic program in mammals

The identification of components of the apoptotic program in the nematode *C. elegans* was the starting point for the discovery of homologous genes or proteins in mammals (Thompson, 1995; Green, 1998; Green and Reed, 1998; Raff, 1998). During apoptosis in *C. elegans*, at least 14 different genes are involved with specific roles in regulation and performance of apoptosis. It was shown that different apoptotic signalling pathways are involved, depending on stimulus and cell type, and these meet in a common effector pathway. Central components of this effector pathway are a certain class of proteases, the caspases (Kraut, 2001).

1.2.1. Caspases: the executioners of apoptosis

Most of the morphological changes that were observed are caused by a set of cysteine proteases that are activated specifically in apoptotic cells (Hengartner, 2000). These death proteases are homologous with each other (Muzio, 1998). Caspases are highly conserved through evolution and can be found from humans all the way down to insects, nematodes and hydra (Srinivasula *et al.,* 1996).

1.2.1.1. The structure and activation of caspases

Caspases are a kind of cysteine containing, aspartic acid-specific protease. They exist as zymogens in the soluble cytoplasm, mitochondrial intermembrane space and nuclear matrix of virtually all cells (Chandra *et al.,* 2000). For example, caspase-3 is a single-chain consisting of four domains: a NH_2-teminal prodomain of variable length, P20 and P10 domains and a linker region connecting these catalytic subunits. The linker region is missing in some caspase family members. The cleavage of the caspase precursors results in the mature caspase, a heterotetramer containing the P20/P10 heterodimers and two active sites (Wolf and Green, 1999) (Figure 1.2).

Caspases are typically divided into three major groups based on the structure of the prodomain and their function: inflammatory caspases which have large prodomains, initiator caspases and effector caspases with a short prodomain. The prodomain of apoptotic caspases contains a structure called the death domain subfamily which includes the death domain (DD), the death effector domain (DED), and the caspase recruitment domain (CARD) (Fesik, 2000). Each of these motifs interacts with other proteins by homotypic interactions. For example, DD and CARD contacts are based on electrostatic interactions, while DED contacts are via hydrophobic interactions (Eberstadt *et al.,* 1998). Procaspase-8 and -10 have DEDs in their prodomain. The CARD domain is found in procaspase-1, -2, -4, -5, -9, -11 and -12 (Erstadt *et al.,* 1998; Muzio *et al.,* 1998; Sprick *et al.,* 2002; Fuentes-Prior and Salvesen, 2004; Lamkanfi *et al.,* 2005).

Based on their proapoptotic functions, the caspases can be divided into two groups: initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10 and -14) which are activated via oligomerization-induced autoprocessing (Srinivasula *et al.*, 1998; Yang *et al.*, 1998), while effector caspases (caspase-3, -6 and -7) are activated by other proteases, including initiator caspases and granzyme B. Proteolytic cleavage of cellular substrates by effector caspases largely determines the features of apoptotic cell death (Liu *et al.*, 1998; Sakahira *et al.*, 1998; Zhang *et al.*, 1998). Caspases inactivate proteins involved in DNA repair, DNA replication and mRNA splicing (Cryns and Yuan, 1999; Rheaume *et al.*, 1997). They destroy the nuclear lamina, which results in the degradation of focal adhesion kinase (FAK) (Wen *et al.*, 1997).

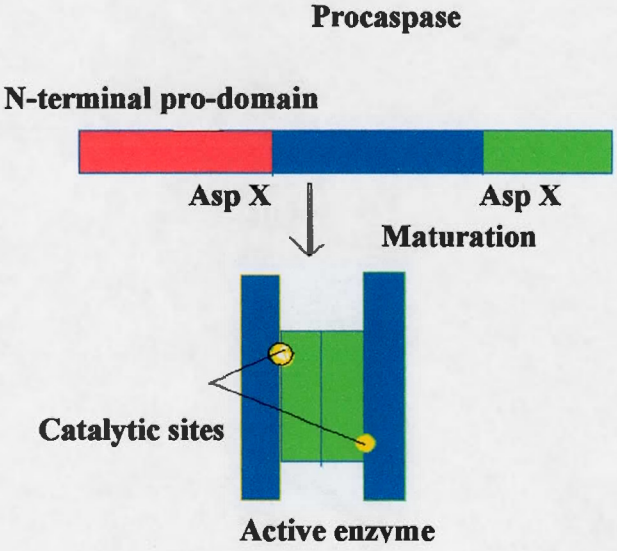

Figure 1.2: Structure and activation process of caspases. Caspases can be activated either by autoproteolysis or by other caspases. During the activation, the procaspase is cleaved after the aspartic acid residues, then the N-terminal pro-domain is discarded and the other two fragments reassemble to form a tetramer with two active sites (Adapted from Kraut, 2001).

1.2.1.2. The regulation of caspases

Seven members of the mammalian inhibitors of apoptosis protein (IAP) family, such as FADD-like ICE-inhibitory protein (c-FLIP), bifunctional apoptosis regulator (BAR) and apoptosis repressor with CARD (ARC) are able to down regulate the activity of caspases. All of the IAP family proteins share a specific baculoviral IAP repeat (BIR) region of about 70 amino acid residues, which allows an anti-apoptotic effect. The IAPs can bind to active forms of some caspases such as caspase -3 and -7 but not to their precursors (Liston *et al.,* 2003). c-FLIP, BAR and ARC can interrupt proapoptotic signal transduction by preventing death receptors from targeting an apical caspase, such as caspase -8. In addition, BAR protects cells from death via mitochondria, death receptors, or endoplasmic reticulum (ER) stress (Roth *et al.,* 2003).

1.2.2. P53 - a guardian of the genome

The tumor suppressor gene p53 has multiple roles in DNA repair, senescence and apoptosis (Fridman and Lowe, 2003). On the one hand, p53 is attributed a role in apoptosis as a transcription factor. It can promote the expression of genes involved in the apoptotic process ranging from death receptors, Bcl-2 family and the response to DNA damage. P53 mediates cell death through down regulation of the anti-apoptotic genes Bcl-2, microtubule-associated protein 4 (MAP4) and survivin, and up-regulation of the pro-apoptotic genes, Bax, insulin-like growth factor binding protein 3 (IGF-BP3), death receptor 5 (DR5), Fas, apoptotic protease activating factor-1 (Apaf-1) and various other components of the apoptosome (Slee *et al.,* 2004).

On the other hand, the transcriptional-independent apoptosis mediated by p53 requires the involvement of Bax, cytochrome c and caspase activation (Chipuk *et al.,* 2004; Chipuk and Green, 2004). Although p53 does not directly release cytochrome c from mitochondria, it is capable of performing a function analogous to that of Bcl-2 homology domain (BH3)-only proteins. Apoptosis induced by p53 is dependent on Bax and Bak. P53 has been reported to bind to Bcl-2 and Bcl-xL, which might allow

for the release of Bax, Bak, Bid and Bim from their inhibitors (Baptiste and Prive, 2004; Erster *et al.,* 2004; Mihara *et al.,* 2003). The targeting of mitochondria by p53 is supported by the finding that wild-type p53 translocates to mitochondria following γ-irradiation in murine thymocytes (Mihara *et al.,* 2003). Furthermore, endogenous Bcl-2 and Bcl-xL directly bind to wild-type p53 that targets mitochondria, and these interactions are dependent on the DNA binding domain of p53. This specifically involves amino acids 239–248, because p53 without these amino acids is no longer able to bind to Bcl-xL (Mihara *et al.,* 2003). The mitochondria-targeted effect of p53 is inhibited by Bcl-2, as demonstrated by the finding that siRNA introduced to down-regulate Bcl-2 caused p53-dependent apoptosis in the absence of genotoxic stress (Jiang and Milner, 2003). In summary, p53 can dictate a cell's fate through direct caspase-activating functions in the cytoplasm, in coupling or uncoupling of its transcriptional effects.

1.3. Death signal transduction in apoptosis

Three major pathways have been identified in apoptosis according to their initiator caspase: the death receptor pathway involving caspase-8 (Medema et al., 1997), the endoplasmic reticulum stress pathway attributed to activation of caspase-12 (Nakagawa et al., 2000) and the mitochondrial pathway, in which various signals can trigger the release of pro-apoptotic proteins from mitochondria into the cytoplasm, leading to activation of caspase-9 and downstream cleavage and activation of caspase-3, -7 or -6 (Grutter, 2000; Li et al., 1998; Luo et al., 1998) (Figure 1.3).

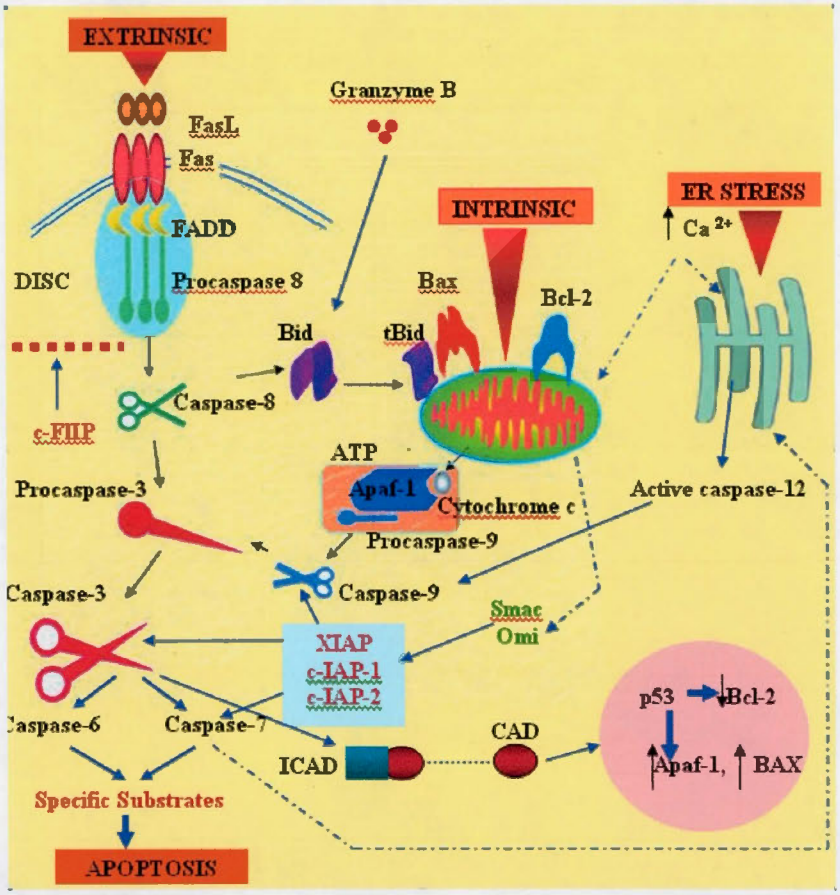

Figure 1.3: Apoptosis signal transduction pathways. In the death receptor pathway, the death receptors (e.g. FasL) recruit an adaptor protein (e.g. FADD) and procaspase-8 to form a death-inducing signaling complex (DISC). The oligomerization of procaspase-8 results in its activation. Activated caspase-8 then cleaves and activates caspase-3. The release of cytochrome c from mitochondria initiates the mitochondrial pathway. Cytochrome c activates formation of the apoptosome complex composed of cytochrome c, Apaf-1 and caspase-9. Activated caspase-8 or granzyme B can cleave a pro-apoptotic Bcl-2 family member Bid, and the truncated form of Bid is able to activate the caspase-9 dependent apoptosis pathway. In the ER stress pathway, the increase in intracellular Ca^{2+} causes the translocation of caspase-7 to the ER surface and the subsequent activation of caspase-12, which will then activate caspase-9 and caspase-3. On the other hand, the increased cytosolic Ca^{2+} might be taken up by mitochondria and initiate the release of cytochrome c. The activity of caspases is negatively regulated by IAPs, c-FLIP and the anti-apoptotic members of Bcl-2 family proteins. Smac and Omi are pro-apoptotic proteins released from mitochondria, which antagonize the inhibitory effect of IAPs. P53 regulates apoptosis by its transcriptional and non-transcriptional activity. Eventually the cleavage of ICAD by caspase-3 results in the liberation of CAD, which is responsible for the internucleosomal DNA fragmentation (Philchenkov, 2004; Gottlieb, 2000).

1.3.1. Death receptor signaling

The best characterized death receptors are CD95 (also called Fas or Apo1) and tumor necrosis factor receptor 1 (TNFR1) (also called p55 or CD120a) (Gruss and Dower, 1995). Additional death receptors are avian cerevisiae arginase 1 (CAR1) (Brojatsch *et al.,* 1996), death receptor 3 (DR3) (also called Apo3, WSL-1, TRAMP or LARD) (Kitson *et al.,* 1996; Chinnaiyan *et al.,* 1996; Screaton *et al.,* 1997; Bodmer *et al.,* 1997; Marsters *et al.,* 1996), DR4 and DR5 (also called Apo2, TRAIL-R2, TRICK 2, or KILLER). Human Fas receptor belongs to the same family of cell surface receptors as those for tumor necrosis factor (TNF) and nerve growth factor (NGF). The human Fas receptor is a 325 amino acid polypeptide (Nagata and Golstein, 1995) and has a sequence in the intracellular domains called the death domain (Ware *et al.,* 1996). When the engagement of Fas receptor and FasL, a transmembrane ligand of 40 kDa expressed on the surface of many cells (Suda *et al.,* 1995) occurs, the Fas receptor interacts with Fas associated death domain protein (FADD). FADD recruits the signaling complex FADD-associated ICE (FLICE). The recruitment of FLICE, or procaspase-8, causes its auto-activation and its subsequent release into the cytosol. Caspase-8 then cleaves and activates caspase-3. Activated caspase-3 eventually results in DNA fragmentation and apoptosis, by cleaving various cellular proteins. Activated caspase-8 or granzyme B can cleave a pro-apoptotic Bcl-2 family member Bid, and the truncated form of Bid is able to activate the caspase-9 dependent pathway of apoptosis (Muzio *et al.,* 1998).

1.3.2. ER-Stress mediated apoptosis

The endoplasmic reticulum (ER) is the organelle where the translated proteins are modified to form their proper tertiary structure and then translocate to the outer cell membrane (Kaufman, 1999). Many stimuli such as the inhibition of glycosylation, reduction of disulfide bonds, calcium depletion from the ER lumen, impairment of protein transport to the Golgi, and expression of mutated proteins in the ER will cause unfolded proteins to accumulate in the lumen of the ER and

eventually decide the fate of the cell (Harding *et al.*, 1999). This response is known as ER stress, which can cause cell-cycle arrest in G1/S phase, and a decrease in protein synthesis to prevent mal-folded protein aggregation and accumulation, the chaperone and folding induction and the degradation of ER-associated proteins to eliminate unwanted aggregates (Brewer and Diehl, 2000; Prostko *et al.*, 1992; Van Laar *et al.*, 2001). The three sensors of ER-stress are Ire1 (α and β), activating transcription factor 6 (ATF6) and phosphorylated extracellular signal-regulated protein kinase (PERK/PEK), which are localized on the ER membrane and trigger ER-stress. PERK/PEK and Ire1 (α and β) both share a homologous N-terminal region which is thought to sense protein aggregation (Tirasophon *et al.*, 1998; Shi *et al.*, 1998). These three sensor molecules have combined effects to upregulate genes encoding proteins that are involved in the secretory pathway including ER-resident chaperones and proteins required in the ER-associated protein degradation (Shen *et al.*, 2001).

All of the factors mentioned above are involved in the signaling response to maintain the homeostasis of the ER. If the stress cannot be resolved, the cell may then die by apoptosis. Two apoptotic pathways have been reported: the transcription factor- and caspase-dependent pathways. In the transcription factor pathway, Ire1 is thought to upregulate the transcription factor growth-arrest and DNA damage (GADD 153) homologous protein (CHOP) and alter the balance between Bcl-2 and Bax (Wang *et al.*, 1998; Ghribi *et al.*, 2001). Ire1 may also recruit tumour necrosis factor receptor-associated factor 2 (TRAF2) adaptor molecules to activate the c-jun N-terminal kinase (JNK) pathway (Yoneda *et al.*, 2001).

The ER can also induce apoptosis through activation of caspase 12. This pathway is independent of the mitochondrial and death receptor pathways. Caspase 12, which is localized on the ER membrane, plays a central role in inducing apoptosis in response to ER stress (Nakagawa, 2000). Caspase-12 belongs to the group I family of caspases including caspases-1, -4, -5, -11 and -13 (Van de Craen *et al.*, 1997) and shares a long pro-domain and two catalytic subunits. Functional caspase-12 has been cloned from the mouse and rat, but whether a human isoform of caspase-12 exists

remains unclear (Fischer *et al.,* 2000). When activated, caspase-12 translocates from the ER to the cytosol and then cleaves procaspase-9, which, in turn, activates the effector caspase, caspase-3 (Morishima *et al.,* 2002).

It has been reported that the ER and mitochondria are linked closely because the Ca^{2+} released from the ER eventually accumulates in mitochondria (Nakamura *et al.,* 2000). ER stress also causes oxidative stress and mitochondrial changes can be blocked by overexpresion of Bcl-2. Besides the transcription- and caspase-mediated cell death pathways in the ER, disruption of the Ca^{2+} balance leads to calpain activation which, through the cleavage of Bid and procaspase-12, contributes to caspase-9 activation (Hacki *et al.,* 2000).

1.3.3 The role of mitochondria in apoptosis

Mitochondria are important intracellular organelles for producing energy from adenosine 5'-triphosphate (ATP). Many factors can induce mitochondrial mediated apoptosis including anti-cancer drugs, irradiation, growth factor deprivation and oxidative stress. Recently, research has been focused on mitochondrial dysfunction caused by changes in mitochondrial membrane permeabilization (MMP) (Green and Kroemer, 2004). Alteration of MMP is responsible for the release of small molecules from the intermembrane space, including cytochrome c (Liu *et al.,* 1996), apoptosis inducing factor (AIF) (Joza *et al.,* 2001), second mitochondria-derived activator of caspase (Smac), also known as direct IAP-binding protein with low pI (DIABLO) (Verhagen *et al.,* 2000; Du *et al.,* 2000), Omi, also known as high temperature requirement protein A2 (HtrA2) (Suzuki *et al.,* 2001; Martins *et al.,* 2002; Hegde *et al.,* 2002; Verhagen *et al,* 2002; van Loo *et al.,* 2002), endonuclease G (Li *et al.,* 2001) and a subpopulation of pro-caspases (-2, -3, -8, -9) in certain cell types (Mancini *et al.,* 1998; Samali *et al.,* 1998; Susin *et al.,* 1999; Zhivotovsky *et al.,* 1999; Qin *et al.,* 2001). Members of the Bcl-2 family control this process tightly (Iwama *et al.,* 2001).

Bcl-2 is a member of a protein family involved in regulation of the apoptotic program in mammalian cells (Adams and Cory, 1998; Reed, 1997). At present, at least 15 members are known of the Bcl-2 family, which can have a negative, or a positive effect on initiation of the apoptotic program (Kraut, 2001). According to their functions, Bcl-2 proteins are divided into two groups: the first group includes anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, while the second group consists of pro-apoptotic proteins such as Bad and Bax. The sensitivity of cells to apoptotic stimuli can depend on the balance between pro- and anti-apoptotic Bcl-2 proteins. When there is an excess of pro-apoptotic proteins, the cells are more sensitive to apoptosis. When there is an excess of anti-apoptotic proteins, the cells will tend to be less sensitive (Tsujimoto, 2003).

Proapoptotic Bcl-2 proteins such as Bax are activated upon receiving apoptotic signals resulting in outer mitochondrial membrane permeabilization. However, anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, can prevent this process by heterodimerization with Bax-like proteins. Other Bcl-2 proteins such as Bad, Bid, Bim, Bmf and Noxa will impede the function of Bcl-2 or Bcl-xL or they will activate Bax-like proteins by direct binding.

A second mechanism of permeabilization of the outer mitochondrial membrane is the opening of a permeability transition pore (PTP) in the inner mitochondrial membrane upon a variety of stimuli. This allows water and small molecules to pass through, leading to swelling of the intermembrane space and rupture of the outer mitochondrial membrane (Green and Kroemer, 2004). Bcl-2 family proteins play a pivotal role in regulating the PTP, which is composed of the voltage-dependent anion channel (VDAC) (Shimizu, 1999), cyclophilin D and adenine nucleotide translocator (ANT) (Vyssokikh, 2003). The first protein released from mitochondria is cytochrome c, which plays an essential role in caspase-dependent apoptotic cell death: its release triggers formation of the apoptosome (Acehan *et al.*, 2002) composed of Apaf-1, ATP and procaspase-9. This leads to activation of caspase-9, which activates effector caspase-3 and caspase-7, leading to oligonucleosomal DNA

fragmentation (Baliga *et al.,* 2003). In contrast, AIF acts in a caspase-independent pathway that produces large-scale DNA fragmentation leading to apoptotic cell death (Cregan, 2004). The protective, anti-apoptotic abilities of IAPs can be inhibited by several proteins released from mitochondria, such as Smac/DIABLO and Omi/HtrA2. The Smac and Omi proteins both contain IAP binding motifs (IBMs) (Saelens *et al.,* 2004). Cells with overexpression of Smac or Omi/HtrA2 have enhanced sensitivity to UV radiation (Jia *et al.,* 2003; Martins *et al.,* 2002).

1.4. Mediation of apoptosis by poly (ADP-ribose) polymerase-1

Poly (ADP-ribose) polymerases (PARP) are enzymes that catalyze the post-translational modification of proteins. The PARP family of enzymes is involved in DNA repair, replication, transcription and cell death. The best known member is PARP-1, a 113 kDa nuclear protein involved in DNA repair. PARP is synthesized by utilizing NAD^+ as a substrate to catalyze the polymers of ADP-ribose. In general, PARP has three domains: the DNA binding domain (DBD, 46 kDa), the automodification domain (22 kDa) and the C-terminal catalytic domain (54 kDa) (Burkle, 2001; Smith, 2001; Chiarugi, 2002). When the cells receive environmental stimuli such as oxidative stress, PARP functions as a protector against DNA damage. Upon DNA damage, PARP-1 binds to DNA strand breaks and generates polymers of ADP-ribose bound to chromatin-associated proteins. The negative charge of these polymers allows PARP to dissociate from DNA, thus allowing the enzymes to repair DNA (Burkle, 2001).

PARP-1 can be cleaved by effector caspases such as caspase-3 (Lazebnik, 1994). During this process, PARP-1 is cleaved into two fragments: 24 and 89 kDa. It is assumed that the 24 kDa fragment containing the DBD may prevent the DNA repair enzyme from binding to the fragmented chromatin. The 89 kDa fragment contains the automodification and catalytic domains, but it cannot be stimulated by DNA strand breaks. As a result, cleaved PARP-1 loses the nick-sensor function and is incapable of performing its DNA repair function, thus promoting apoptosis. Poly

(ADP-ribosyl)ation is a high energy consuming process, which can also lead to the depletion of cellular energy and initiation of necrosis (Berger, 1985). Cleaved PARP-1 also has a role in blocking energy depletion-dependent necrosis since the consumption of NAD^+ is prevented (D'Amours *et al.*, 2001).

It has been reported that the translocation of AIF from mitochondria to the nucleus requires PARP-1 activity and is dependent on poly (ADP-ribosyl)ation and seems to trigger caspase-independent cell death (Yu *et al.*, 2002; 2003; Wang *et al.*, 2004). However the exact mechanism is unknown.

1.5. The role of DFF40/CAD endonuclease in apoptosis

An important feature of apoptosis is the fragmentation of genomic DNA (Arends *et al.*, 1990). Several different endonucleases have been thought to be responsible for apoptotic DNA fragmentation. One of these is DNA fragmentation factor (DFF) (Liu *et al.*, 1997), or caspase–activated DNase (CAD) (Enari *et al.*, 1998) or caspase–activated nuclease (Halenbeck *et al.*, 1998). DFF45 is a regulatory subunit of DFF while DFF40 is a catalytic subunit (Lugovskoy *et al.*, 1999; Otomo *et al.*, 2000). Both of these subunits reside in the cell nucleus (Liu *et al.*, 1998). DFF40 and DFF45 have a conserved domain of 80 amino acids at their N-terminal. DFF/CAD is a heterodimer composed of 40 and 45kDa protein subunits in an inactive form. DFF45 carries two caspase-3 – recognition sites, aspartate residues 117 and 224 (Liu *et al.*, 1997; 1998; Enari *et al.*, 1998; Halenbeck *et al.*, 1998).

DFF45 binds to DFF40 to inhibit its catalytic activity. Activated caspase-3 can cleave DFF45, releasing DFF40. DFF45 can be also cleaved by caspase-7 and granzyme B, but not by other caspases. The expression of DFF40 requires the presence of DFF45; otherwise DFF40 might form inactive aggregates (Liu *et al.*, 1997; 1998; Enari *et al.*, 1998). The nuclease activity of DFF40/CAD can be further activated by histone H1, HMGB1/2 and topoisomerase II (Durrieu *et al.*, 2000; Liu *et al.*, 1998; 1999; Widlak *et al.*, 2000). When DFF40 is synthesized in the cytoplasm, DFF45 binds to its nascent chain and promotes its correct folding. The catalytically

inactive complex of DFF40/DFF45 is then transferred to nuclei. DFF40 is specific for double-stranded DNA and depends on magnesium cations and can be inhibited by zinc cations. DFF40 attacks chromatin in the internucleosomal linker DNA, generating mono and oligonucleosomal fragments (Widlak *et al.*, 2000).

1.6. Oxidative stress

1.6.1 Sources of oxidative stress

The intrinsic balance of cells between life and death can be influenced by several environmental stresses such as irradiation, hyperthermia or certain chemotherapeutic drugs (Chandra *et al.*, 2000). Generation of oxidative stress in response to a variety of external stimuli has been implicated in the activation of transcription factors and in the triggering of apoptosis. In the apoptotic process, initial damage does not kill cells directly; rather it triggers an apoptotic signalling program that eventually leads to cell death (Gabai *et al.*, 1998). Oxidative stress has been defined as a disturbance in the pro-oxidant/antioxidant balance resulting in potentially lethal cell damage (Sies, 1999). Oxidative stress is a putative mediator of apoptosis by decreasing intracellular glutathione, the major buffer of the cellular redox status and by increasing cellular reactive oxygen species (ROS) (Suzuki *et al.*, 1998; Fridovich *et al.*, 1995). Cells are then subjected to a chronic redox imbalance leading to metabolic oxidative stress (Halliwell, 1999; Aruoma, 1996). ROS are widely generated in biological systems and include hydroxyl radicals ($^{\cdot}OH$), superoxide anions ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). H_2O_2 at low doses induces apoptosis via production of $^{\cdot}OH$ radicals and alteration of the oxidant/antioxidant balance (Wiseman and Halliwell, 1996).

1.6.2. Oxidative damage to macromolecules

If oxidative stress persists, oxidative damage to critical macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins and DNA

accumulates and eventually results in several biological effects. These range from alterations in signal transduction and gene expression to mitogenesis, transformation, mutagenesis and cell death (Hunt *et al.,* 1998; Mills *et al.,* 1998). Depending on the severity of the injuries, cell death can occur by either apoptosis or necrosis. Apoptotic cell death can be switched to necrosis during oxidative stress by two possible mechanisms: 1) inactivation of caspases due to oxidation of their active site thiol group by oxidants (Samali *et al.,*1999), or s-nitrosylation (Melion *et al.,*1997), which can lead to a necrosis-like cell death in fatally damaged cells, and 2) a drop in cellular levels of ATP due to the failure of mitochondrial energy production by oxidants (Leist *et al.,*1999; Tsujimoto *et al.,*1997).

1.6.3. Antioxidant defense mechanisms

To avoid the consequences of ROS-induced damage, cells have an antioxidant defense system that limits the production and accumulation of ROS. A wide array of enzymatic and nonenzymatic antioxidant defenses exists. Among these, three major antioxidant defense pathways are superoxide dismutase (SOD), catalase (CAT) and the glutathione redox cycle. The latter two are the major enzymatic pathways for H_2O_2 detoxification (Ahmad, 1995).

SOD destroys the reactive superoxide radical by converting it into the less reactive hydrogen peroxide (H_2O_2), which can be detoxified by catalase or glutathione peroxidase and the glutathione redox cycle. Catalase is mainly peroxisomal and catalyzes the reduction of H_2O_2 to H_2O and O_2 (Melion *et al.,* 1997). Glutathione peroxidases (GPx) catalyse the reduction of a variety of hydroperoxides using reduced glutathione (GSH) as substrate, thereby protecting mammalian cells against oxidative damage and reducing, among others, cellular lipid hydroperoxides. The glutathione peroxidases are divided into two groups, selenium–independent and selenium-dependent enzymes. Selenium-dependent enzymes can decompose H_2O_2 and various hydro- and lipid peroxides by the glutathione redox cycle. In this cycle, GSH is used as a cosubstrate to metabolize H_2O_2, resulting in H_2O and glutathione

disulfide (GSSG). The major source of H_2O_2 is the biochemical conversion of superoxide anion ($O_2^{\bullet -}$) by the action of SOD. GSSG can be reduced back to GSH by the enzyme GSH reductase (GR), in a reaction requiring NADPH regenerated by glucose 6-phosphate dehydrogenase (G6PDH). The capacity to recycle GSH makes the glutathione redox cycle a pivotal antioxidant defence mechanism for cells and prevents the depletion of cellular thiols (Ahmad, 1995).

In addition, several non-enzymatic antioxidants such as vitamin C and vitamin E are among the important antioxidants. Vitamin E is an antioxidant within membranes and vitamin C is within extracellular fluids. Both of these are general scavengers of free radicals.

1.6.4. ROS and apoptosis

ROS can be considered as major mediators of apoptosis (Gottlieb *et al.*, 2000). Several studies found that the addition of ROS or the depletion of endogenous antioxidants can promote cell death (Carmody *et al.*, 1999; Kane *et al.*, 1993; Guénal *et al.*, 1997). For instance, catalase can prevent spontaneous and H_2O_2 induced apoptosis in neutrophils (Kasahara *et al.*, 1997). Furthermore, several antioxidants can protect cells against apoptosis (Iwata *et al.*, 1997; Greenlund *et al.*, 1995). The thiol antioxidant N-acetyl-cysteine can scavenge and inhibit activation of caspases and subsequent steps, and then block or delay apoptosis in some systems (Mayer and Noble, 1994). The overexpression of MnSOD in cells can restore the mitochondrial transmembrane potential (Majima *et al.*, 1998) and could protect the cell from death caused by inhibition of the respiratory chain (Kiningham *et al.*, 1999). Increased levels of Cu/Zn SOD in the cells can delay apoptosis by scavenging $O_2^{\bullet -}$ (Greenlund *et al.*,, 1995) and preventing early release of cytochrome c (Fujimura *et al.*, 2000). Increased intracellular glutathione levels also prevented Fas receptor-mediated apoptosis in these cells (Watson *et al.*, 1997). Overexpression of the antioxidant phospholipid hydroperoxide glutathione peroxidase can also inhibit apoptosis (Nomura *et al.*, 1999).

1.6.4.1. Mechanisms of ROS signaling in apoptosis

Through activation of proteins such as tyrosine kinase and mitogen-activated proteins, ROS are also involved in signal transduction pathways in different physiological processes. It was reported that ROS are used as messengers by different stimuli to activate transcription factors, such as AP-1 and nuclear factor kappa B (NF-κB), to induce gene expression (Pinkus *et al.,* 1996). Data from many studies converge to the hypothesis that increases in ROS in cells are the consequence of an impairment of the mitochondrial respiratory chain. An interesting phenomenon is that the depletion of GSH, a non-enzymatic cellular antioxidant, is an event which takes place at the very beginning of the apoptotic process (van den Dobbelsteen *et al.,* 1996; Bojes *et al.,* 1997). The mechanisms for activation of apoptosis have been suggested as follows.

ROS can trigger death receptor-mediated apoptosis. The first implication that ROS could be involved in signal transduction arose from studies of TNF-α-induced cytotoxicity (Lancaster *et al.,* 1989, Schulze-Osthoff *et al.,* 1992). Fas receptor/Fas, which also belong to the tumor necrosis factor /nerve growth factor (TNF / NGF) receptor family, can be activated by ROS and results in a signal transduction pathway leading to apoptosis (Um *et al.,* 1996; Chiba *et al.,* 1996). In certain cell lines, the expression of FasL can be unregulated by hydrogen peroxide and the involvement of NF-κB (Bauer *et al.,* 1998; Vogt *et al.,* 1998). For a long time, apoptosis was considered to be under the control of nuclear events, but more recently, mitochondria are a central point of control (Mignotte and Vayssière, 1998; Desagher and Martinou, 2000; Ferri *et al.,* 2000). It has been suggested that ROS can activate the mitochondrial uncoupling protein-2 (UCP-2) located at the inner membrane of mitochondria. UCP-2 is a homolog of ANT and plays an important role in the maintenance of mitochondrial membrane potential (Arsenijevic *et al.*, 2000; Casteilla *et al.,* 2001; Echtay *et al.*, 2002; Voehringer *et al.,* 2000). Recent evidence also indicates that VDAC, another PTP complex protein located on the outer

mitochondrial membrane, can be targeted by $O_2^{\cdot-}$ and results in PTP opening (Madesh and Hajnoczky, 2001). Low doses of oxidants can induce apoptosis by triggering the mitochondrial membrane permeability transition and the release of both cytochrome c (Cai *et al.,* 1999) and AIF (Susin *et al.,* 1996). On the other hand, high doses of oxidants can damage mitochondrial energetic functions, causing a large decrease in cellular ATP levels and an acute energetic failure (Liu *et al.,* 1996). The depletion of ATP might delay the activation of caspases and switch cell death from apoptosis to necrosis. As mentioned previously, the Bcl-2 family of proteins can control the process of apoptotic cell death both positively and negatively (Gross *et al.,* 1999). Interestingly, the anti-apoptotic proteins Bcl-2 and Bcl-xL have been associated with protection against oxidants and a shift of the cellular redox potential to a more reduced state (Hockenbery *et al.*, 1990; Kane *et al.,* 1993). These findings suggest a role for ROS in the process of apoptosis by the mitochondrial pathway.

There is no evidence to indicate the involvement of ROS in the autocatalytic cascade of caspases (Cai and Jones, 1998) and the redox state of cytochrome c was not relevant to its ability to initiate the activation of caspases (Kluck *et al.,* 1997; Hampton *et al.,* 1998).

1.7. Hyperthermia

Hyperthermia refers to various techniques of heat application to already established strategies in cancer treatment, such as radiotherapy and chemotherapy. Heat treatment cannot replace the established therapies, but it can enhance the cell killing effects of cytotoxic drugs and radiation (Dahl, 1994). Furthermore hyperthermia has been used in gene therapy (Gerner *et al.,* 2000; Huang *et al.,* 2000) and stem cell purging (Moriyama *et al.,* 1986).

1.7.1 Hyperthermia in cancer treatment

Hyperthermia has been used in cancer treatment during the past 10 to 15 years. Generally, there is no intrinsic difference between the sensitivity of normal and

tumour cells to hyperthermia. However, *in vivo*, the enhanced tumour cell killing effect of hyperthermia has been observed at temperatures in the range of 40 to 44°C. This may be due to the fact that the architecture of the vasculature in solid tumours is chaotic, as well as the low pH and hypoxic tumour environments (Reinhold and Endrich, 1986; Song *et al.,* 1995; Vaupel and Kelleher, 1995).

1.7.1.1 Combination of radiotherapy and hyperthermia

Hyperthermia may increase radiosensitivity *in vivo* by causing an increased blood flow, which may result in an improvement in tissue oxygenation (Song *et al.,* 1997) Hyperthermia also potentiates radiation effects *in vitro*. The most important mechanism is that the effect of hyperthermia interferes with the cellular repair of radiation-induced DNA damage, probably by an effect on cellular proteins (Kampinga and Dikomey, 2001).

1.7.1.2 Combination of chemotherapy and hyperthermia

When chemotherapy is combined with hyperthermia, heat potentiates cytotoxic effects of many drugs including Adriamycin, melphalan, BCNU, bleomycin and cisplatin, both *in vitro* and *in vivo* (Honess, 1985; Raaphorst *et al.,* 1996; Orlandi *et al.,* 1995; Bates and Mackillop, 1986,1990,; Dahl, 1994; Bates *et al.,* 1985). Several of the reasons for an interactive effect are 1) the increase in intracellular drug uptake; 2) the enhancement of DNA damage and 3) higher intratumoral drug concentrations due to the increase in blood flow.

1.7.1.3. Methods to increase tumor temperatures

In the clinical application of hyperthermia, the three distinct techniques used are whole-body hyperthermia (WBH), hyperthermic isolated limb perfusion (HILP) and hyperthermic peritoneal perfusion (HPP).

Localised hyperthermia is used mainly to increase the tumour temperature in a targeted region. The temperature distribution depends on the energy distribution as

well as on thermal tissue characteristics and blood flow. The reduced blood flow in tumour tissue allows the tumour tissue to be heated more easily (Myerson *et al.*, 1997).

Regional hyperthermia is applied by perfusion of a limb, organ or body cavity with heated fluids (Schlemmer *et al.*, 2004; Petrovich *et al.*, 1989). When it is combined with cytostatic drugs, the temperature has to be lower than 43°C to avoid unacceptable toxicity.

For whole-body hyperthermia, the energy is introduced into the body and at the same time the energy loss is minimized (Robins *et al.*, 1992). The approach is to increase the temperature to about 40°C for several hours, and to use heat in combination with cytokines and cytotoxic drugs (Bull, 1996).

1.7.2. Heat shock-induced cell death

Thermal induced cell death has been observed in mammalian cells at temperatures between 41 and 47°C. At lower temperatures (41-43°C), cell death is much less than at higher temperatures above 43°C. Different cell types exhibit varying susceptibilities to hyperthermia. At temperatures between 41°C and 42°C, human tumour cells are less heat sensitive than rodent cells, and a potential therapeutic advantage can be achieved with prolonged heating at these non-lethal temperatures (Armour *et al.*, 1993). The sensitivity of cells to heat also varies with phase of the cell cycle, where cells in S phase and mitosis are the most heat sensitive (Yuguchi *et al.*, 2002).

Hyperthermia induces alterations involving inhibition of DNA, RNA and protein synthesis at molecular levels (Laszlo, 1992). The synthesis and polymerization of both RNA and DNA molecules are decreased at temperatures between 42 and 45°C. When heat exposure terminates, the syntheses of RNA and proteins recover more rapidly than the synthesis of DNA (Streffer, 1988; Hahn, 1982; Henle and Leeper, 1979). The thermal energy dose required to induce cell death is closely correlated to that required for cellular protein denaturation. The denaturation of cytoplasmic and

membrane proteins is thought to be involved in the cytotoxic effects of hyperthermia. There is a consistency between thermal damage and protein denaturation (Laszlo, 1992; Dewey, 1989). Protein unfolding occurs during heat shock and is responsible for thermal damage. Proteins aggregate when cells are heated (Roti Roti *et al.,* 1979). The protein aggregates contain denatured protein and also a large quantity of native protein (Borrelli *et al.,* 1996).

During moderate to severe heat shock, protein synthesis is inhibited temporarily. However, milder temperatures induce thermotolerance by induction of synthesis of heat shock proteins (Kregel, 2002). The heat shock factor (Hsf1) is believed to be responsible for this process through regulating stress gene activation (Voellmy, 2004).

Hyperthermia has also been reported to be cytotoxic in drug-resistant cells, including the multidrug resistant (MDR) Chinese hamster ovary cell line (CH^RC_5), overexpressing P-glycoprotein (Bates and Mackillop, 1986). Similar results were obtained for MDR human cervical adenocarcinoma (HeLa) cells, overexpressing multidrug-resistance protein-1 (MRP1) (Souslova and Averill-Bates, 2004).

1.7.3. Induction of apoptosis by heat shock

Hyperthermia can induce both apoptosis and necrosis in a temperature dependent manner. At higher temperatures, it is more likely to induce necrosis rather than apoptosis, but this varies in different cell lines (Harmon *et al.,* 1990; Allan *et al.,* 1998; Baxter and Lavin, 1992; Gabai *et al.,* 1996).

There is little knowledge about the signalling pathways that mediate the apoptosis-inducing effects of heat. It is commonly believed that environmental stresses, such as UV, gamma-radiation, drugs, toxins or heat shock, induce the release of heat shock proteins (HSPs), such as HSP60/HSP10 from mitochondria, which accelerates the release of cytochrome c and the subsequent activation of caspases in the mitochondrial pathway of apoptosis. In addition, heat shock or certain anti-cancer drugs can cause the production of ROS, whose accumulation causes the release of

cytochrome c from mitochondria, which leads to apoptosis through the activation of caspase-9 and caspase-3. The stress-activated protein kinase (SAPK) or c-JNK also results in activation of the caspase-dependent apoptotic pathway. Finally, the DNA damage caused by various stress can increase the Fas/FasL ligation process, which, in turn, leads to apoptosis involving the activation of caspase-8 and caspase-3 (Beere *et al.,* 2000; Meriin *et al.*, 1999; Gabai *et al.*, 1997). The tumour suppressor gene p53 can also be activated as a consequence of stress exposure, resulting in the increased transcription of some p53 target genes and the decreased transcription of other genes, such as c-fos or MDR genes (Amundson *et al.,* 1998; Miyashita and Reed, 1995; Agoff *et al.,* 1993; Elkeles *et al.,* 1999).

The balance between death and survival in cells during heat shock is determined by the expression of pro and anti-apoptotic proteins. Such proteins include Bcl-2 family members, the IAP members and HSP family members (Adams and Cory, 1998; Lacasse *et al.,* 1998; Jaattela, 1999). HSP70 has been shown to inhibit apoptosis by preventing the recruitment of procaspase-9 to the apoptosome complex, thereby preventing the assembly of a functional apoptosome (Beere *et al.,* 2000). HSP70 can also act on the apoptotic pathway by inhibiting JNK activation (Gabai *et al.*, 1997), either through targeting of JNK itself or the regulators of JNK. HSP70 may prevent apoptotic cell death by inhibiting the activation of effector caspases, such as caspase-3 (Mosser *et al.,* 1997; Jaattela *et al.,* 1998). Another important mediator of heat shock-induced apoptosis is HSP27, which can block apoptosis induced by heat, Fas ligand and anticancer drugs (Trautinger *et al.,* 1997; Richards *et al.,* 1996). Apoptosis can be observed when HSP synthesis is inhibited, although further research is required to clarify the underlying mechanisms (Buchner, 1996; Jaattela, 1999; Ciocca *et al.,* 1993; Fuller *et al.,* 1994; Kaur and Ralhan, 2000; Samali *et al.,* 1999).

1.8. Research project

1.8.1. Introduction

The hypothesis of the project is that heat shock could promote an increase in oxidative stress, thus creating a redox imbalance in favor of pro-oxidants. This could arise by a heat-induced increase in the generation and reactivity of oxidants such as superoxide and H_2O_2 and/or by inactivating cellular antioxidant defenses. As a consequence, heat could induce oxidative changes in cells leading to cell damage and eventually cell death.

It was previously reported that the antioxidants N-acetyl-L-cysteine (NAC), sodium pyruvate and catalase were able to protect against cytotoxicity induced by heat shock and/or exogenous H_2O_2 in Chinese hamster ovary cells (Lord-Fontaine *et al.*, 2001). In addition, the depletion of antioxidant defenses such as glutathione and catalase enhanced the cytotoxicity of heat and/or hydrogen peroxide in these cells. Cytotoxicity was evaluated by the inhibition of cell proliferation using a clonogenic cell survival assay. However, the role of oxidative stress as a mediator of heat shock-induced apoptosis has not been evaluated. This study will investigate the role of superoxide in mediating heat shock-induced cell death by apoptosis.

1.8.2 Objectives

Objective 1: To determine the ability of antioxidants to protect against heat shock-induced apoptosis.

Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) is a cell–permeable SOD mimetic which is able to enter the cells and can detoxify superoxide at the intracellular level. The ability of MnTBAP to protect cells against apoptosis induced by heat shock will be investigated.

Objective 2: To determine the ability of inhibitors of the antioxidant defense system to potentiate heat-induced apoptosis.

The inhibitors of certain antioxidants can be used to allow determination of the involvement of certain ROS in the induction of apoptosis by different stresses such as heat shock. Diethyldithiocarbamate (DDC) was selected because it is a strong pro-oxidant which can enter the cell and promote significant generation of superoxide by inhibiting SOD (Renoux, 1984). The ability of DDC to enhance heat shock induced apoptosis will be determined.

1.8.3. Experimental approach

The effects of MnTBAP and DDC on heat shock-induced apoptosis will be evaluated in Chinese hamster ovary cells through the exploration of the mitochondrial pathway by measuring:

1) The translocation of Bax from the cytosol to mitochondria followed by depolarization of mitochondrial membrane potential and the consequent release of cytochrome c from mitochondria into the cytosol, by Western blotting and flow cytometry (Tanel and Averill-Bates, 2005);

2) The enzymatic activities of caspases-3 and 9, by a fluorimetric assay (Souslova and Averill-Bates, 2004);

3) The condensation of chromatin in the nucleus of cells using the fluorochrome Hoechst 33258 and cellular incorporation of propidium iodide, by fluorescence microscopy, for the induction of apoptosis and necrosis, respectively (Souslova and Averill-Bates, 2004); and

4) The cleavage of ICAD and PARP by caspase 3, using Western blotting (Tanel and Averill-Bates, 2005).

CHAPTER 2: EXPERIMENTAL RESULTS

1-Preface

The content of this chapter is as follows:

A manuscript describes the results of the experiments that I carried out in the laboratory of Dr. Diana Averill for my Master's research project. This manuscript will be submitted for publication in the near future to the journal Archives of Biochemistry and Biophysics. It was written by Zhenghui Wang and was revised by Dr Diana Averill. The content of the manuscript includes the effect of MnTBAP, a SOD mimetic and DDC, an inhibitor of SOD, on hyperthermia-induced cell death by apoptosis in Chinese hamster ovary cells.

2- Manuscript

The Role of Oxidative Stress in Heat Shock-induced Apoptosis in Chinese Hamster Ovary Cells

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Keywords: Apoptosis, Hyperthermia, Oxidative stress, Mitochondria, Chinese hamster ovary cells (CHO), Manganese (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP), Diethyldithiocarbamate (DDC).

Abbreviations: AFC: 7-amino-4-trifluoromethylcoumarin; AMC: amino methylcoumarin; ANOVA: analysis of variance; Apaf-1: apoptotic protease-activating factor-1; CAD: caspase activated DNase; CHAPS: 3-[(3- cholamidopropyl) dimethylammonio]–2–hydroxyl-1-propanesulfonic acid; CHO: Chinese hamster ovary; DDC: diethyldithiocarbamate; DFF: DNA fragmentation factor; DTT: dithiothreitol; FBS: fetal bovine serum; FWE: family wise error; GPx: glutathione peroxidase; GR: GSH reductase; GSH; reduced glutathione; HRP: horseradish peroxidase; ICAD: inhibitor of caspase-activated DNase; JC-1: 5,5',6,6' tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide; α-MEM: minimum essential medium-Alpha; MMP: mitochondrial membrane permeabilization; MnTBAP: Mn(III) tetrakis (4–benzoic acid) porphyrin chloride; PARP: poly (ADP-ribose) polymerase-1; PBS: phosphate-buffered saline; PI: propidium iodide; PIPES: piperazine-N,N'-bis(2-ethanesulfonic acid); PMSF: phenylmethylsulfonyl fluoride; PVDF: polyvinylidene difluoride; ROS: reactive oxygen species; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM: standard error of the mean; SOD: superoxide dismutase.

ABSTRACT

Apoptosis is a mode of cell death that is different from necrosis. As a way to remove damaged cells, apoptosis occurs during severe pathological situations in multicellular organisms and is characterized by DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of apoptotic bodies (membrane enclosed vesicles). Apoptosis can also arise following exposure of cells to various types of stress, including radiation, oxidative stress, heat shock, environmental pollutants and chemotherapeutic agents. The mechanism of heat shock-induced cell death is not understood. It appears that heat shock could promote an increase in oxidative stress, thus creating a redox imbalance in favour of peroxidants. This could arise by increasing generation and reactivity of oxidants such as superoxide and H_2O_2 and by inactivating cellular antioxidant defences. As a consequence, heat is likely to induce oxidative changes in cells leading to cell damage and eventually cell death. This study evaluates the role of oxidative stress in heat shock-induced apoptosis by altering the level of antioxidant defenses against superoxide. The ability of the antioxidant Mn(III)tetrakis(4–benzoic acid) porphyrin chloride (MnTBAP) to decrease heat shock-induced apoptosis and the ability of inhibitor of the antioxidant defence system diethyldithiocarbamate to increase heat-induced apoptosis is investigated in Chinese hamster ovary cells. MnTBAP can protect cells from heat shock-induced apoptosis through blocking the translocation of the pro-apoptotic proteins Bax and cytochrome c between mitochondria and the cytosol, and by inhibiting the activity of caspase-9 and caspase-3. There was a decrease in cleavage of inhibitor of caspase-activated DNase and poly (ADP-ribose) polymerase-1 by MnTBAP. Superoxide dismutase inhibitor diethyldithiocarbamate (DDC) decreased apoptosis via the mitochondrial pathway, which was associated with an increase in heat shock-induced necrosis. In conclusion, these results suggest that oxidative stress is involved in heat shock-induced apoptosis.

Introduction

Hyperthermia refers to various techniques of heat application which can be combined with established strategies of cancer treatment. The combination of hyperthermia with radiation or chemotherapeutic agents is a very promising strategy, implied both from cellular and clinical studies (Kampinga and Dikomey, 2001; Honess, 1985; Song *et al.,* 1997; Raaphorst *et al.,* 1996; Orlandi *et al.,* 1995; Dahl, 1994; Bates *et al.,* 1985; Bates and Mackillop, 1986,1990). Further more hyperthermia has been used in gene therapy (Gerner *et al,* 2000; Huang et al., 2000) and stem cell purging (Moriyama *et al.,* 1986).

Based on animal and cellular studies, it has been shown that elevated temperatures (40-45°C) can enhance the cell-killing effect of cytotoxic drugs and radiation to tumour cells in *vitro* as well as in *vivo* (Dahl, 1994). Hyperthermia has been shown to increase the cytotoxic effect of numerous anticancer drugs such as AAPH, cisplatin (CDDP), VP-16, CPT-11 and doxorubicin (DOX) *in vitro* and *in vivo* (Takahashi *et al.,* 2002; Sumiyoshi *et al.,* 2002; Rowe *et al.,* 1999). At present, the use of hyperthermia in clinical cancer treatment is increasing (van der Zee, 2002). Hyperthermia has a tumor-selective effect because the hypoxic and low pH environments make the tumor cells more sensitive to heat killing than normal cells (Reinhold and Endrich, 1986; Song *et al.*, 1995; Vaupel and Kelleher, 1995). The increased blood flow resulting from hyperthermia improves the tissue oxygenation (Song *et al.,* 1997), increases drug uptake, enhances DNA damage and leads to higher intratumour drug concentrations (van der Zee, 2002). Clinical studies show that the combination of radiotherapy, chemotherapy and hyperthermia was effective in patients with advanced cervical carcinoma (Westermann *et al.,* 2005). A significant local control benefit was observed in patients with superficial tumors when radiation and hyperthermia was applied (Jones *et al.,* 2005).

There is very little knowledge about the signalling pathways that mediate the cytotoxic effects of heat. It has been suggested that heat shock could cause alteration

of the pro-oxidant/antioxidant balance, thereby promoting an increase in oxidative stress, thus creating a redox imbalance in favor of pro-oxidants. This could arise by an increase in the generation and reactivity of oxidants such as superoxide and H_2O_2 at high temperatures and/or by the inactivation of cellular antioxidant defenses (Lord-Fontaine and Averill-Bates, 1999). As a consequence, heat can induce oxidative changes in cells, leading to cellular damage and eventually cell death.

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are generated by endogenous metabolic processes in biological systems. Many enzymatic and non-enzymatic antioxidant defenses exist, thereby protecting mammalian cells against oxidative damage (Jose and Francisca, 2000). Among these, major antioxidant defenses are superoxide dismutase (SOD), catalase and the glutathione redox cycle (Ahmad, 1995). SOD detoxifies the superoxide radical by converting it into the less reactive H_2O_2, which can be detoxified by catalase or glutathione peroxidase and the glutathione redox cycle (Lindquist and Craig, 1998; Li and Nussenzweig, 1996). Catalase is mainly peroxisomal and catalyzes the reduction of H_2O_2 to H_2O and O_2 (Melino *et al.*, 1997). In the glutathione redox cycle, glutathione peroxidases (GPx) catalyse the reduction of H_2O_2 and a variety of hydroperoxides, using reduced glutathione (GSH) as substrate. GSH is oxidised to GSSG, which can be reduced back to GSH by the enzyme glutathione reductase (GR). This reaction requires NADPH, which is generated by glucose metabolism through the pentose phosphate cycle. The capacity to recycle GSH from GSSG makes the glutathione redox cycle a pivotal antioxidant defence mechanism for cells and prevents the depletion of cellular thiols (Ahmad, 1995).

Superoxide anion is very stable in an aqueous environment at neutral pH. Its toxicity is mainly based on generation of reaction products such as H_2O_2 (De Grey, 2002). This can result from conversion of $O_2^{\cdot-}$ in the mitochondrial matrix by MnSOD (Melov, 2000). H_2O_2 can be converted to the highly reactive hydroxyl radical $^{\cdot}OH$ by transition metal-catalysed reactions (Droge, 2002, Gutteridge and Halliwell, 2000), mainly by Fe^{2+} in the Fenton reaction. ROS disturb the oxidant/antioxidant balance,

leading to a state of oxidative stress (Wiseman and Halliwell, 1996). The persisting disturbance of "ROS homeostasis" leads to oxidative damage to critical biomolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins and DNA. The ROS accumulate and eventually result in several biological effects. These range from alterations in signal transduction and gene expression to mitogenesis, transformation, mutagenesis and cell death (Hunt *et al.,* 1998; Mills *et al.,* 1998). Depending on the severity of the injuries, cell death can occur by either apoptosis or necrosis (Samali *et al.,* 1999; Melion *et al.,* 1997).

Apoptosis is a highly regulated type of cell death characterized by DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of apoptotic bodies (membrane enclosed vesicles), which are eventually engulfed by phagocytes to avoid inflammation. During cell death by necrosis, cells swell and then rupture their membrane, releasing their contents and thereby causing an inflammatory response in adjacent cells and tissues (Wyllie *et al.,* 1980). A family of cytosolic cysteine proteases, caspases, exists in most cells as zymogens and play an essential role in the execution of apoptosis. The caspases are divided into initiator (-2, -8, -9, and -10) and executioner subsets (-3, -6, and -7) (Salvesen and Dixit, 1997).

Mitochondria are important intracellular organelles for producing energy from adenosine 5'-triphosphate (ATP). Many factors can activate mitochondrial mediated apoptosis including anticancer drugs, irradiation, growth factor deprivation and oxidative stress (Cai *et al.,* 1999). Mitochondrial dysfunction appears to be mediated by mitochondrial membrane permeabilization (MMP) (Green and Koremer, 2004). MMP induces the membrane transition pore to release small molecules from the intermembrane space, including cytochrome c (Liu *et al.,* 1996). This process is tightly controlled by the anti-apoptotic members of the Bcl-2 protein family (Chan and Yu, 2004). Proapoptotic Bcl-2 proteins such as Bax are activated upon receiving apoptotic signals, resulting in outer mitochondrial membrane permeabilization. The release of cytochrome c plays an essential role in caspase-dependent apoptotic cell death: its release into the cytosol triggers formation of the apoptosome (Acehan *et al.,*

2002) composed of apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9. This leads to activation of caspase-9 and subsequently effector caspase-3 and caspase-7. The effector caspases cleave a range of cellular substrates such as the DNA repair enzyme, poly (ADP-ribose) polymerase-1 (PARP) (Lazebnik, 1994). Another protein that can be cleaved by caspase-3 is DNA fragmentation factor (DFF) (Liu *et al.,* 1997), also known as inhibitor of caspase–activated DNase (ICAD) (Enari *et al.,* 1998; Halenbeck *et al.,* 1998). The cleavage of these proteins results in oligonucleosomal DNA fragmentation and eventually the cells die (Baliga *et al.,* 2003).

Hyperthermia can induce both apoptosis and necrosis in a temperature dependent manner (Harmon *et al.,* 1990). However, the mechanisms involved are not known. This study determines whether oxidative stress induced by heat shock has a role in the induction of apoptosis in Chinese hamster ovary (CHO) cells. The possible involvement of superoxide in heat shock-induced apoptosis is investigated by modulation of SOD levels. When the level of SOD is elevated, cells could be protected from heat shock-induced apoptosis. The inhibition of SOD activity could potentiate the heat shock-induced apoptosis in the cells. The cell–permeable SOD mimetic, Mn (III) tetrakis (4–Benzoic acid) porphyrin chloride (MnTBAP) was used to detoxify superoxide at the intracellular level. Diethyldithiocarbamate (DDC) was used to inhibit the activity of SOD. The ability of these modulators of intracellular levels of superoxide to alter cellular response to apoptosis was investigated at different levels, both upstream and downstream of mitochondria.

Materials and Methods

Cell culture

CHO cells (AuxBl) (Ling and Thompson, 1974) were grown in monolayer in minimum essential medium-Alpha (α-MEM) plus 10% fetal bovine serum (FBS) (Invitrogen Canada, Burlington, ON) and 1% penicillin (50 units/mL)-streptomycin (50 μg/mL) (Flow Laboratories, Mississauga, ON, Canada), 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 were 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, pH 7.4), washed by centrifugation (1000 *g*, 3 min) and resuspended in PBS-1% BSA-2 mM glucose for experimental studies.

Modulation of antioxidants

CHO cells were pretreated with MnTBAP (50 μM) for 1 h or DDC (5 mM) for 2 h in α-MEM with 10% FBS at 37°C, relative to controls without any modulator. Then, the modulator was removed and cells were washed, harvested and resuspended in 1 mL of PBS-1% BSA-2 mM glucose. Cells, either with or without MnTBAP or DDC pretreatment, were subsequently heated at different temperatures (37, 42 and 43°C) for 1 h. DDC can inhibit the enzymatic activity of SOD to about 20% of its initial level in CHO cells (Khadir *et al.,* 1999).

Morphological analysis of apoptosis

To visualize nuclear morphology and chromatin condensation by fluorescence microscopy (Bettaieb and Averill-Bates, 2005), cells were seeded and cultured to near confluence in tissue culture dishes containing 5 ml of α-MEM and 10% FBS. Cells were incubated with MnTBAP (50 μM) for 1 h or DDC (5 mM) for 2 h, and then

modulators were removed. Cells were then heated for 1.5 h in PBS-1% BSA-2 mM glucose at different temperatures (37, 42 and 43°C). Dishes were washed twice with PBS and Hoechst (33258) (0.06 mg/ml) was added for 15 min at 37°C to stain apoptotic cells. The dishes were washed with PBS and 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. 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 200 cells was counted per dish.

Determination of caspase activity by fluorescence spectroscopy

Following treatment with modulators (MnTBAP or DDC) and heat, CHO cells (1 x 10^6) were washed three times with cold PBS by centrifugation (1000 *g*, 3 min). The cells were resuspended in 50 µl of PBS and 25 µl were deposited into 96-well plates and lysed by freezing at -20°C for 20 minutes. Fifty µl of reaction buffer (20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% 3-[(3- cholamidopropyl) dimethylammonio] – 2 – hydroxyl-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) was added and stabilized at 37°C (Stennicke and Salvesen, 1997). The kinetic reaction was started after addition of 25 µl of the appropriate caspase substrate (200 µM) at 37°C using a 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-9 activity was measured by cleavage of the substrate Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl-coumarin to produce 7-amino-4-trifluoromethylcoumarin (AFC) with λmax excitation at 400 nm and λmax emission at 505 nm.

Flow cytometry analysis of mitochondrial membrane potential

To measure mitochondrial membrane potential ($\Delta\psi m$), the fluorescent probe 5,5',6,6' tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) was used. Following treatment with modulators (MnTBAP or DDC) and heat, the cells were washed three times with cold PBS by centrifugation (1000 *g*, 3 min). The cells were resuspended with PBS and then incubated with the lipophilic cationic dye JC-1 (5 μM) for 30 min at 37°C (Mancini et al., 1997). Cells were washed three times by centrifugation (2,500 *g*, 3min) and resuspended in 1 ml of cold PBS. Prior to analysis, 10 μg/ml of PI was added to stain necrotic cells. Data were collected using a FACS scan flow cytometer equipped with an argon laser emitting at 488 nm and analyzed using Cell Quest software (Becton-Dickinson, Menlo Park, CA). Forward and side scatters were used to establish size gates and exclude cellular debris from the analysis. JC-1 emission was collected on FL-1 and FL-2 channels at 530 ± 20 nm and 590 ± 20 nm, respectively. The PI emission was collected on the FL-3 channel (620 ± 15 nm). Ten thousand cells were analyzed for each sample. Under normal conditions, JC-1 exists in a monomeric form and stains as green fluorescence (FL-1). JC-1 forms J-aggregates in the presence of increasing mitochondrial potential. These J-aggregates give a red fluorescence (FL-2). By comparing the red/green fluorescence ratio, after removing PI-stained necrotic cells, one can determine the changes in $\Delta\psi m$.

Subcellular fractionation and immunodetection of Bax, cytochrome c, caspases, ICAD and PARP

Following treatment with MnTBAP or DDC and heat shock, 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 centrifugation at 2500 *g* for 10 min. The supernatant was centrifuged further at 25,000 *g* for 15 min. The resulting supernatant was designated as the cytosolic fraction, which was used for detection of cytosolic cytochrome c and Bax. The resulting pellet was designated as the mitochondrial fraction, which was used for detection of mitochondrial cytochrome c and Bax.

For immunodetection of ICAD, PARP and procaspase-3, whole cell lysates were used. Cells were lysed in 500 µl of lysis buffer (Samali *et al.,* 1999) containing 100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4, 0.1 mM DTT, 5% freshly added percoll, 0.01% digitonin, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 100 µl/10 ml of cocktail of protease inhibitors. Unbroken cells and nuclei were pelleted by centifugation at 2500 *g* for 10 min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins was carried out according to Laemmli (1970). Proteins (30 µg) were quantified according to Bradford (1976) and then solubilised in Laemmli sample buffer. The samples were boiled for 5 min at 100°C and loaded onto a SDS-polyacrylamide 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) (Turcotte and Averill-Bates, 2001). 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, pH 7.4 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 following primary antibodies (1:1000): anti-cytochrome c (BD Biosciences Canada, Mississauga, ON), anti-caspase-3, anti-Bax, anti-ICAD, anti-PARP and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T, 1% 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, Bax, caspase-3, ICAD and PARP 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-actin antibody and by coloration of the gels using Coomassie blue. Protein expression was quantified using a scanning laser densitometer, relative to actin (Molecular Dynamics, Sunnyvale, CA).

Statistics

Data are presented as means ± standard error of the mean (SEM) from at least 3 independent experiments performed with multiple estimations per point. For each series of data, we compare the average values of cells without modulators obtained at 42 and 43°C, relative to a control value at 37°C, designated as 1. The control value was subtracted from each observation and a one way analysis of variance (ANOVA) was performed to test H_0: mean = 0 versus H_1: mean different from zero (bilateral test) for each temperature. A Bonferroni-Holm (sequentially rejective method) adjustment was performed to control for the family wise error (FWE) rate at 5%. For the comparisons between cells without and with modulators, a bilateral student t-test

was performed with the hypothesis that with modulators at 37°C the value is equal to 1. For the comparisons at 42 and 43°C, we use the bilateral student t-test for independent samples.

Results

Initially, the types of cell death induced by heat shock were investigated in CHO cells by fluorescence microscopy. Apoptotic cells were identified by the fluorescent probe Hoescht 33258 (blue) which binds to condensed chromatin in the nucleus. The fluorescent probe PI (red) was used to identify necrotic cells. There was a significant increase in apoptotic cell death induced by 1 h of heat shock at 42 and 43°C in the absence of SOD modulators (Fig 2.1A, 2.1D), compared to the control cells at 37°C. Very few necrotic cells were observed (Fig 2.1A, 2.1D, 2.1G). When cells were pretreated with the superoxide scavenger MnTBAP for 1 h prior to heat shock, apoptotic cell death was significantly reduced by about 50% at 42°C and 38% at 43°C, compared to the controls (Fig 2.1B, 2.1C). Again, there were very few necrotic cells. When the cells were pretreated with the SOD inhibitor DDC for 2 h prior to heat shock at 42 and 43°C, the apoptotic cell death was also decreased (Fig 2.1E, 2.1G). However, DDC increased heat shock-induced cell death by necrosis. These graphs show that MnTBAP can protect CHO cells from heat shock-induced apoptosis, and that DDC caused an increase in necrosis rather than apoptosis.

Further investigations were carried out to determine whether the SOD modulator effects are at the level of the mitochondrial pathway. Molecular events involved in apoptosis were evaluated at the pre-mitochondrial, mitochondrial and post-mitochondrial levels. At the pre-mitochondrial level, the effects of MnTBAP and DDC effect on Bax translocation from the cytosol to mitochondria were determined by immunodetection. Cells were pretreated with MnTBAP or DDC as above and were heated at 42 or 43°C for 1 h. Heat shock caused a gradual loss of Bax in the cytosol (Fig 2.2A, 2.2B), due to its translocation to mitochondria (Fig 2.2E, 2.2F). At 43°C, the level of Bax decreased by about 30% in the cytosol (Fig 2.2C). When cells were treated with MnTBAP, the heat shock-induced translocation of Bax from cytosol (Fig 2.2A, 2.2C) to mitochondria (2.2E, 2.2G) was reduced at 42 and 43°C, relative to cells heated without MnTBAP. In DDC administered cells, the heat shock-

induced translocation of Bax from cytosol (Fig 2B, 2D) to mitochondria (Fig 2G, 2H) was also inhibited. These experiments with DDC are consistent with a change in the mode of cell death from apoptosis to necrosis (Fig 2.1).

Translocation of pro-apoptotic protein Bax to the mitochondria leads to mitochondrial membrane changes. FACS analysis of JC-1 was used to determine the ability of MnTBAP and DDC to alter the heat shock-induced changes in mitochondrial membrane potential ($\Delta\psi m$). JC-1 emission was collected on FL-1 and FL-2 channels. A significant decrease in the FL-2/FL-1 (red: green) ratio was observed in cells when temperature increased (56% at 42°C, 64% at 43°C) (Fig 2.3A, 2.3D). Exposure to MnTBAP (Fig 2.3C, 2.3D) slightly reduced damage to the mitochondrial membrane caused by 1 h of heat shock in cells. For DDC (Fig 2.3B, 2.3E), there was significant increase in the FL-2/FL-1 ratio relative to untreated cells at each temperature (32% at 37°C, 52% at 42°C). This indicates an antagonistic effect of DDC on heat shock-induced alteration of $\Delta\psi m$. The collapse of mitochondrial membrane potential is considered an important reason for the release of pro-apoptotic proteins like cytochrome c. Immunodetection showed that 1 h of hyperthermia (42 and 43°C) caused loss of cytochrome c from mitochondria (Fig 2.4A, 2.4B), and corresponding gain of cytochrome c in the cytosol (34% at 42°C, 68% at 43°C) (Fig 2.4E, 2.4F). A decrease in cytochrome c translocation from mitochondria (Fig 2.4A, 2.4C) to cytosol (Fig 2.4E, 2.4G) was observed in MnTBAP treated cells at 42 and 43°C, relative to corresponding heated cells without MnTBAP. A similar decrease in cytochrome c translocation was obtained in DDC treated samples (Fig 2.4B, 2.4D, 2.4F, 2.4H).

The effects of the superoxide modulators were subsequently investigated at the post-mitochondrial level. An important apoptotic event downstream of cytochrome c release is the activation of caspases, such as caspase-9 and caspase-3. One hour of hyperthermia induced activation of caspase-9 relative to unheated control cells at 37°C. MnTBAP treatment significantly lowered the activity of caspase-9 (36% at

37°C, 50% at 42°C, 50% at 43°C) (Fig 2.5A), relative to cells heated at different temperatures without MnTBAP. For cells pretreated with DDC (Fig 2.5B), there was decrease in caspase-9 activity, relative to corresponding untreated cells that were heated at 42 and 43°C.

Heat shock caused activation effector caspase-3, which was detected by caspase enzymatic activity and immunodetection of pro-caspase cleavage. Hyperthermia induced activation of caspase-3 relative to unheated control cells at 37°C by 6 fold at 42°C and 9-10 fold at 43°C (Fig 2.5C). For the cells pretreated with MnTBAP, caspase-3 activity was inhibited at 42°C to 43°C (Fig 2.5C). DDC also inhibited heat shock-induced caspase-3 activation at each temperature (50% at 42°C, 60% at 43°C) (Fig 2.5D). The immunodetection of procaspase-3 cleavage further confirmed the above experiments, in which heat induced the cleavage of procaspase-3 compared to its untreated controls (Fig 2.6A. 2.6B). Procaspase-3 cleavage was inhibited by treatment with both MnTBAP (Fig 2.6A, 2.6C) and DDC (Fig 2.6B, 2.6D).

Caspase-3 can cleave various protein substrates such as PARP and ICAD, which are involved in the final stages of apoptotic damage to DNA. Immunodetecton of PARP and ICAD cleavage was applied to determine the effects of MnTBAP and DDC on heat shock-induced DNA impairment. One hour of heat shock diminished the level of PARP by 22% at 42°C and 35% at 43°C (Fig 2.7A, 2.7B), and the ICAD level by 22% at 42°C and 28% at 43°C (Fig 2.8A, 2.8C). MnTBAP decreased PARP (Fig 2.7A, 2.7C) and ICAD (Fig 2.8A, 2.8C) cleavage caused by 1 h of heat shock. In DDC treated cells, both heat shock-induced cleavage of PARP (2.7B, 2.7D) and ICAD (2.8B, 2.8D) were also decreased.

Discussion

Heat shock can potentiate the cytotoxicity of radiation and certain chemotherapy drugs (Hahn, 1982; Liu and Wilson, 1998), but the mechanisms involved are not fully understood. It has been previously reported that more superoxide radical is generated in cells treated with hyperthermia (Lin *et al.,* 1991) and that levels of antioxidants such as superoxide dismutase, catalase, and glutathione redox cycle can affect the cellular sensitivity to heat (Khadir *et al.*, 1999; Lord-Fontaine and Averill-Bates, 1999). When managanese superoxide dismutase was overexpressed in human T-cell lines, the cell death induced by hyperthermia was diminished (Wong *et al.,* 1991). Overexpression of managanese superoxide dismutase could also protect human breast cancer cells (MCF-7) against hyperthermia (Li and Oberley, 1997).

Based on these previous findings, experiments were designed with the hypothesis that heat shock could promote an increase in oxidative stress, thus creating a redox imbalance in favour of peroxidants. It has been suggested that during heat shock, superoxide or its reaction product H_2O_2 are generated and can accumulate in cells. It is likely that high levels of superoxide produced during heat shock could have a detrimental effect on the cells. To identify if the heat shock-induced cell death in CHO cells was mediated by oxidative stress, we studied the role of superoxide in this process. This was achieved either by decreasing the superoxide production caused by heat shock or by inhibiting the activity of antioxidant defence system which detoxifies it.

In this study, we show that heat shock-induced cell death is modulated by oxidative stress. This was supported by the finding that 1) MnTBAP, a potent cell permeable SOD mimetic, could protect against apoptotic cell death by detoxifying superoxide caused by heat shock; 2) DDC, a strong cell permeable pro-oxidant, can promote increased generation of superoxide by inhibiting SOD (Renoux, 1984) and eventually induce necrotic death in cells. The mechanisms of heat shock-induced cell

death were investigated by exploration of the mitochondrial apoptotic pathway, involving Bax translocation, the alteration of mitochondrial membrane potential, the release of cytochrome c, the subsequent activation of downstream caspases and the cleavage of essential substrates leading to DNA damage.

Heat shock alone

We showed that 1 h of heat shock could induce apoptosis in CHO cells, especially at 43°C. This was confirmed by the condensation of nuclear chromatin through morphological analysis. Under these conditions, necrotic cell death was not induced during heat shock.

Apoptosis can occur through different pathways: the death receptor pathway (Medema *et al.,* 1997), the mitochondrial pathway (Grutter, 2000; Li *et al.,* 1998; Luo *et al.,* 1998) and the ER mediated pathway (Nakagawa *et al.,* 2000). Our earlier results confirmed that apoptotic events caused by heat shock occurred at the mitochondrial level (Bettaieb and Averill-Bates, 2005). At the mitochondrial level, Bax can facilitate cytochrome c release either by interacting with the permeability transition pore complex (Brenner *et al.,* 2000) and/or by forming oligomers as channels that trigger cytochrome c release (Antonsson *et al.,* 2000). Our findings suggest that the heat induced production of ROS caused opening of the PTPs and the loss of $\Delta\psi m$ (Madesh and Hajnoczky, 2001). Our results suggest that the translocation of Bax was essential in altering outer membrane permeabilization caused by heat shock in CHO cells.

At the post-mitochondrial level, cytochrome c release occurred due to the opening of the PTP. As a pivotal event for the formation of the apoptosome complex, heat shock-induced release of cytochrome c caused the activation of caspase-9 and the downstream effector caspase-3 (Grutter, 2000; Li *et al* 1998; Luo *et al.,* 1998).

The heat shock-induced damage to DNA was confirmed by the cleavage of PARP and ICAD by effector caspases. Caspase-3 can eventually cleave PARP (Lazebnik, 1994) and ICAD (Liu *et al.,* 1997). However it has been reported that

caspase-7 also caused cleavage of ICAD and PARP (Germain *et al.,* 1999; Wolf *et al.,* 1999).

The antioxidant function of MnTBAP

According to several reports, MnTBAP can detoxify superoxide to H_2O_2 (Hildeman *et al,* 1999) and prevent neural cells and endothelial cells from apoptosis caused by oxidative stress (Day *et al.,* 1995; 1997; Patel *et al.,* 1996; Patel, 1998). We treated cells with 50 μM of MnTBAP for 1 h. Our results showed heat shock-induced apoptosis was suppressed by MnTBAP. This indicates that MnTBAP can prevent apoptosis of cells caused by heat, probably by detoxifying superoxide.

Our results also showed that MnTBAP blocked the loss of mitochondrial membrane potential, possibly by preventing superoxide as a signaling molecule at the mitochondrial level. The possible pathways could involve down-regulation of Bcl-2, which antagonizes Bax (Tsujimoto, 2003). A possible candidate is NF-κB, which can be activated by ROS, and represses the expression of Bcl-2 (Bauer *et al.,* 1998; Voget *et al.,* 1998).

DDC- a SOD inhibitor

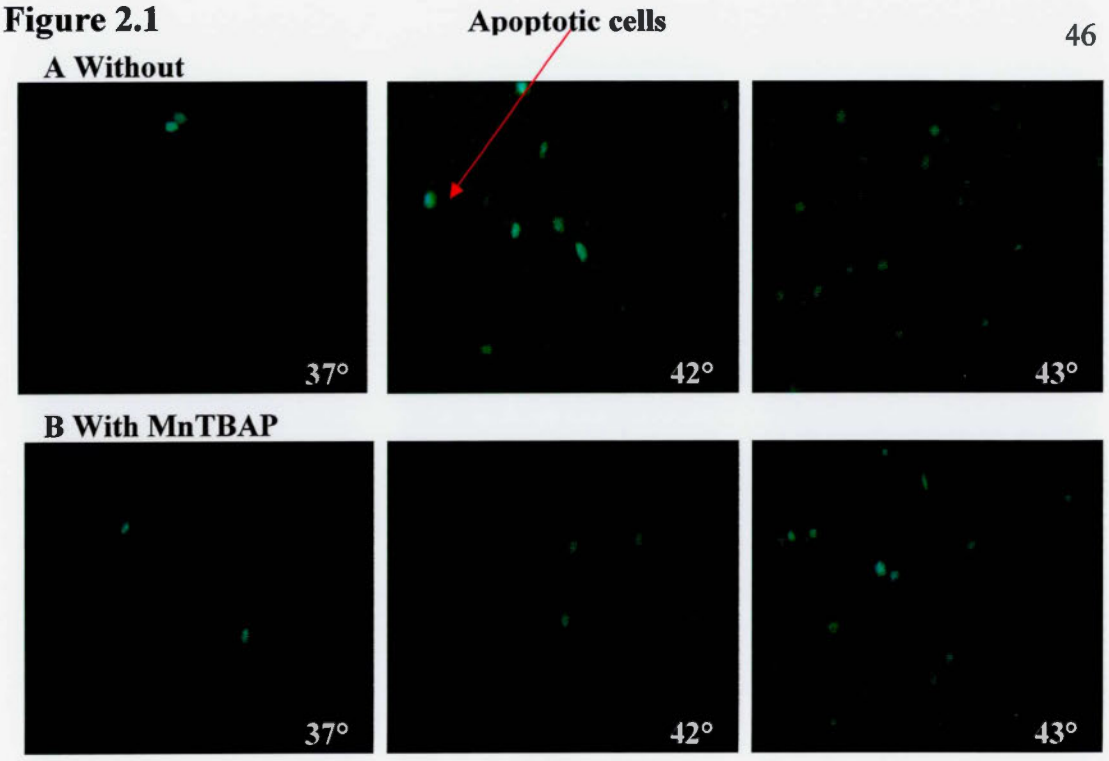
DDC can severely disturb the redox balance by inhibiting the activity of SOD in heated cells, which would result in the accumulation of superoxide. DDC was previously reported to enhance the cell killing effect of hyperthermia and the chemotherapeutic agent Bleomycin (Khadir *et al.,* 1999). As a potent pro-oxidant, DDC was able to induce both apoptosis and necrosis in human promyelocytic leukemia cells, depending on the concentrations (Kimoto-Kinoshita *et al.,* 2004). An interesting discovery of our study is that the combination of DDC and heat shock induces necrosis rather than apoptosis, in cells. This might due to DDC stimulation of the production of superoxide by inactivation of copper-zinc superoxide dismutase (Heikkila *et al.,* 1976). However DDC can also inhibit the copper-dependent cytochrome c oxidase in the mitochondria. The resulting high-dose oxidants

eventually damage mitochondrial energetic function, causing a dramatic decrease of cellular ATP level and acute energetic failure (Liu *et al.*, 1996). Depletion of ATP and accumulation of ROS can switch cell death from apoptosis to necrosis (Eguchi *et al.*, 1997). Moreover, there are also reports that PARP activity provokes necrosis (Ha and Snyder, 1999; Watson *et al.*, 1995) due to DNA strand break-dependent activation of PARP. PARP consumes NAD and in consequence also affects the ATP pool (Lee and Shacter, 1999; Filipovic *et al.*, 1999).

Conclusion

This study provides further evidence that heat shock increases oxidative stress in cells. This fundamental research gives a very promising profile of the modifier of cell death and might be useful in cancer control, prevention and cancer therapy.

Figure 2.1


Apoptotic cells
A Without
37°
42°
43°
B With MnTBAP
37°
42°
43°

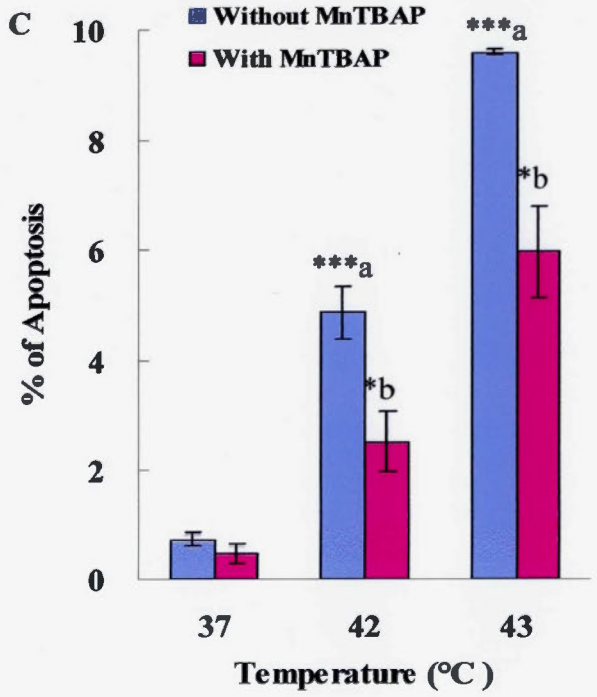
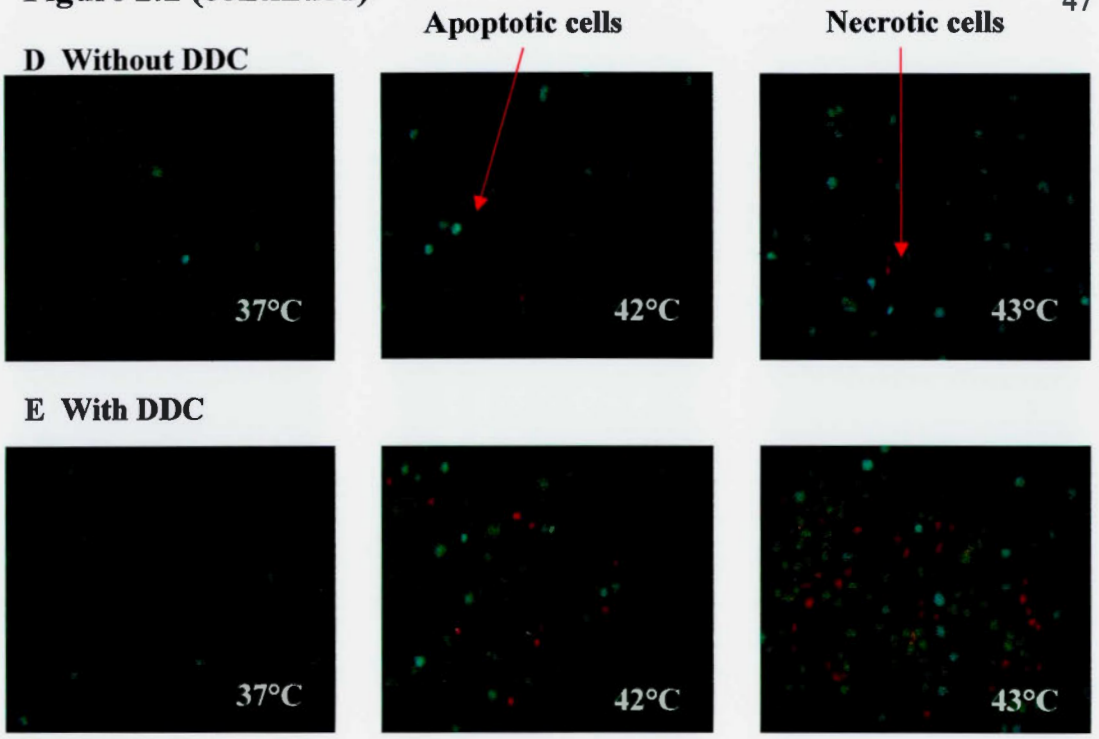

C
Without MnTBAP
With MnTBAP
% of Apoptosis
10
8
6
4
2
0
***a
*b
***a
*b
37
42
43
Temperature (°C)

Figure 2.1 (continued)



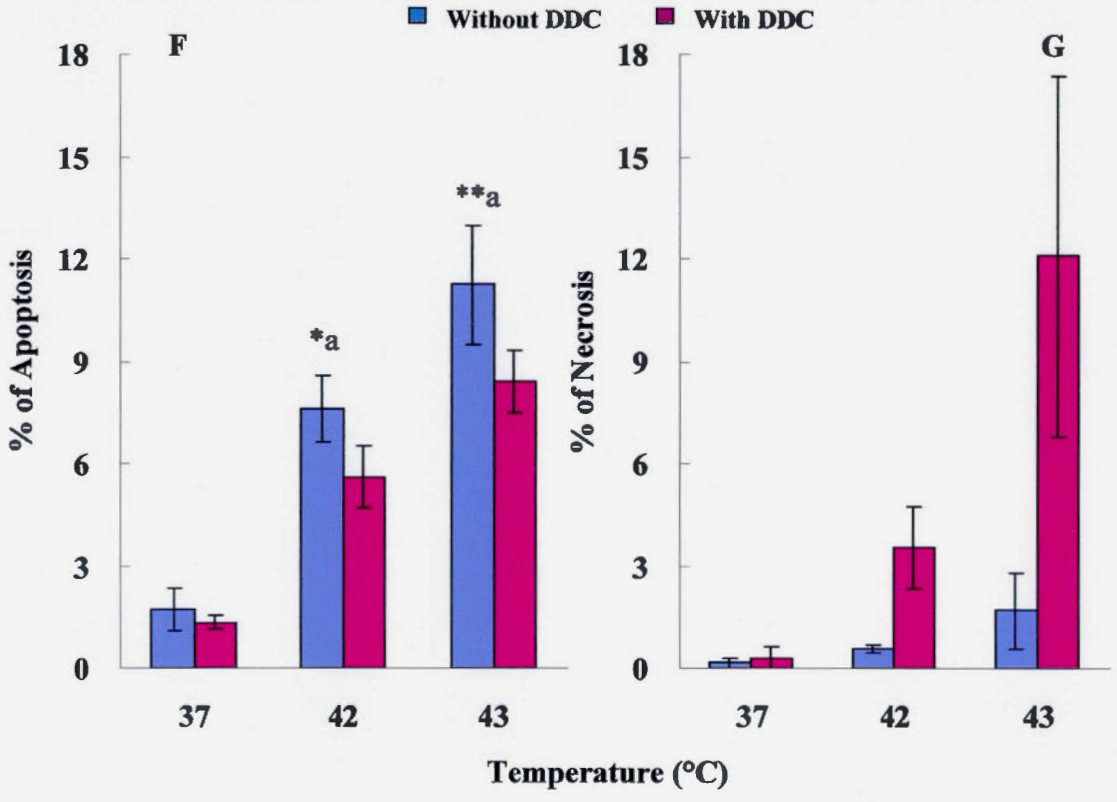


Figure 2.1: Morphological analysis of apoptosis and necrosis in cells following exposure to heat shock: modulation by MnTBAP or DDC. Cells (0.3 $x10^6$) were seeded and cultured for two days to near confluence in tissue culture dishes containing α-MEM and 10% FBS at 37°C. Cells were pretreated with MnTBAP (50 μM) for 1 h or DDC (5 mM) for 2 h, relative to respective controls without any modulator. The modulator was then removed and cells were heated in PBS-1% BSA-2 mM glucose at different temperatures (37, 42 and 43°C) for 1.5 h. Cells were stained with Hoechst and PI and visualised by fluorescence microscopy (magnification 320 X). The percentages of apoptotic and necrotic cells are given relative to total cells. Representative photographs (A and B for control and MnTBAP; D and E for control and DDC) are shown. Data represent means and SEM from 3 independent experiments (C for MnTBAP, F and G for DDC) performed with multiple estimations per point. a, significantly different for heated cells from corresponding control (no modulators) at 37°C. b, significantly different for treatment with or without modulators, at 42 and 43°C. $p<0.05$ (*), $p< 0.01$ (**) or $p<0.001$ (***).

Figure 2.2

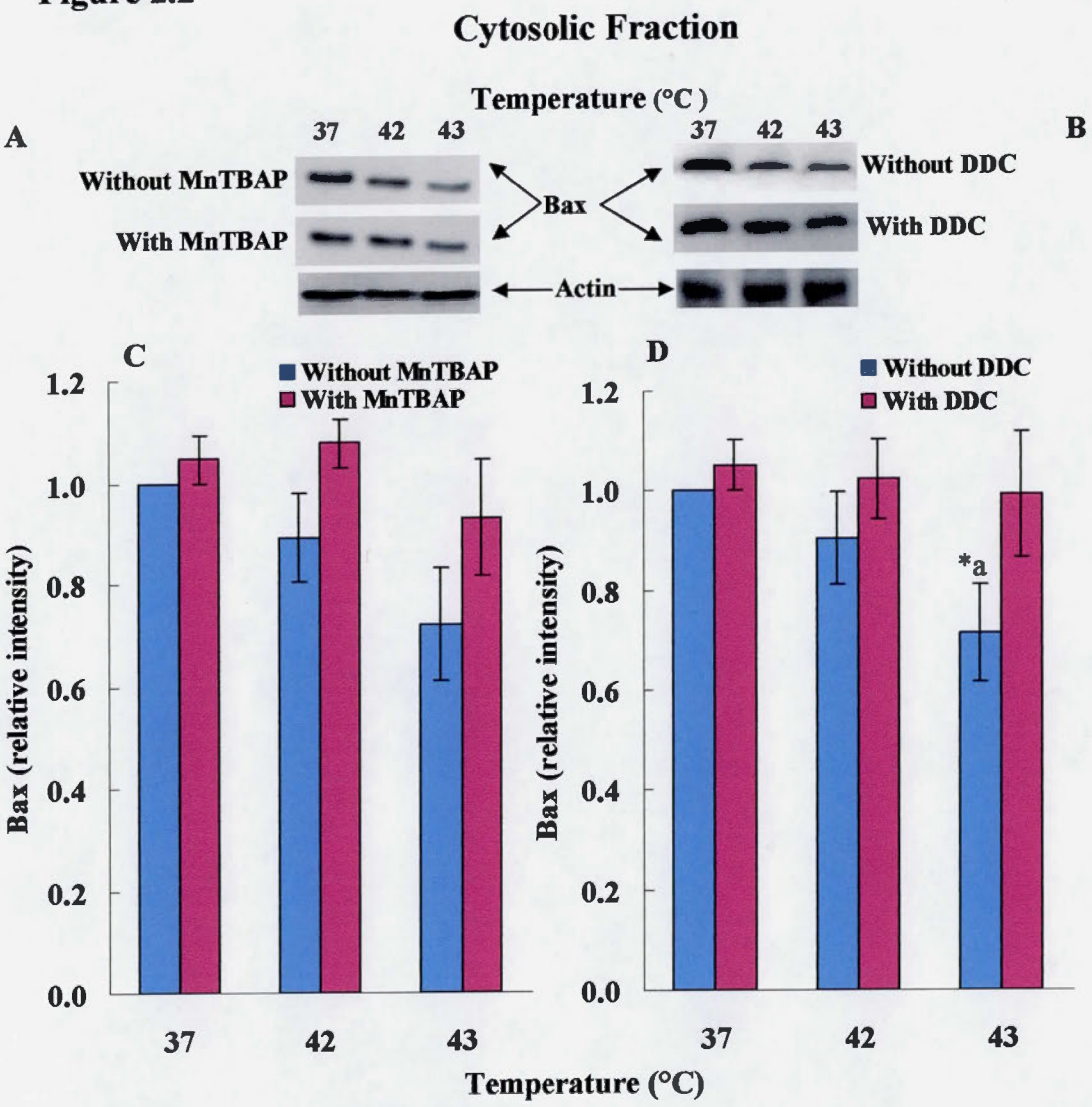

Cytosolic Fraction
Temperature (°C)
A
37 42 43
Without MnTBAP
With MnTBAP
Bax
Actin
B
37 42 43
Without DDC
With DDC
C
Without MnTBAP
With MnTBAP
Bax (relative intensity)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
37 42 43
Temperature (°C)
D
Without DDC
With DDC
*a
Bax (relative intensity)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
37 42 43

Figure 2.2 (continued)

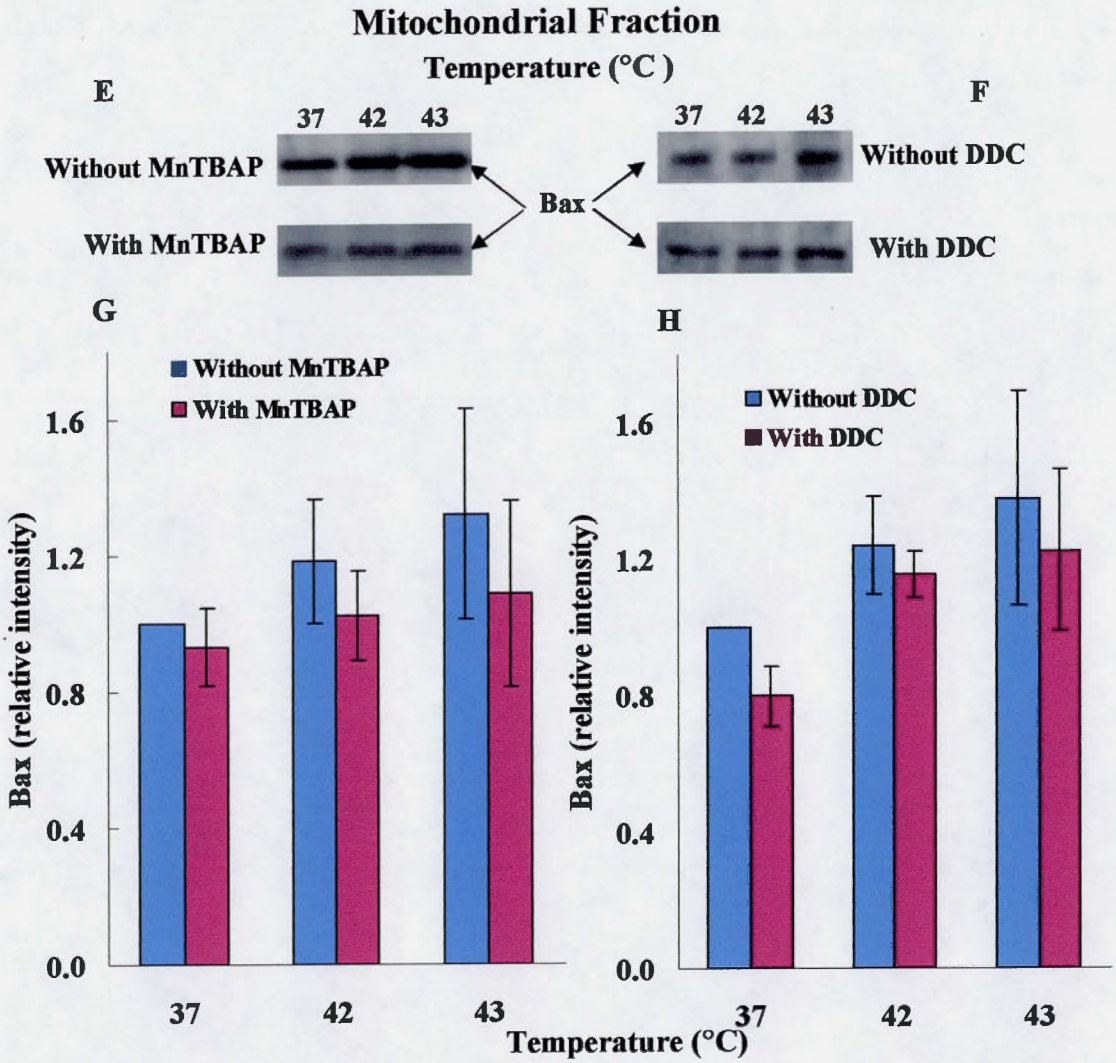
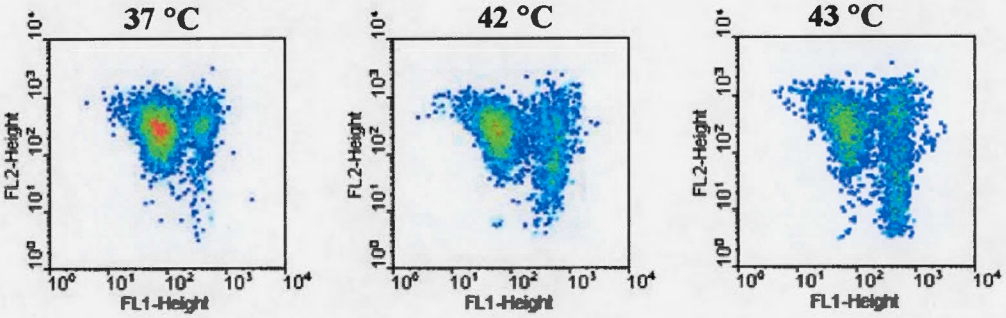


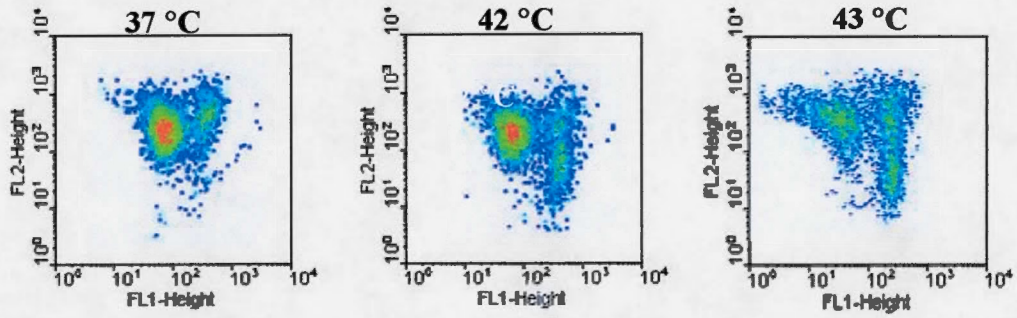
Figure 2.2: Inhibition of heat shock-induced translocation of Bax from cytoplasm to mitochondria by DDC or MnTBAP. Following treatment with modulators and heat shock, immunodetection of Bax was carried out in cytoplasmic (A-D) and mitochondrial (E-H) fractions. Representative blots are shown (A and E for control and MnTBAP; B and F for control and DDC). Data represent means and SEM from 4 independent experiments for controls and MnTBAP (C, G) and 3 independent experiments for DDC (D, H). Expression of Bax was relative to untreated control cells at 37°C, designated as 1. a, significantly different for heated cells from corresponding control (no modulators) at 37°C. $p<0.05$ (*).

Figure 2.3

A Without Modulator



B With DDC



C With MnTBAP

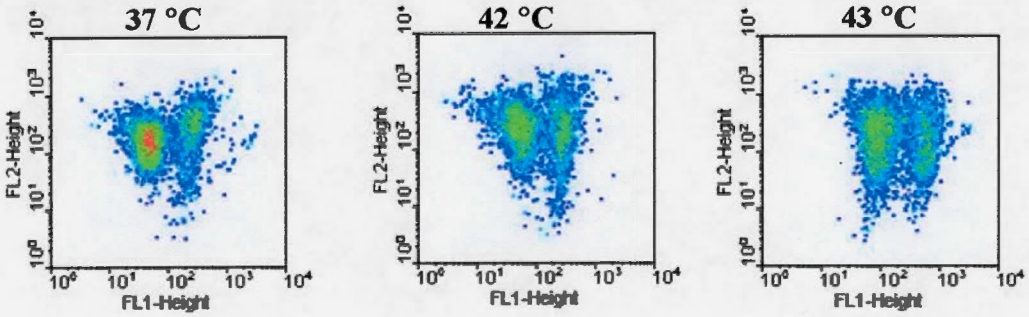


Figure 2.3 (continued)

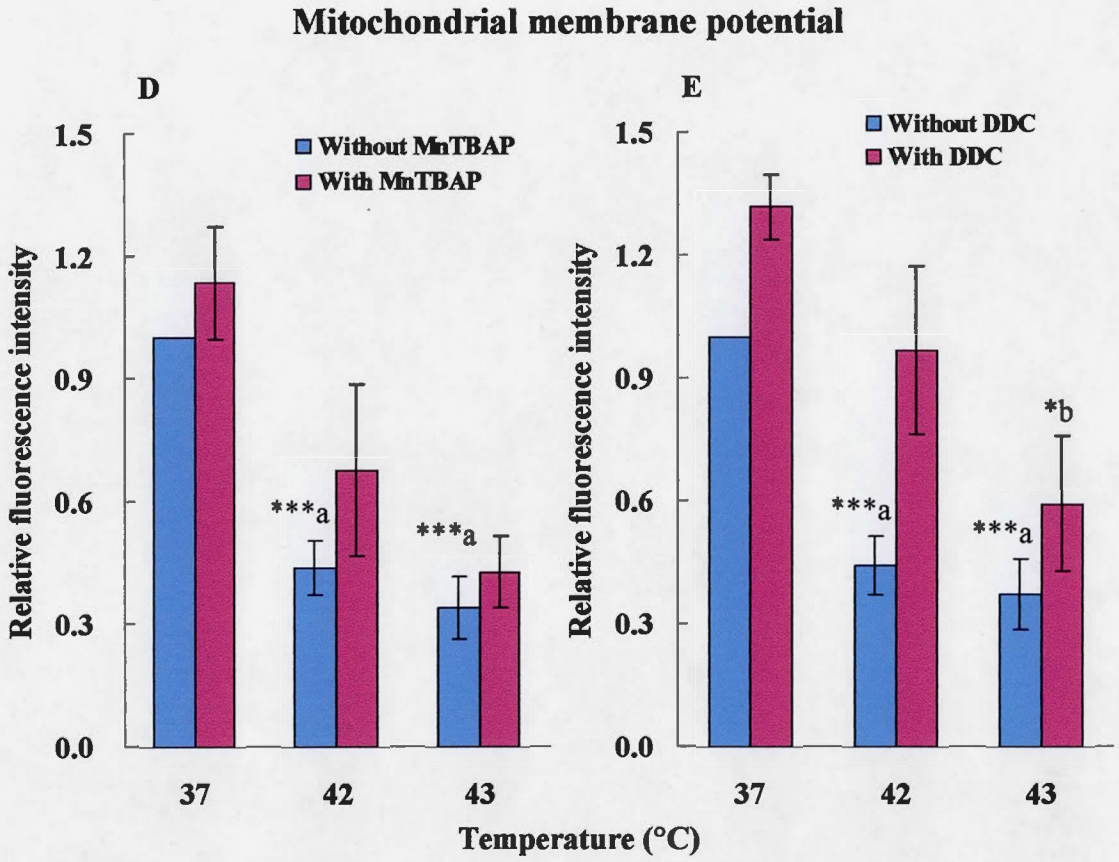


Figure 2.3: Heat shock causes a decrease in mitochondrial membrane potential: effects of MnTBAP or DDC. Following treatment with modulators and heat shock, cells were analysed by flow cytometry using JC-1. Representative Facs Scan dot blots are shown (A-C). Data represent means and SEM of relative fluorescence intensity for JC-1 from 8 or 9 independent experiments for cells pretreated with MnTBAP (D) or DDC (E), respectively. a, significantly different for heated cells from corresponding control (no modulators) at 37°C. b, significantly different for treatment with or without modulators, at 42 and 43°C. $p<0.05$ (*), or $p<0.001$ (***).

Figure 2.4

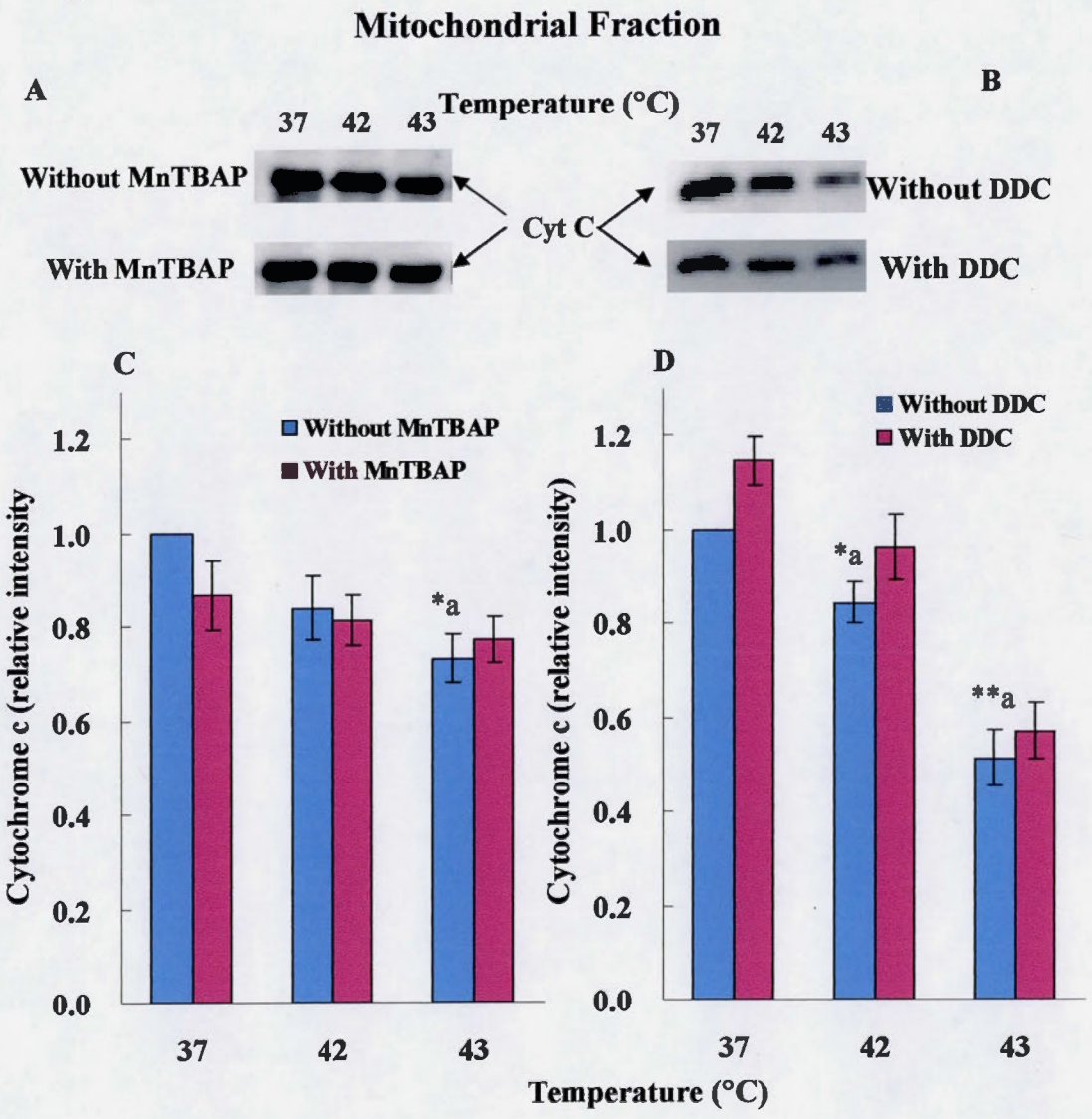

Mitochondrial Fraction
A
B
Temperature (°C)
37 42 43
37 42 43
Without MnTBAP
With MnTBAP
Cyt C
Without DDC
With DDC
C
D
Without MnTBAP
With MnTBAP
Without DDC
With DDC
Cytochrome c (relative intensity)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*a
**a
37
42
43
Temperature (°C)

Figure 2.4 (continued)

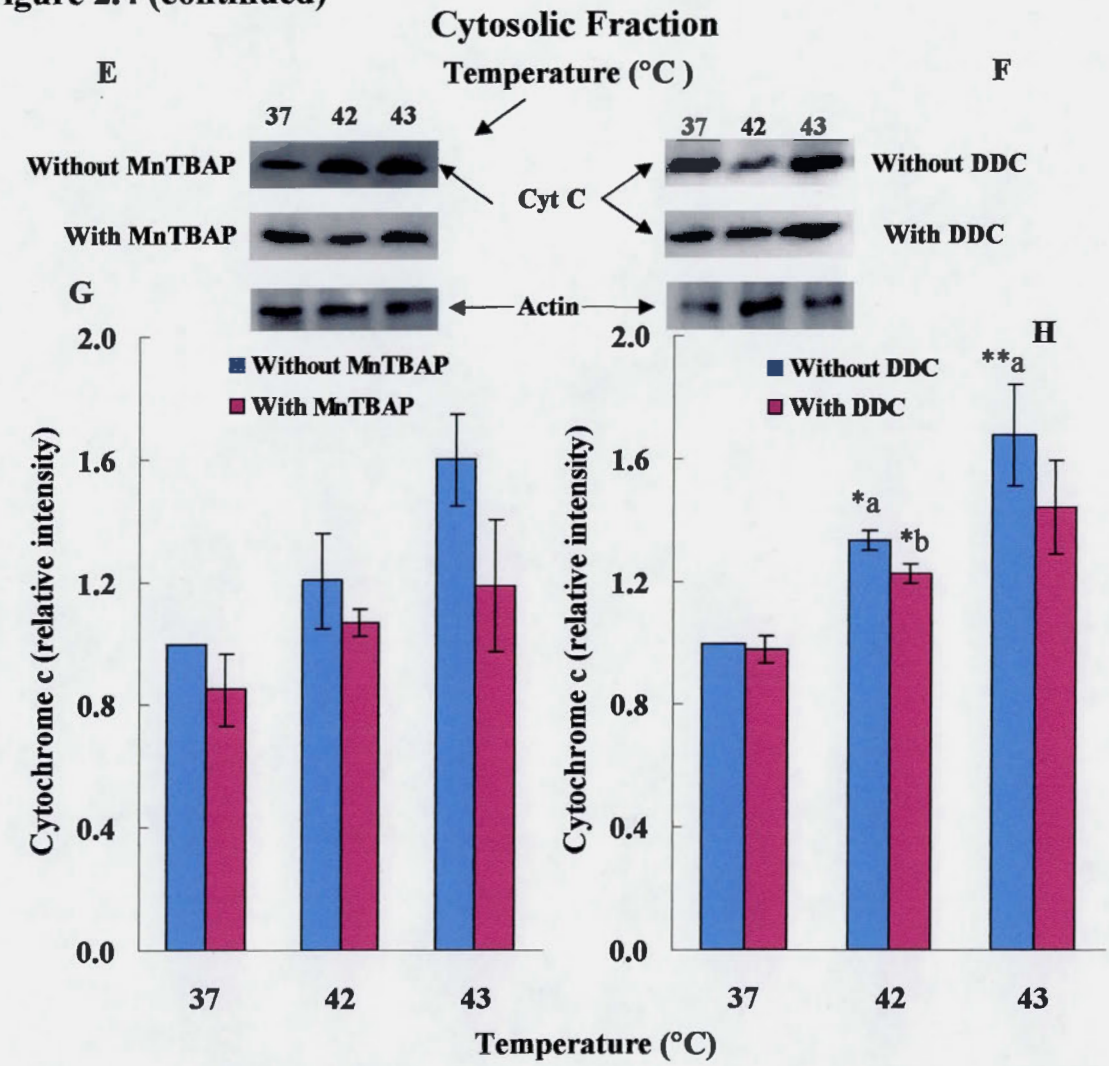


Figure 2.4: Inhibition of heat shock-induced release of cytochrome c from mitochondria into cytoplasm by modulators of SOD. Following treatment with modulators and heat shock, immunodetection of cytochrome c was carried out in cytosolic and mitochondrial fractions. Representative blots are shown (A and E for control and MnTBAP; B and F for control and DDC). Data represent means and SEM from 3 or 4 independent experiments for cells pretreated with MnTBAP (C and G) or DDC (D and H), respectively. Expression of cytochrome c was relative to untreated control cells at 37°C, designated as 1. a, significantly different for heated cells from corresponding control (no modulators) at 37°C. b, significantly different for treatment with or without modulators, at 42 and 43°C. $p<0.05$ (*), or $p< 0.01$ (**).

Figure 2.5

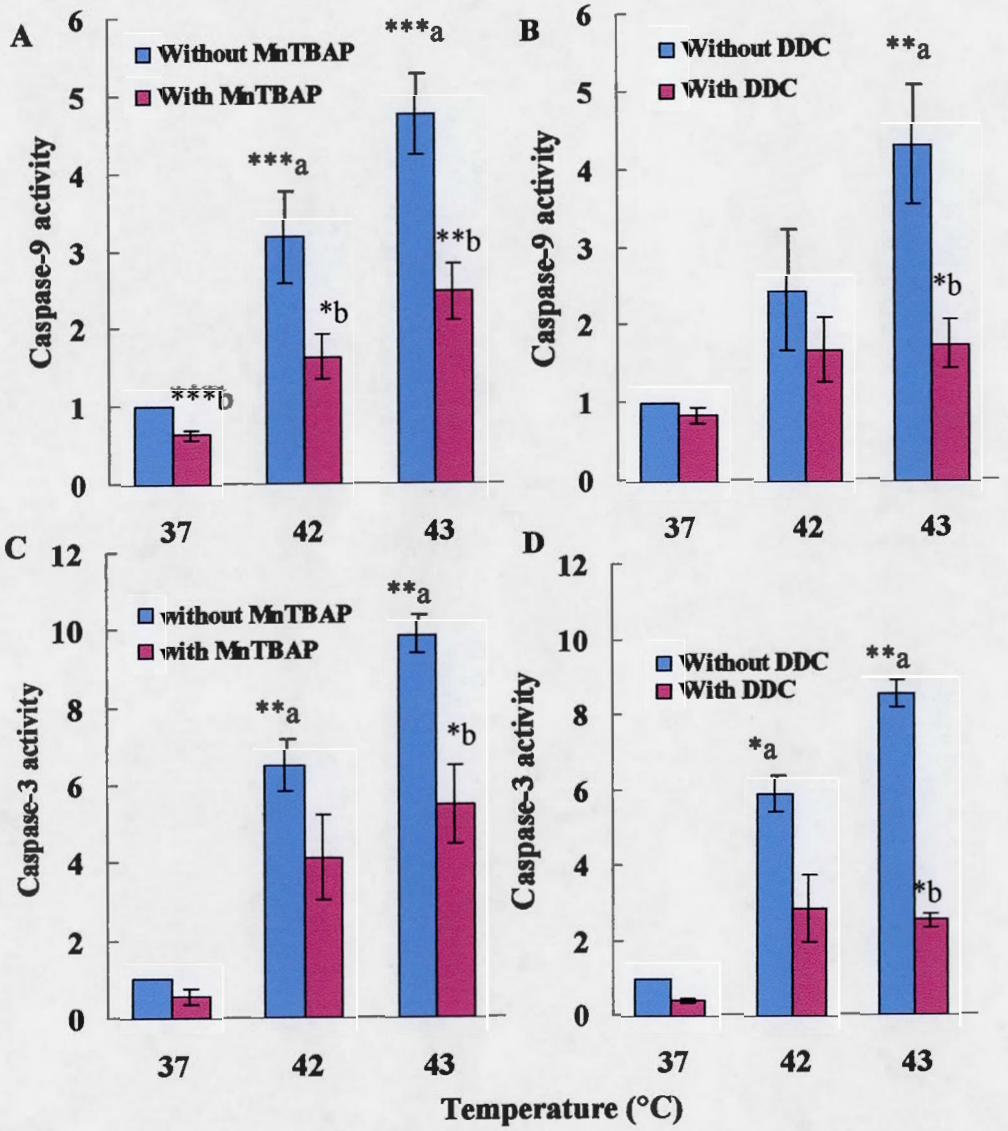


Figure 2.5: MnTBAP or DDC attenuate activation of caspase-9 and caspase-3 by heat shock. Following treatment with modulators and heat shock, caspase-9 (A, B) or caspase-3 (C, D) activity was measured in cell lysates using appropriate fluorescent substrates. Caspase-9 or caspase-3 activity was expressed relative to untreated control cells at 37°C, designated as 1. Data represent means and SEM from 6 (caspase-9) or 3 (caspase-3) independent experiments for cells pretreated with MnTBAP (A, C) or DDC (B, D). a, significantly different for heated cells from corresponding control (no modulators) at 37°C. b, significantly different for treatment with or without modulators, at 42 and 43°C. $p<0.05$ (*), $p< 0.01$ (**) or $p<0.001$ (***).

Figure 2.6

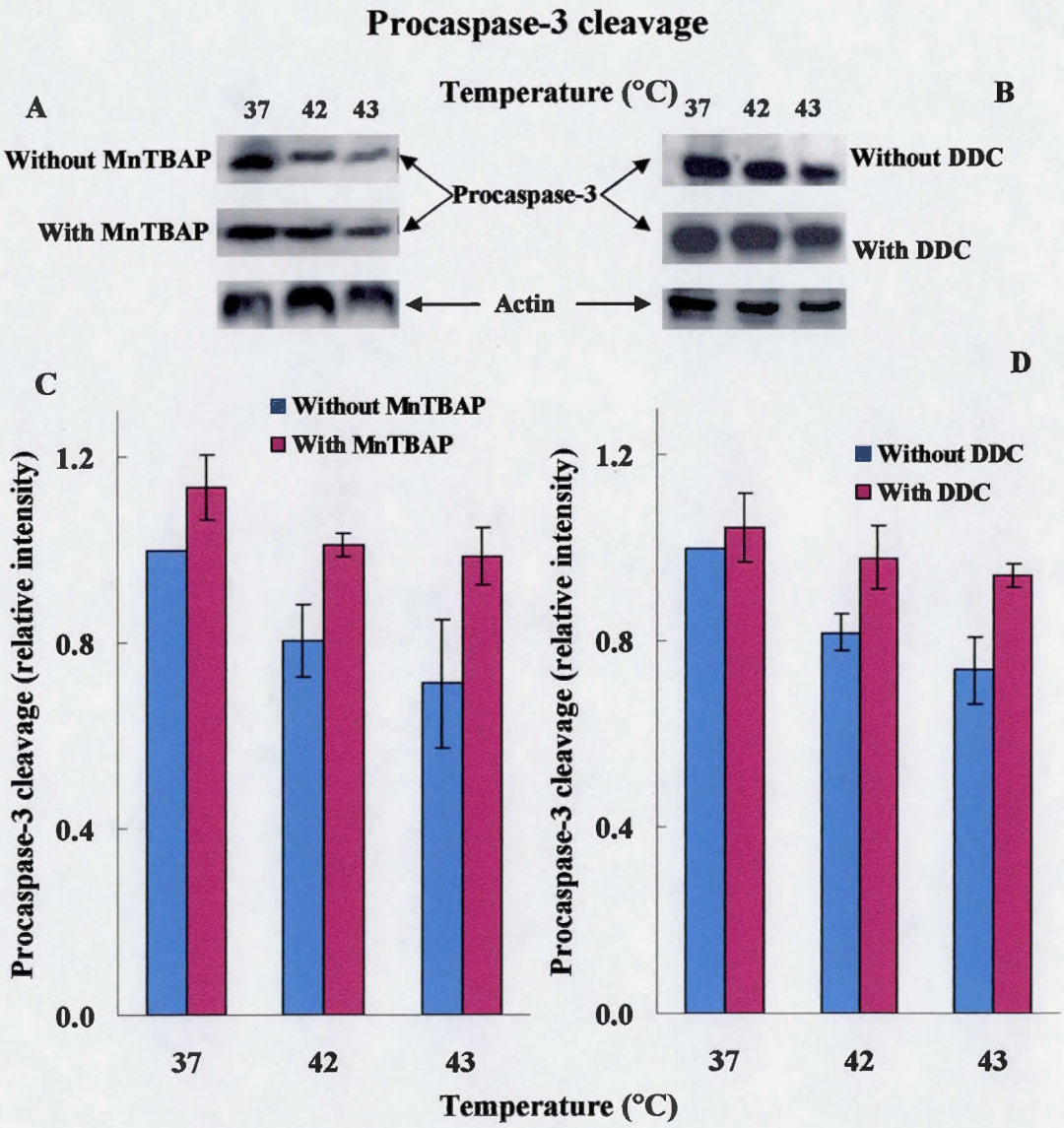


Figure 2.6: MnTBAP or DDC decrease heat shock-induced cleavage of procaspase-3. Following treatment with modulators and heat shock, immunodetection of procaspase-3 was carried out. Representative blots are shown for MnTBAP (A) or for DDC (B). Data represent means and SEM from 4 independent experiments for MnTBAP (C) or for DDC (D). Expression of procaspase-3 was relative to the untreated control cells at 37°C, designated as 1.

Figure 2.7

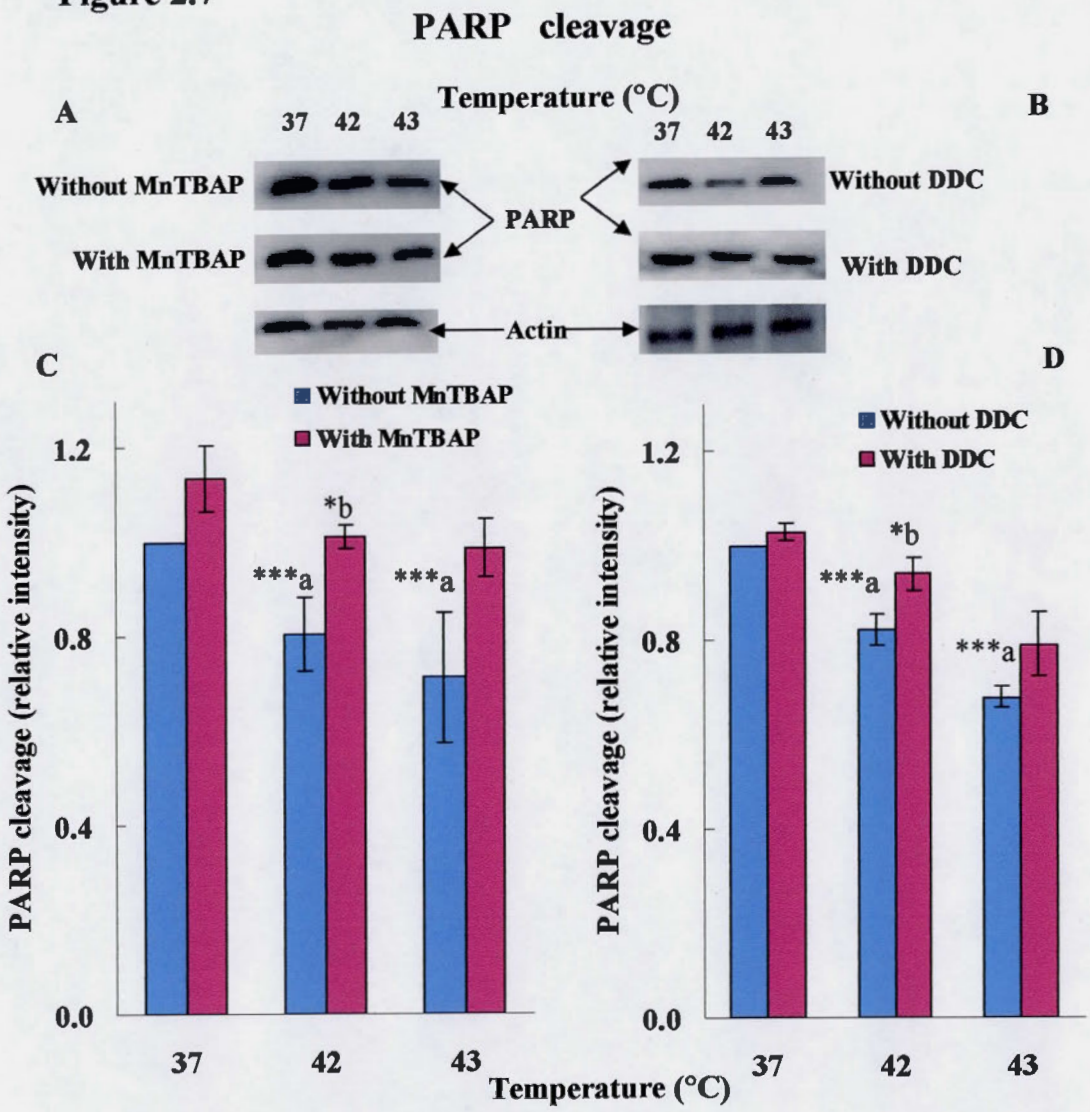


Figure 2.7: MnTBAP or DDC decrease heat shock-induced cleavage of PARP. Following treatment with modulators and heat shock, immunodetection of PARP was carried out. Representative blots are shown for MnTBAP (A) or for DDC (B). Data represent means and SEM from 4 independent experiments for MnTBAP (C) or for DDC (D). Expression of PARP was relative to the untreated control cells at 37°C, designated as 1. a, significantly different for heated cells from corresponding control (no modulators) at 37°C. b, significantly different for treatment with or without modulators, at 42 and 43°C. $p<0.05$ (*), or $p<0.01$ (**).

Figure 2.8

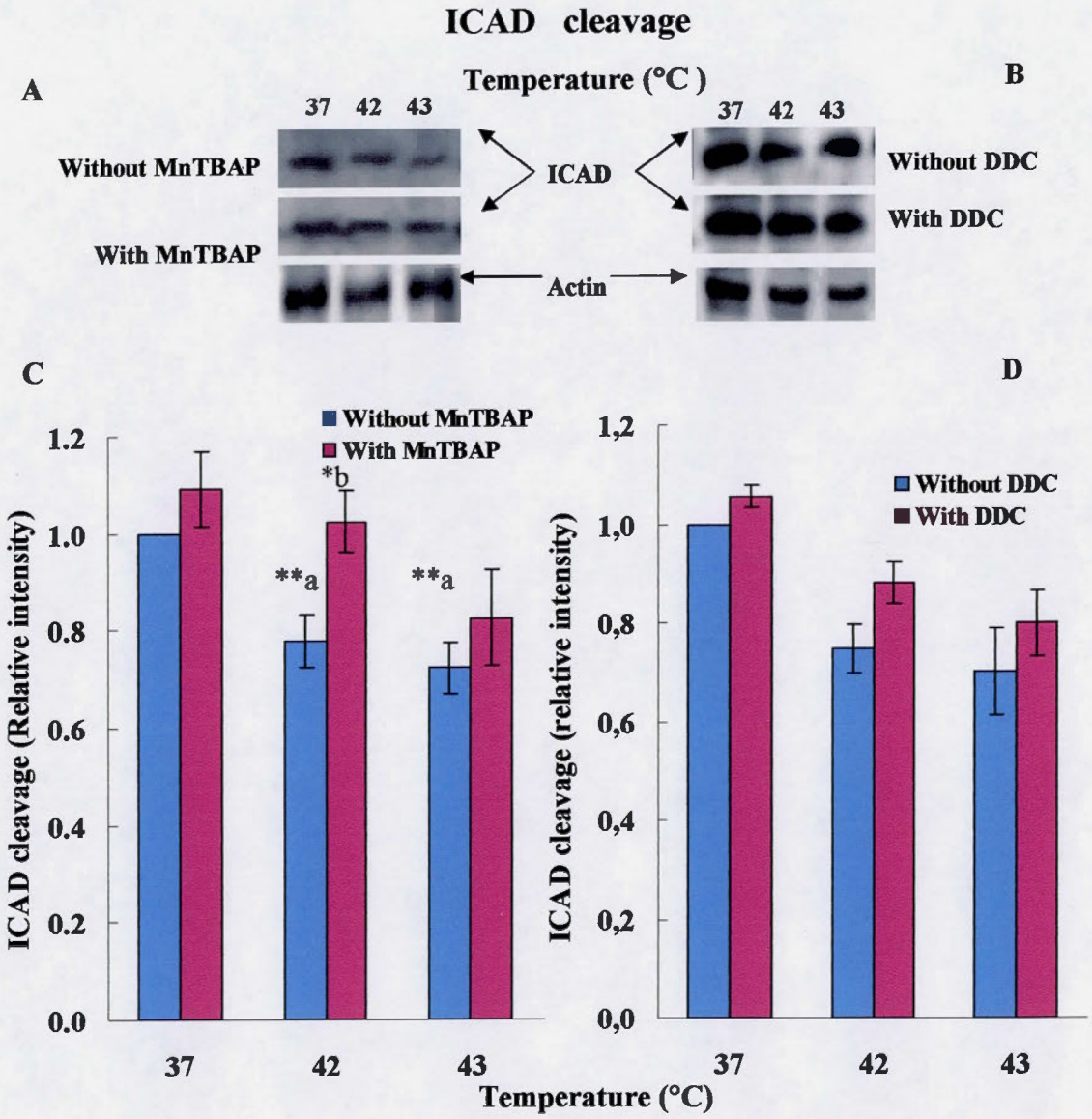


Figure 2.8: Heat shock-induced ICAD cleavage is decreased by modulators of SOD. Following treatment with modulators and heat shock, immunodetection of ICAD was carried out. Representative blots are shown from 4 independent experiments for MnTBAP (A) or for DDC (B). Data represent means and SEM from 4 independent experiments for MnTBAP (C) or for DDC (D). Expression of ICAD was relative to the untreated control cells at 37°C, designated as 1. a, significantly different for heated cells from corresponding control (no modulators) at 37°C. b, significantly different for treatment with or without modulators, at 42 and 43°C. $p<0.05$ (*), or $p<0.001$ (***).

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CHAPTER 3: CONCLUSION

Hyperthermia is a recently developed technique clinically proven to be valuable in cancer therapy. Its cell death inducing effect was considered as a promising field in cancer therapy. When hyperthermia is combined with traditional chemotherapy, it has surprising effects by damaging and killing cancer cells, leading to the shrinkage of the tumors (Dahl, 1994). The underlying mechanisms of heat shock-induced cell death usually involve inhibition of DNA, RNA and protein synthesis (Laszlo, 1992). Identification of the molecular pathways leading to cell death caused by hyperthermia will be very important in the development and clinical application of this technique.

It has been previously reported that increased levels of superoxide radical are generated in cells treated with hyperthermia (Lin *et al.,* 1991). Furthermore, heat increased the cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells (Lord-Fontaine and Averill-Bates, 1999). The antioxidant, SOD can detoxify superoxide (Fridovich, 1975) and can be inhibited by DDC (Heikkila et *al.,* 1976). Based on all these findings, the study was designed with the hypothesis that heat shock could promote an increase in oxidative stress, thus creating a redox imbalance in favour of peroxidants. This could be achieveed either by increasing generation and accumulation of pro-oxidants or by inhibiting the activity of the antioxidant defence system. To determine the role of oxidative stress in heat shock-induced apoptosis, MnTBAP, a cell-permeable SOD mimetic was used to detoxify superoxide at the intracellular level and DDC, was used as an inhibitor of SOD.

This study showed that MnTBAP could protect the cells from heat shock-induced apoptosis via the mitochondrial pathway and that DDC could induce necrosis instead of apoptosis, in CHO cells. By exploring the mitochondrial pathway of apoptosis, we determined the involvement of Bax in causing the mitochondrial membrane collapse, the liberation of some essential proteins from mitochondria, such as cytochrome c and

the consequent activation of caspases and the cleavage of PARP and ICAD in the process of DNA damage.

The project initially examined the morphological analysis of cell death by condensation of nuclear chromatin, a later event of apoptosis. A significant increase in apoptotic cell death was induced by heat shock (42 and 43°C) compared to the control at 37°C. No significant increase in necrotic cell death was observed. When cells were treated with MnTBAP before heat shock, the apoptotic cell death was significantly reduced compared to corresponding controls. These results indicate that heat shock can induce apoptosis and not necrosis in cells and that MnTBAP can protect cells from heat-shock induced apoptosis.

It is well known that apoptosis can occur by different pathways: the receptor pathway, the mitochondrial pathway and the ER mediated pathway. As the mitochondria are the major sites of ROS production in the cell, this study focused on the mitochondrial pathway.

At the post-mitochondrial level, the release of cytochrome c is an essential event for formation of the apoptosome complex composed with cytochrome c, Apaf1 and procaspase-9. The exact mechanism of cytochrome c release is not fully understood, but it is believed that the opening of the PTP and the formation of channels on the mitochondrial membrane are involved. The mechanism is that Bax can be activated upon receiving apoptotic signals resulting in outer mitochondrial membrane permeabilization. The data indicate that the translocation of Bax is involved in altering the mitochondrial membrane potential during hyperthermia (42 and 43°C). The translocation of Bax was slightly inhibited by MnTBAP at elevated temperatures compared to the corresponding control.

The mitochondrial membrane potential was altered dramatically in cells treated by heat shock. MnTBAP reduced the damage to mitochondrial membrane. The decrease of cytochrome c in mitochondria at 42°C and at 43°C was consistent with the increase in caspase-9 activity during heat shock. When MnTBAP was administered to cells, the activation of caspase-9 was reduced. These findings imply

that caspase-9 activation is essential for the later events in heat shock–induced apoptosis in CHO cells. In the MnTBAP treated cells, the activation of caspase-3 was also inhibited. These results are consistent with the morphological analysis data in which MnTBAP protects cells at each temperature (42°C and 43°C). However, caspase-3 is not the only executioner of the late phase of apoptosis. There are reports that in some cell lines, even in the absence of caspase-3, the cells still undergo apoptosis. Researchers found caspase-9 is able to cleave procaspase-7 instead of procaspase-3 and leads to the subsequent events of apoptosis (Degterev *et al.,* 2004; Slee *et al.,* 1999). When cells were treated with heat shock, the decrease of PARP and ICAD, due to cleavage by caspase-3 was observed. MnTBAP inhibits the cleavage of PARP and ICAD at 42 and 43 °C. But there are reports that caspase -7 have been also involved in the cleavage of ICAD and PARP (Germain *et al.,* 1999; Wolf *et al.,* 1999). Further research needs to be done to determine the role of caspase-3, caspase-7 and caspase-6 in the post-mitochondrial events in heat shock–induced apoptosis. The inhibitors of these caspases could be used to clarify their roles mechanism in the execution phase of apoptosis.

The second part of the study was with DDC, an inhibitor of SOD. From the hypothesis, we were expecting an increase in the apoptosis-inducing effect. Surprisingly, from the morphological analysis, DDC induced necrosis rather than apoptosis. This is probably due to stimulation of the production of superoxide by inactivation of copper-zinc superoxide dismutase by DDC (Heikkila *et al.,* 1976). The resulting higher-dose of oxidants generated eventually damage mitochondrial energetic function, causing a dramatic decrease in cellular ATP levels and an acute energetic failure (Liu *et al.,* 1996). The depletion of ATP might delay the activation of caspase and switch cell death from apoptosis to necrosis.

Data from the immunodetection of Bax suggested that the inhibitory effect of DDC is a rather early event. Bax translocation to mitochondria was lower in DDC treated cells, and the collapse of mitochondria at 42°C and 43°C was decreased. The following event, release of cytochrome c, was also inhibited at 42°C and at 43°C

compared to the corresponding control. The enzymatic activity of caspase-3 and caspase-9 were decreased in cells treated with DDC. This could be due to oxidation of the thiol active site of the caspases.

In conclusion, this study demonstrates clearly that oxidative stress has a role in hyperthermia induced apoptosis. MnTBAP, as a SOD mimetic, can protect cells from heat shock-induced apoptosis by detoxifying superoxide and by blocking the mitochondrial pathway of apoptosis involving cytochrome c release from the mitochondria and the activation of caspases in CHO cells. When combined with heat shock DDC induces necrosis rather than apoptosis in this cell line. These fundamental studies give a very promising profile of this modifier of cell death and might be useful in cancer control, cancer prevention and cancer therapy.

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