IDENTIFICATION OF ANOXIA-INDUCED EXPRESSION OF HSP70 IN *CHRYSEMYS PICTA BELLI* HEPATOCYTES

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
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I ABSTRACT

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This study examines the expression of the 70 kDa heat shock protein (HSP70) in hepatocytes from *Chrysemys picta belli* in response to various stresses, namely heat shock, anoxia, and exposure to heavy metals and peroxide. The effect of the A1 receptor agonist, N^6-cyclopentyladenosine (CPA), and antagonist, 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX), on the expression of HSP70 during heat shock and anoxia were also investigated. HSP70 mRNA and protein were detected at temperatures ranging from 28°C-48°C. Temperatures above 48°C were injurious to the cells. Both heavy metal and peroxide exposure resulted in the expression of HSP70 mRNA and protein. Importantly, 1 to 6 h anoxia induced the expression of HSP70 mRNA and protein. DPCPX blocked the expression of both HSP70 mRNA and protein during anoxia. However, CPA failed to induce the expression of HSP70 during normoxia. DPCPX did not block the expression of HSP70 during heat shock. These data also suggest that the induction of HSP70 during anoxia may be A1R mediated, indicating that the expression of HSP70 can be induced via different pathways.

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III LIST OF ABBREVIATIONS

A1R	A1-adenosine receptor
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BGG	Bovine gamma globulin
BIP	Family of HSP70 present in the endoplasmic reticulum
BSA	Bovine serum albumin
Ca^{++}	Calcium
CA1	*Calcar avis* 1 (a defined region of hippocampus)
CO_2	Carbon dioxide
$CdCl_2$	Cadmium chloride
CPA	N^6-cyclopentyladenosine
CTP	Cytidine triphophate
DAG	Diacylglygerol
DNA	Deoxy-ribonucleic acid
DnaK	Bacterial analogue of HSP70
DPCPX	8-Cyclopentyl-1, 3-dipropylxanthine
ER	Endoplasmic reticulum
Flou-3 AM	Florescence calcium indicator dye
g	Gravitational constant

GAM-AP	Goat anti rabbit antibody (conjugated with alkaline phosphates)
GroEL	Bacterial homologue of HSP60
GRP 75	Mitochondrial homologue of HSP70
GRP94	Glucose regulated protein
h	Hour
H_2O_2	Hydrogen peroxide
HPLC	High pressure liquid chromatography
HSC70	Constitutive form of heat shock protein 70
HSE	Heat shock element
HSF	Heat shock factor
HSP60	Mitochondrial heat shock protein 60
HSP70	Inducible form of heat shock protein 70
HSP90	Heat shock protein 90
HSP100	Heat shock protein 100
HSP72	Inducible isoform of heat shock protein 70
HSC73	Constitutive isoform of heat shock protein 70
IP_3	Inositol 1,4,5-triphophate
K^+	Potassium
kb	Kilobase
kg	Kilogram
kDA	Kilodalton
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate

min	Minute
ml	Milliliter
mM	Millimole
M	Molar (concentration)
Na^{++}	Sodium
NaCl	Sodium chloride
N_2	Nitrogen
°C	Degree Celsius
$\alpha^{32}P$	Radioactive isotope of phosphorus
PI	Propidium Iodide
PKC	Protein kinase C
$^{86}Rb^{+}$	Radioactive isotope of rubidium
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase- polymerase chain reaction
SDS	Sodium dodecyl sulfate
8 SPT	8-P-Sulfophenyl theophylline
TTB	Tris buffer saline
TTBS	Tris buffer saline with Tween 20
μM	Micromole

IV LIST OF FIGURES AND TABLES

V ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Les Buck, for his support and guidance throughout this project. I also would like to thank Dr. Ringuette for his continuos scientific input into this project.

I would like to thank my friend Mae for all her help during my experiments. Many thanks to Damian for providing the means to write this thesis and for all the fun in the lab, along with Himesh.

Most of all I would like to thank my mother and sister Gayathri for their unconditional love and support throughout my life, without them I would not have been nearly as far as I am now.

CHAPTER 1 INTRODUCTION

1.1 Turtles and anoxia tolerance:

Living without oxygen is a common adaptation among invertebrate species; however, among vertebrates this adaptation is extremely rare. One of these vertebrates is the Western-painted turtle, *Chrysemys picta belli*, which can survive up to 6 months of oxygen deprivation and has even been referred to as a "facultative anaerobe" (Storey and Storey, 1990).

The unique adaptations of this turtle species to survive anoxia have been well studied by various investigations when these cells were challenged with anoxia (Buck *et al.*, 1993b; Land *et al.*, 1993; Buck *et al.*, 1993a). They also showed that ATP concentrations of normoxic and anoxic cells were not significantly different from each other. A decrease in ATP production implies that ATP utilization must also decrease. To further demonstrate this acute anoxic reduction, a study using microcalorimeter showed that turtle hepatocytes underwent a 65% decrease in heat flux in response to anoxia alone, and a 95% decrease when anoxia was combined with decreased temperature (Buck *et al.*, 1993a). The large-scale reduction in ATP turnover rates and heat flux indicate that, under physiologically over-wintering conditions (anoxia at 4°C), turtle hepatocytes can undergo marked metabolic down regulation.

There are many important energy consuming cellular processes that exist during the normal physiology of cells. Several of these have been studied in the turtle hepatocytes, such as the Na^+/K^+-ATPase activity which functions to maintain the cell membrane ionic gradient, protein synthesis, and urea synthesis. In order to understand

the mechanism of anoxia tolerance in turtle hepatocytes, it is instructive to consider how these energy-consuming processes are modified during anoxia.

Under normoxic conditions, Na^+/K^+-ATPase activity consumes 6-40% of the total cellular energy budget in hepatocytes (Nobes *et al.*, 1989). Using the ouabain inhibitable fraction of $^{86}Rb^+$ transport, Buck and Hochachka (1993) demonstrated a 75% reduction in this pump's activity upon exposure to anoxia; an action which has been shown to be ATP sparing during stressful conditions.

Biosynthesis of proteins requires the hydrolysis of four ATP equivalents/peptide bonds and 1 additional ATP for amino acid transport. Therefore, in animals such as turtles, where ATP production is depressed during long-term anoxia, sustaining rates of protein synthesis would be an expensive process. To investigate this, Land et al (1993) examined the ability of hepatocytes to suppress protein synthesis and urea production during anoxia. They showed that while protein and urea synthesis utilize between 15-30% of the total ATP turnover, these values decrease to 2-8% during anoxia (Land and Hochachka, 1994). This reduction in energy consumption may act as another important ATP sparing mechanism during anoxia.

Considering the high energy demand of protein synthesis, it is of interest to determine if selective gene expression occurs during stressful conditions, as they may play vital roles in promoting anoxia tolerance. Such an occurrence has in fact been found in anoxia- or heat shock-exposed turtle hepatocytes where the expression of 5 types of protein has been shown to increase (Land and Hochachka, 1994; Hochachka *et al.*, 1996). We suspect that these upregulated proteins are stress proteins, commonly known as heat shock proteins (HSP), which have been shown to be evolutionarily conserved and present

in all organisms studied (Morimoto, 1993). Thus far, many mammalian studies have focused on the mechanisms of induction of HSPs during anoxia, while little has been investigated about their roles in anoxia tolerant models.

1.2 Heat shock proteins

Heat shock proteins are evolutionarily conserved cellular proteins that are expressed not only during heat shock, as the name implies, but also under a variety of stressors, such as, ischemia, anoxia, hypoxia, elevated Ca^{++} levels, protein kinase C stimulators and intense exercise (Kiang and Tsokos, 1998). Several types of HSP families have been identified: HSP100, HSP90, HSP70, HSP60, as well as the small HSPs. There are a number of common features across the different types of HSPs. For instance, all of these proteins have chaperoning activities, thus they recognize unfolded, misfolded and aggregated proteins and they require ATP for their chaperoning function (Xu and Lindquist, 1993; Welch, 1992). HSP90, 70, and small HSPs interact with cytoskeletal proteins and accumulate inside the nucleus upon heat shock. HSP100 and HSP70 are involved in protein degradation pathways. Each group, however, has specific characteristics, which will be outlined below.

(I) **HSP100:** With molecular weights ranging from 104 kDa to 110 kDa, these proteins are found in both prokaryotes and eukaryotes. Each member of the family possesses ATPase activity and contains at least two ATP-binding domains. These proteins are mostly heat inducible, but a constitutive form also exists (Parsell *et al.*, 1994). Evidence of HSP100s providing cytoprotection is still unclear. At least some members of the family are involved in promoting degradation of damaged proteins.

(ii) **HSP90:** These proteins have been shown to have a regulatory function associated with several protein kinases and other proteins such as steroid hormone receptors, transcription factors, actin, tubilin and calmodulin (Welch, 1992). HSP90 is also known to decrease activity of several protein kinases, transcription factors and glucocorticoid receptors. HSP90 itself is a substrate to protein kinases and seems to have ATPase activity (Legagneux *et al.*, 1991). One of the major functions of HSP90 is in regulation of the cytoskeleton, including cell shape and motility. This protein can bind to actin and tubulin, and is associated with the plasma membrane. In higher eukaryotes glucose regulated protein (GRP94) is also a member of the HSP90 family that is present in the endoplasmic reticulum (ER) and aids in protein secretion.

(iii) **HSP60:** In eukaryotes, this group of proteins is present in the matrix of the mitochondria. HSP60 has a weak K^+ dependent ATPase activity and exhibits chaperone properties. In mammals, HSP60, which is present both constitutively and induced upon stress, has been shown to be necessary for normal mitochondrial functioning (Agsteribbe *et al.*, 1993). In prokaryotes, the bacterial homologue of HSP60 is known as the groEL. Along with other chaperones, i.e., groES (HSP10) and DnaK (HSP70), groEL regulates protein folding and assembly (Gething and Sambrook, 1992; Georgopoulos and McFarland, 1993).

1.3 HSP70

HSP70 is known to be the major molecular chaperone in both eukaryotes and prokaryotes and has been shown to be present in almost all cellular compartments and organelles (Gething and Sambrook, 1992). HSP70 proteins are highly conserved with a 60-78% identity among eukaryotic cells and 40-60% identity between eukaryotic and

prokaryotic cells. In prokaryotes, the bacterial analogues of HSP70 are known as DnaK (Lindquist and Craig, 1988).

The inducers of the HSP70 gene can be divided into 3 general categories: 1) environmental stress, 2) pathophysiological stress, and 3) non-stressfull conditions (See Fig. 1) (Morimoto *et al.*, 1992). The first report of HSPs appeared in 1962; after *Drosophila* larvae salivary gland cells were exposed to 37°C for 30 min and then returned to their normal temperature at 25°C, a chromosomal puffing was observed in these cells (Ritossa *et al.*, 1962). In 1974, Tissieres and co-workers showed that this heat induced puffing was accompanied by an increase in expression of proteins with the molecular weight of 70 kDa (Tissieres *et al.*, 1974). Now it is generally accepted that genes, which are expressed during stressful conditions such as heat shock, anoxia, exposure to heavy metals, and various other stressors encode special proteins possessing cytoprotective roles. These inducible proteins can be grouped in a family of so called "heat shock proteins" (HSPs) or "stress proteins" or "molecular chaperones".

The HSP70 proteins have ubiquitous chaperoning activity, with highly conserved nucleotide binding N-terminal domain, and a relatively variable peptide binding C-terminal domain. This protein is the most widely studied of the HSPs and belongs to a multigene family. In unstressed cells, the constitutive form of this protein (HSC70 or cognate) is expressed. The major role of HSC70 in unstressed cells is to facilitate the folding of nascent proteins, preventing their aggregation, and chaperon proteins to various sites. Following heat shock or other stressors, however, HSC70 and HSP70 are involved in refolding denatured proteins (Gething and Sambrook, 1992; Welch, 1992; DeLuca-Flaherty *et al.*, 1990).

Another HSP70 isoform, the 78 kDa protein GRP78 or BIP, is present in the ER and is expressed constitutively. Its synthesis can be enhanced by heat shock, hypoxia, increased Ca^{++} levels and various other stressors (Lindquist and Craig, 1988; Gething and Sambrook, 1992; Welch, 1992). In mitochondria, the HSP70 family is represented by a 75 kDa chaperoning ATPase (GRP75) which is made in the nucleus and transported to the mitochondrial matrix where it plays an important role in processes of folding and assembly of proteins destined for mitochondria (Bhattacharyya *et al.*, 1995).

(i) HSP70 gene regulation

Under physiologically stressful conditions, such as heat shock, the inducible HSP70s are produced to repair damaged proteins. The steps involved in the induction of HSP70 are many. An element involved in the initial steps of HSP induction has been recognized to be a transcription factor known as heat shock factor (HSF). In unstressed cells, this HSF is present in the cytoplasm and the nucleus, where it is synthesized constitutively and stored in monomeric form (Perisic *et al.*, 1989). In such monomeric form, it is unable to bind to DNA. In response to stress, however, HSF trimerizes and migrates to in the nucleus (Westwood and Wu, 1993) (See Fig. 2).

Three types of HSF are known: heat shock factor 1 (HSF1), heat shock factor 2 (HSF2), and heat shock factor 3 (HSF3). All three types have similar molecular organizations: a DNA binding domain at the NH_2 terminal with hydrophobic amino acid repeats (leucine zipper), followed by amino acid repeats at the carboxyl terminal. The induction of transcription requires formation of trimers, which are depended on hydrophobic repeats located at this terminal (Rabindran *et al.*, 1991).

HSF1 is activated upon exposure to increased temperatures, heavy metals, amino acid analogues, or oxidative stress. HSF2 is activated during differentiation or development. The factors leading to HSF3 activation, however, are still under investigation (Sarge and Cullen, 1997). As shown in Fig. 2, following multiple activation processes, HSF binds to a specialized region known as the Heat Shock Element (HSE) within the promoter region of the HSP70 gene and initiates the transcription of HSP70 mRNA. This mRNA then moves to the cytoplasm where it is translated to heat shock proteins (Larsen *et al.*, 1995).

How is the DNA binding affinity of HSF regulated? Monomeric forms of HSF bind to DNA more poorly than the trimeric forms (Clos *et al.*, 1990). Therefore, induction of HSF-DNA binding involves the formation of trimers which are dependent on hydrophobic repeats located in the carboxy terminal of the HSF protein. Leucine zippers located at the COOH terminal maintain the monomeric form of the protein under normal conditions (Rabindran *et al.*, 1993).

1.4 HSP70 and stress tolerance (preconditioning)

Once an unstressed cell or organism undergoes direct exposure to an extreme stress, cellular damage or necrosis may occur. However, if the cell or organism was previously exposed to the milder form of that stress, it can become more tolerant to a subsequent extreme exposure. This is a general phenomenon for induction of tolerance or preconditioning in response to any type of stress. Thermo-tolerance was first seen in Yeast *(Saccharmyces cerevisiae)*, where the survival of cells exposed directly to a high temperature (50°C) was compared to the survival of those that were first pretreated at a milder temperature (37°C) for 30 min before exposure to 50°C. It was observed that

pretreatment improved survival at 50°C more than 1000-fold. Interestingly, pretreated groups also showed elevated levels of HSP70 (Sanchez and Lindquist, 1990).

The same phenomenon is observed in ischemic/hypoxic preconditioning. This type of preconditioning can be defined as an adaptive change occurring early after a period of brief, sublethal ischemia that confers a state of tolerance upon an organ or tissue. This can result in resistance to a subsequent more lethal ischemic injury. However, it has been difficult to formulate a definite mechanism for hypoxic/ischemic preconditioning due to the fact that the time frame for preconditioning of different organs is different.

The heart was the first organ where the participation of HSPs in protecting against ischemia was demonstrated by over-expressing HSP70 in transgenic animals. Mestril and colleagues (1995) used an embryonic heart derived cell line (H9c2) that constitutively over expressed human inducible HSP70. Ischemic-like conditions were initiated by hypoxia and glucose deprivation. HSP70 over-expression did confer resistance to the simulated ischemia. Furthermore, in the parental cell line of H9c2 cells, previous heat shock resulted in the accumulation of inducible HSP70. They concluded that HSP70 played a major role in protecting cardiac cells against ischemic injury (Mestril and Dillmann, 1995).

In 1990, Kitagawa and co-workers were the first to discover the preconditioning phenomena in brain. Gerbils subjected to global ischemia through occlusion of bilateral common carotid arteries for 5 min showed marked neuronal death of CA1 slices (a defined region of hippocampus) after 7 days of recovery. Pretreatment of the gerbils with 2 min of ischemia 1-2 days prior to the 5 min ischemic insult, protected the neurons from

cell death (10-fold increase in neuronal survival). Kirino et al (1991) extended this study and showed accumulation of HSP70 in the gerbil CA1 region after 2 min of ischemia. Kitagawa (1990) confirmed and further studied this hypoxic/ischemic induction of HSP70. These studies concluded that HSP70 is induced in the brain as a result of ischemia/hypoxia, and its expression might be necessary for preconditioning (Kirino *et al.*, 1991; Kitagawa *et al.*, 1990; Aoki *et al.*, 1993).

In the rat liver, researchers observed that short term ischemia (15 min) increased HSP70 expression after 48 h and afforded significant protection of hepatic function and survival following a longer ischemic insult (Kume *et al.*, 1996). Furthermore, Saad and coworkers (1995) studied the effect of heat pretreatment on ischemic tolerance in rat liver, by first exposing rats to whole-body heat shock (42°C, 15 min) which induced transcription and translation of HSP70. Two days later, *in situ* livers were subjected to warm ischemia for 30 min and subsequent reperfusion for 40 min and little damage was observed (Saad *et al.*, 1995). This was similar to the results observed in the HSP70 over-expressing heart model.

HSP70 has been shown to play a significant role in the mechanism of preconditioning in various organs. However, what triggers the production of HSP70 at the time of stress is under much debate. Recent studies have shown that the endogenous neuromodulator adenosine is an important mediator of preconditioning (Rudolphi *et al.*, 1992a; Sweeney, 1997). Thus, in understanding different mechanisms of preconditioning and anoxia tolerance, it is important to investigate the relationship between the presence of adenosine and HSP70 production.

1.5 Effects of adenosine on preconditioning

Adenosine may be best characterized as a homeostatic cell signal, which tends to maintain the cellular resting state and to counteract functional or metabolic activation. Its physiological and metabolic functions include vasodilation, increased glycolysis, and decreased neurotransmitter release and neuronal excitability. It is well established that adenosine plays a protective role in the mammalian brain during the initial period of energy deficit caused by hypoxia or ischemia (Rudolphi *et al.*, 1992a; Sweeney, 1997). This protection, however, is only temporary, as after 2-3 min of anoxia the mammalian brain starts to fail. This is in contrast to the western painted turtle brain where more than 24 h of anoxia can be tolerated at room temperature (Lutz and Kabler, 1997; Bickler and Buck, 1998). Adenosine which is released during anoxia has been recognized as an important candidate in providing such protection (Nilson *et al.*, 1992; Lutz and Kabler, 1997). The exact mechanism involved, however, is still unclear.

(i) Function of Adenosine Receptors:

The A1 and A2 receptors are both G-protein coupled. The A1 receptor is coupled to a pertussis toxin sensitive Gi-protein and can act through effectors other than adenylate cyclase, including potassium channels, voltage sensitive Ca^{++}-channels (decrease N- and Q-channels activities), phospholipase A2 or C, and guanylcyclase (Olsson *et al.*, 1978; Fredholm, 1997). A2 receptors are associated with Gs-proteins and therefore ligand binding and activation of these receptors cause the activation of adenylate cyclase and perhaps also activation of some types of voltage sensitive Ca^{++}-channels, especially the L-channel (Fredholm, 1997). Thus, A1 and A2 receptors have partly opposing actions at

the cellular level. This is of interest since these two types of receptor are co-expressed in cells such as hepatocytes.

In different *in vivo* and *in vitro* models of ischemia, blockage of adenosine receptors have been shown to increase ischemic damage, whereas the reinforcement of adenosine action by adenosine receptor agonists conferred neuroprotection. For instance, in rat cerebral ischemic preconditioning, activation of the A1 receptor was shown to provide extensive protection during subsequent lethal ischemia (Heurteaux *et al.*, 1995). Similarly, in hippocampal slices, transient perfusion with adenosine analogues prior to an anoxic insult significantly improved recovery of evoked potentials, this indicative of survival. This type of protection was blocked in the presence of an A1-receptor antagonist, DPCPX. Similar results have been found in other systems. For instance, adenosine was shown to decrease myocardial oxygen demand by causing negative inotropy and chronotropy and by increasing oxygen supply through coronary vasodilation. This was shown by perfusing the heart with an adenosine analogue, which produced a protection equivalent to that induced by ischemic preconditioning. This protection was abolished in response to a non-selective adenosine receptor antagonist, 8SPT (8-p-sulfophenyl theophylline) (Liu *et al.*, 1991).

1.6 HSP70 and Adenosine

The important role of adenosine in ischemic preconditioning has been established. HSP70 has also been shown to have protective effects during stressful conditions such as ischemia. These findings suggest that a relationship may exist between the presence of adenosine and its possible mediation of HSP70 production. This forms the focus of this project.

We hypothesize that:

1) There is an increase in heat shock protein levels in turtle hepatocytes during anoxia and it is an important contributor to long term anoxia tolerance in this model.

2) Adenosine receptor agonists and antagonists modulate the expression of HSP70.

Specific Aims:

1) To characterized the baseline HSP70 levels in *Chrysemys picta belli* hepatocytes.

2) To determine whether the constitutively high levels of HSP70 contributes to protection against anoxia, or whether, HSP70 is induced during anoxia.

3) To understand the regulatory role of adenosine in the induction of HSP70.

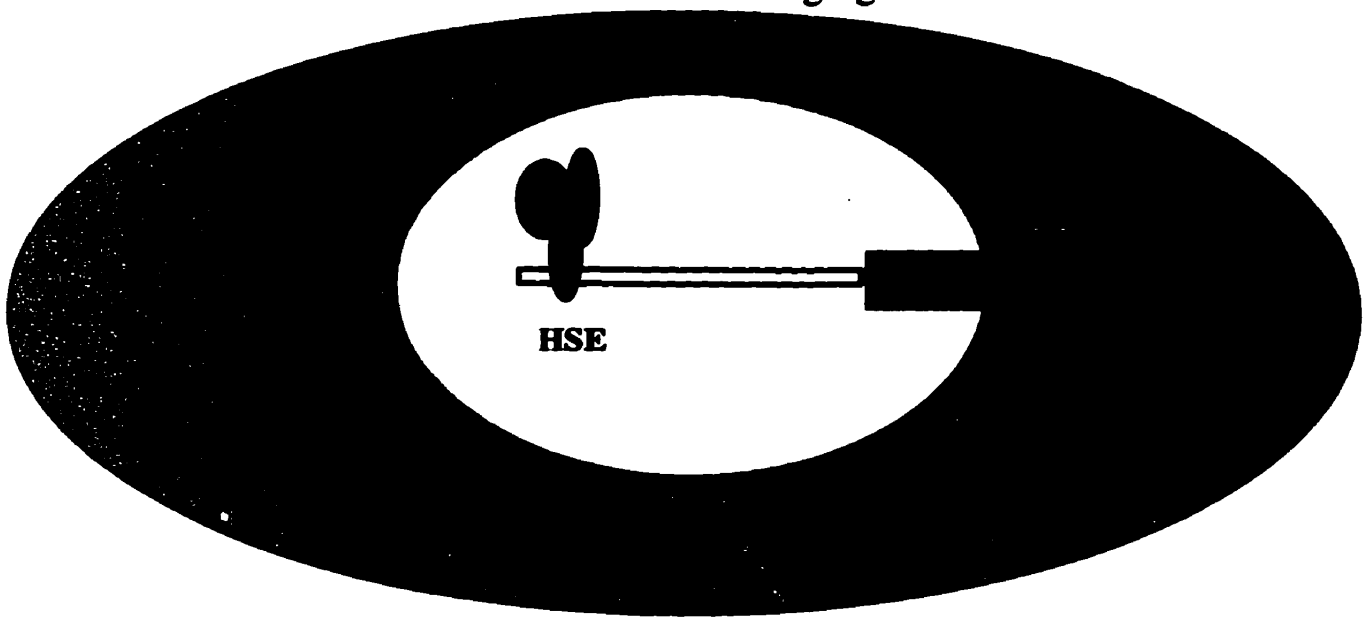
Fig. 1. Cellular stress response: Representation of three classes of conditions that induce the expression of HSP70 including (i) environmental stresses, (ii) patho-physiological stresses, and (iii) non-stress full conditions such as growth and development (Morimoto *et al.*,1993).

ENVIRONMENTAL STRESS

Heat shock
Inhibitors of energy metabolism
Exposure to heavy metals

PATHOPHYSIOLOGICAL STRESS

Fever and inflammation
Hypertrophy
Ischemia
Viral and bacterial infection
Aging



NON-STRESSFUL CONDITIONS

Cell cycle
Growth factors
Development and differentiation
Oncogenesis and proto-oncogenesis

Fig. 2. Proposed mechanism of stress induced increase in HSP70

In the unstressed cell, the HSF is maintained in a non-DNA binding form. Upon heat shock or other forms of stress, HSFs assemble into trimers and bind to a specific sequence in the heat shock gene promoter. Transcriptional activation of heat shock gene lead to increased levels of HSP70. Finally, the HSF dissociates from the DNA and is eventually converted to a non-DNA binding monomer.

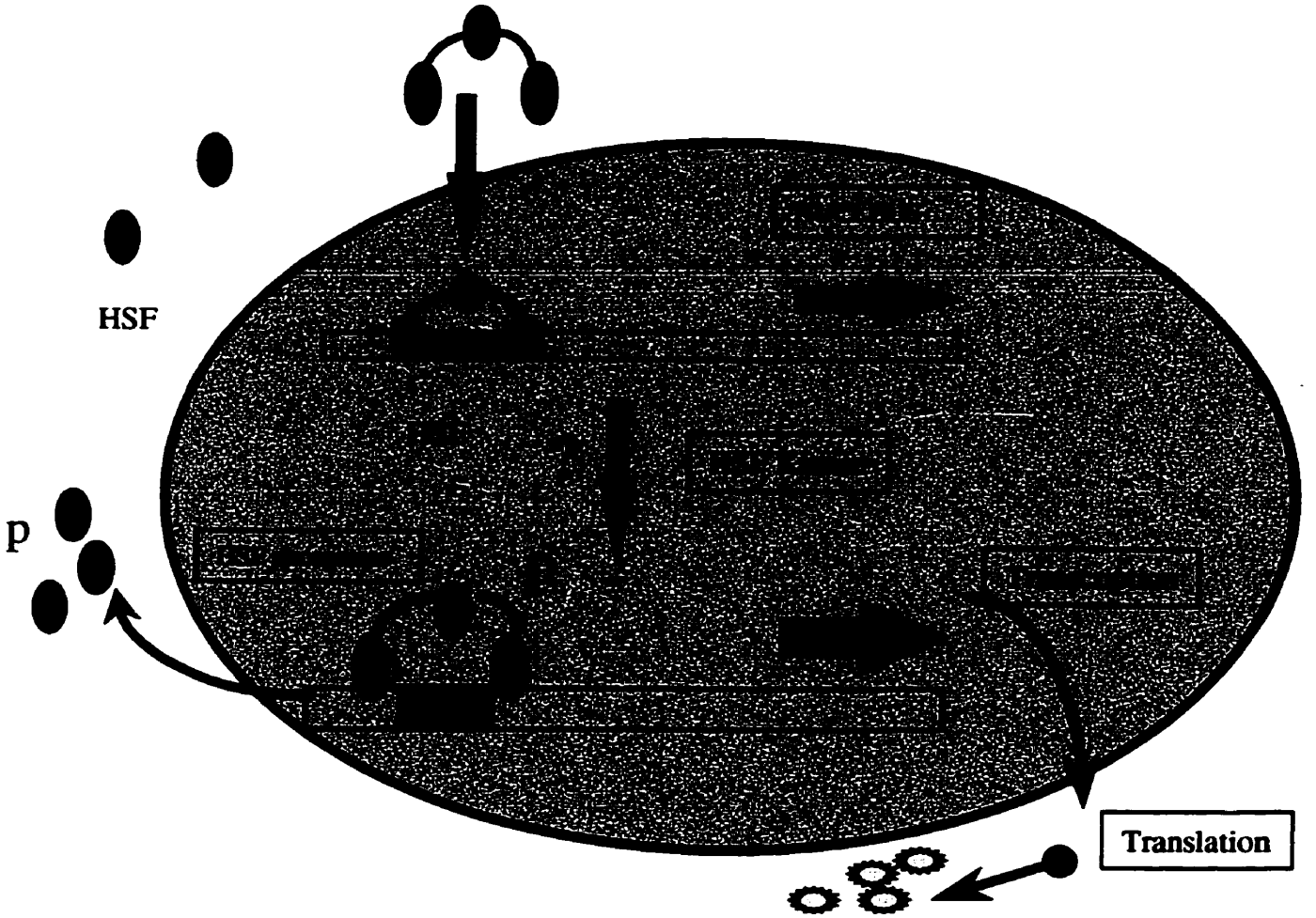


Diagram modified from Morimoto et al 1992

CHAPTER 2 METHODS

2.1 Hepatocyte Isolation

Female turtles (*Chrysemys picta bellii*) weighing between 250-750 gram were obtained from WM.A. Lemberger Co., Inc. Oshkosh, WI and maintained in aquaria at 15-20°C with a flow through dechlorinated water system.

Animals were killed by an intraperitonial injection of sodium pentobarbital (200 mg. kg^{-1} body weight). Following a 20 min period the plastron was quickly removed with an electric bone saw to expose two large abdominal veins. These vessels were quickly cannulated and the liver was perfused at a rate of 3 ml min^{-1} at 20°C with a medium containing 1.4 mM Na_2HPO_4, 2.2 mM KCl, 78.5 mM NaCl, 10 mM Na HEPES and 34.5 mM $NaHCO_3$ at pH 7.5 (Solution A). The initial perfusion continued for 25 min. Following cannulation the liver was dissected from the animal and placed floating in a petri dish containing Solution A. The liver was then perfused for 13 min with Solution B (Solution A with 2 Units.ml^{-1} Sigma Protease XXIV). Following perfusion the liver was minced with a razor blade in ice cold Solution C (Solution A with 2% bovine serum albumin, BSA) and the resulting slurry was poured successively through two nylon filters, a 253 μm filter and then a 73 μm filter. All centrifugations and subsequent steps were carried out at 4°C. The cell suspension was centrifuged for 4 min at 50 x g; the resulting pellet was resuspended in Solution C and centrifuged a second time for 2 min at 50 x g. The pellet was then suspended in Solution D (Solution A with 0.1 mM $MgSO_4$, 3.8 mM $MgCl_2$, 5.8 mM $CaCl_2$ and 4% BSA) and centrifuged at 50 x g for 2 min. The resulting cells were suspended in Solution D, allowed to settle in a flat-

bottomed culture flask and stored at 4°C for a maximum of 24 h. The composition of the liver perfusion media was based on previously measured plasma ion concentrations in turtle (Jackson *et al.*, 1984). All experiments reported here represent independent hepatocyte preparations.

2.2 Anoxic and normoxic experiments

Two 50 ml aliquots of solution D (without BSA) were vigorously gassed, one with 95% air/5% CO_2 (normoxic) and the other with 95% N_2/5%CO_2 (anoxic) for 1 h prior to the experiments. Then 0.5 M sodium cyanide (physiological mimic of anoxic metabolism) was added to the anoxic solution. Hepatocytes were removed from storage and allowed to warm up to room temperature. Supernatants were decanted and the cells were resuspended in either of the above two solutions. Aliquots of cell suspension (20 ml each) were placed in 4 Erlenmeyer flasks and fitted with rubber caps. A short piece of tubing was inserted through the rubber cap and anoxic or normoxic gas mixtures were perfused over the cells. A 25-gauge syringe needle was inserted through the rubber cap to allow the gas to escape. The flasks were placed on a shaker. Experiments were conducted over an 8 h time course (see Buck *et al.*, 1993b).

Serial 0.5 ml samples of cell suspension were taken at 0, 1, 2, 3, 4, 6 and 8 h time (at 23°C) from anoxic/normoxic incubations to measure adenylate concentrations as described below. Serial samples (2 x 1 ml) were taken at 0, 1, 2, 3, 4, 6 and 8 h (at 23°C) for Northern blot and Western blot analyses. Furthermore, an anoxic experiment was performed at 4°C and samples (2 x 1 ml) were taken at 0 and 1 h for RNA and protein isolation. RNA and proteins were extracted from these samples as described below.

Hepatocyte samples were weighed (25 mg/ml) cells were used unless otherwise specified) and resuspended in solution D (without BSA) as described above and placed in Erlenmeyer flasks for one of the following experiments. Controls were maintained at 23°C (room temperature) for 1 h.

2.3 Hyperthermic treatments

For heat shock experiments, a 40 ml hepatocyte sample was divided equally among 10 Erlenmeyer flasks and then heated by immersion of flasks in a water bath for 1 h. A circulating heating unit maintained a constant temperature, at 26, 28, 30, 33, 37, 40, 44, 48 or 50°C for 1 h. Samples (2 x 1 ml) were taken from each flask for Northern and Western analysis. All samples were prepared in duplicates.

2.4 Exposure to Hydrogen peroxide and Cadmium Chloride

A 10ml sample of hepatocyte was divided among 5 Erlenmeyer flasks. Flask 1 and 2 contained hydrogen peroxide at 0.5 M or 0.05 M respectively; flasks 3 and 4 contained Cadmium Chloride at 0.2 M or 0.5 M and flask 5 contained normoxic control. Flasks were placed on a shaker for two hours. Serial (2 x 1 ml) samples were taken from each flask for Northern blot analysis. All samples were prepared in duplicates. Cadmium Chloride and hydrogen peroxide were obtained from Sigma, Scarborough, ON, Canada.

2.5.a. Anoxic and normoxic adenosine experiments

To study the affect of the adenosine antagonist/agonist on the expression of HSP70 during anoxia, hepatocytes were pretreated with either DPCPX or CPA.

(i) CPA experiments

A 40 ml hepatocyte sample was divided among 4 flasks. Flasks 1and 2 contained 100 nM CPA and flasks 3 and 4 contained 1uM CPA. Flasks 1 and 3 were subjected to

anoxic treatments and flasks 2 and 4 were subjected to normoxic treatments. Samples were (2 x 1 ml) taken at 0 and 1 h for RNA and protein analyses.

(ii) DPCPX experiments

A 40 ml hepatocyte sample was divided among 4 flasks. Flasks 1 and 2 contained 100 nM DPCPX and flasks 3 and 4 contained 1uM DPCPX. Flasks 1 and 3 were subjected to anoxic treatments and flasks 2 and 4 were subjected to normoxic treatments. Samples (2 x 1 ml) were taken at 0 and 1 h for RNA and protein analyses.

2.5.b Effect of adenosine A1 receptor activation on HSP70 production during heat shock

To study the effect of DPCPX and CPA on the expression of HSP70 during heat shock, hepatocyte samples were prepared as described above and subjected to heat shock (40°C, 1 h). After 1 h, samples (2 x 1 ml) were subjected to Northern and Western blot analysis. N^6-cyclopentyladenosine and 8-cyclopentyl-1, 3-dipropylxanthine was obtained from Sigma, Scarborough, ON, Canada

2.6 Cell viability

Cell viability was tested following each experimental treatment, i.e., heat shock, free radical exposure, anoxic and adenosine agonist antagonist experiments.

(i) Cell viability assessment using viability indicator dye

To assess cell viability Trypan blue exclusion was used. Two volumes of hepatocyte suspension (during different experimental treatments) were incubated for 5 min with 1 volume of 0.6% Trypan blue and an aliquot counted for staining in a Neubauer Chamber. Red blood cell and melanocyte contamination was also determined at this time and was always less than 1%. Cell death was expressed as a percentage of

total number of cells present. Nonviable cells were also labeled using fluorescent nuclear indicator Propidium Iodide (PI). Cells were incubated in 1ug/ml PI solution for 5 min and an aliquot counted for staining using confocal microscopy (BioRad, MRC 600 Laser Scanning microscope).

In order to assess the functional state of hepatocytes during each experimental treatment, 1 ml of cells was incubated for 2.5 h with 3 ug/ml of calcium indicator stain Flou-3 AM (#F2341, Molecular probes). Ca^{++} indicator fluorescence was detected using confocal microscopy. To ensure that the fluorescence detected is Ca^{++}-depended Flou-3 loaded cells were incubated in (30 uM) Ca^{++} ionophore A23187 (#F2341, Molecular probes).

(ii) Measuring ATP levels using High Pressure Liquid Chromatography- reverse phased (HPLC)

Adenylate samples were obtained by injecting a 450 μL aliquot of cells into an eppendorf tube containing 50 μL of 70% Perchloric acid maintained at -5°C in an ice-saltwater slurry and sonicated for 20 seconds. The resulting material was pelleted by centrifugation in a refrigerated micro-centrifuge at 4°C for 10 min at 10,000 x g. The pellet was discarded and the supernatant fraction neutralized with 0.4 M Tris buffer, 3 M KOH and 0.3 M KCl. A 100 μL sample was taken for adenylate determination by high performance liquid chromatography. To separate these nucleotides, a reverse phase column (150 x 4.6 mm) (Supelco Model LC-18-T, Scarborough, ON, Canada) was used. For mobile phase, 2 buffer solutions were used: Buffer A (0.1M monophosphate monobasic ammonium phosphate (Sigma, ON, Canada) in deionized water and Buffer B (70% Buffer A: 30% Methanol). Buffer solutions were degassed with Helium prior to

use. The gradient elution protocol consisted of an initial perfusion with 100% Buffer A, at 5min Buffer B gradient perfusion began, with the transition to 100% Buffer B occurring at 13 min and lasting to 17 min. At this time the perfusion was switched back to Buffer A (100% at 18 min) and perfused an additional 7 min. Adenylate peaks were eluted at 1.5 ml/min and a variable wavelength ultraviolet-visual spectrophotometer operating at 254 nm was used for detection. The adenylate peak areas were quantified by comparison to known peak areas of ATP, ADP, AMP, and adenosine standards.

2.7 Isolation of total RNA and Northern analysis

Total hepatocyte RNA was isolated using the Trizol method (Gibco BRL). Extracted RNA was routinely checked for quality and degradation. Total RNA was quantified using a spectrophotometer as shown in Table1 (Sambrook et al., 1989). For Northern blot analysis 10 μg of total RNA was separated on a 1.5% agarose gel for 2.5 h at 85 Volts, tranferred to a NYTRAN plus membrane (S&S NYTRAN PLUS, Schleicher & Schuell New Hampshire, USA). Hybridization was performed overnight at 50°C with [α^{32}P] dCTP-labeled complementary DNA probe specific for mouse HSP70 (Gift from Dr. M. Locke, University of Toronto, Canada). After hybridization filters were washed 4 x 30 min with 2 x SSC plus 0.1% SDS at 55°C, for 2 x 20 min with 1x SSC plus 0.1% SDS at 55°C and visualized by auto radiography (BioMax autoradiographic film; Kodak). **Data were normalized to the signal for α-tubulin (Sambrook et al., 1989).**

2.8 Isolation of protein and Western blot analysis

Western blot analyses were done essentially according to Locke and Tanguay (1996). For protein extraction, isolated hepatocytes were homogenized in 10 mM Tris-HCl (pH 7.4) buffer centrifuged at 10,000 g for 10 seconds and supernatant was

transferred to a fresh tube. Protein concentrations were determined using BCA kit (BCA protein assay kit, product #23225, PIERCE, USA) using BSA and BGG as standards. Equal amounts of protein were loaded onto each well of the SDS-polyacrylamide (10%) gel and separated electrophoretically. Where appropriate pre stained molecular weight markers such as 133 kDA BSA (BioRad. USA) was electrophoresed. Proteins were electro-blotted on to nitrocellulose membrane (NitroBind, Westborough, MA USA), using a BioRad. mini trans-blot electrophoretic transfer unit (Towbin *et al.*, 1979). Proteins were blocked for 1.5 h in 5% non- fat dry skim milk in Tween 20-Tris-buffer (TTBS) containing 20 mM Tris, pH 7.5, 500 mM, NaCl and 0.05% Tween 20 (Johnson and Cohen, 1984). The blots were then incubated for 1 h with rabbit polyclonal antibodies against HSP72 (1:10,000) or HSC73 (1:1,000) (Stress Gen, Canada) and washed 2 x 5 min with TTBS. Following the washes, the blot was reacted with affinity purified goat anti rabbit conjugated with alkaline phosphates GAM-AP (1:3000) (BioRad. USA) for 1 h, washed with TTBS and TBS and visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 70% dimethylformamide.

2.9 Statistical Analyses

For viability assay and protein analyses results are expressed as means with their corresponding standard errors of means. To examine the quantity of protein present under each experimental condition, all scanned images were recalled on the screen. An appropriate box size was selected to surround one section of the scanned blot lane and the intensity of the area was calculated using NIH image version 1.55 software (NTIS, USA). Using the same box, measurements for other lanes were subsequently taken. The average areas from each was band then used in comparisons within and between treatments.

Student Minitab and SPSS were used for performing the student's t-test and ANOVA followed by a Tukey's post-hoc analysis (a=0.05).

CHAPTER 3 RESULTS

3.1 Cell viability.

(i) **ATP measurements:** ATP concentrations (Fig. 3) were measured from cells incubated for 8 h under normoxic or anoxic conditions. There was no significant decrease in the concentration of ATP during this time in either group. Moreover, pre-incubation of a separate anoxic group in DPCPX (1uM) did not alter the ATP levels. Cell viability was further confirmed using the viability dyes Trypan blue, Propidium Iodide and the Ca^{++} indicator Fluo-3 AM.

(ii) **Trypan blue staining:** In order to measure cell death, hepatocytes were sampled after 1, 2, 3, 4, 5, 6 and 8 h of anoxic treatment and stained with Trypan blue. Under anoxic conditions labeled cells comprised less than 12% of the total number of cells in each sample (Fig. 4). When cells were heat shocked at temperatures above 50°C, a significant increase in Trypan blue staining was observed ($p<0.05$) (Fig. 5).

(iii) **Propidium Iodide staining:** as Trypan blue labelled cells were difficult to detect using phase contrast microscopy, the fluorescent dye Propidium Iodide (PI), which labels cells with compromised membranes, was also used to label dead cells. Fig. 6a shows that the isolation of cells by itself did not cause extensive cell death, cells were sampled as described above and stained with PI. PI labelling of freshly prepared hepatocytes demonstrated more than 92% cell survival.

Hepatocytes exposed to 1 h anoxia continued to maintain high cellular viability (see Fig. 6b). As seen in Fig. 4, there were low levels of PI labeling in such populations indicating high cell survival. Cells exposed to heat shock (up to 50°C) showed high rates

of survival (see Fig. 6c). Temperatures above 50°C, however, showed a marked increase in cell death as indicated by PI labeling (Fig. 6d). Therefore, all the heat shock experiments were performed at temperatures below 50°C.

(iv) Flou-3 AM: Additionally, in order to confirm the functional state of hepatocytes under each experimental condition, the Ca^{++} indicator Fluo-3 AM was used. Fluo-3 AM, which is non-fluorescent unless hydrolyzed by esterases inside the cells, was loaded into freshly isolated hepatocytes (Fig. 7a and 7b). More than 95% of the cells showed fluorescent labelling. To ensure that the fluorescence detected was Ca^{++} dependent, Fluo-3 loaded cells were exposed to the calcium ionophore A23187. This resulted in a marked increase in the fluorescence detected from the hepatocytes within 50 seconds of exposure to the ionophore (Fig. 8).

3.2 Control experiments

(i) Liver Tissue vs. Isolated hepatocytes

To determine if hepatocyte isolation by itself induced HSP70 production, Western analysis was employed to test the expression of HSP70 in freshly prepared cells. Using antibodies against mammalian HSP70 and HSC70 it was found that HSC70 was present in equal intensities in both liver tissue and hepatocytes while HSP70 was not detectable in either sample (Fig. 9a).

(ii) Incubation of hepatocytes at 4°C for 24 h

Expression of HSP70 and HSC70 in hepatocytes was tested at 4°C (Fig. 9b). No accumulation of HSP70 was found. This provided the advantage that the same population of isolated hepatocytes could be used in various experiments over the period of several days.

3.3 Positive Control Experiments

In mammals, the induction of stress proteins occurs upon exposure to a variety of stressors, including heat shock, heavy metals and oxygen radicals. Therefore, as a positive control, the effect of heat shock, Cadmium Chloride, and Hydrogen Peroxide on the induction of HSP70 were examined in isolated turtle hepatocytes.

(i) Heat shock experiments

In heat shock experiments, turtle hepatocytes were exposed to various temperatures, and in each experiment, HSP70 mRNA and protein levels were examined using Northern blot and Western blot analysis respectively. While incubation of hepatocytes at 23°C did not result in detectable HSP70 mRNA, exposure to 30°C for 1 h resulted in an accumulation of HSP70 mRNA. When the cells were exposed to 33°C, 37°C, 44°C and 48°C the intensity of HSP70 mRNA was similar to the induction seen at 30°C (Fig. 10). At this point, it was important to determine the temperature where the induction of HSP70 started. Thus, using mammalian HSP70 it was determined that HSP70 expression began at 28°C. Furthermore, using Western analysis, the presence of HSP70 at 33°C, 37°C, 44°C was also confirmed (Fig. 11).

(ii) Exposure to Cadmium Chloride and hydrogen peroxide

Exposure to Cadmium Chloride and hydrogen peroxide also resulted in the induction of HSP70. Upon exposure to 0.2 M or 0.5 M $CdCl_2$ (Fig. 12a) and 0.05 M or 0.5 M H_2O_2 induction of HSP70 at mRNA levels was detected (Fig. 12b). These results are in accordance with those from mammalian studies (Curtis *et al.*, 1996).

3.3 Effect of anoxia on the expression of HSP70

Using Northern blot analysis, the effect of anoxia at 0, 1, 2, 4, 6, 8 h on the expression of HSP70 mRNA in turtle hepatocytes was examined (Fig. 13). As in all other Northern analyses the total extracted RNA was first quantified using its absorbance at 260nm and then the purity was calculated using spectrophotometer according to Sambrook *et al.* (1989) (see Table 1). In addition, when the samples were separated on a 1.5% agarose gel, 28s and 18s ribosomal RNA bands were clearly visible. These indicate that equal amounts of RNA were loaded in each lane and that the loaded RNA was of high quality, with no detectable degradation. Using a mammalian probe for HSP70, Northern hybridization was performed to determine the effect of anoxia on the expression of HSP70 mRNA in turtle hepatocytes. Fig. 13 shows that in the control groups (normoxic, t=0 h, at 23°C) HSP70 mRNA expression was not detectable. Exposure to 1-6 h anoxia lead to the expression of HSP70 mRNA close to 2.4 kb (n=3). However, at 8 h this expression decreased significantly (see Fig. 23). In order to confirm these results at the protein level, Western analysis was also performed (see Fig. 14).

Thus far, all anoxic experiments were performed at 23°C. However, since turtles over-winter at colder temperatures it was important to determine the expression of HSP70 at physiological over-wintering conditions, i.e., anoxia at 4°C (Fig. 14). Anoxic treatment of hepatocytes at 4°C induced HSP70 and at the same temperature the expression of HSP70 was not detected in the control groups (4°C, normoxia).

3.4 HSC70 and Anoxia

To investigate the presence of HSC70 during anoxia, Western blot analysis was performed on samples that were exposed to different periods of anoxia. Using mammalian HSP70 antibody it was found that HSC70 was present in all the anoxic treatments and the intensity of the band was similar to the control groups (Fig. 15b).

3.5 Effect of adenosine on HSP70 expression

To determine if the expression of HSP70 is affected by adenosine agonists and antagonists, hepatocytes were incubated with 100 nM and 1μM CPA and 100 nM and 1 μM DPCPX and subjected to normoxic and anoxic treatments.

(i) The effect of DPCPX and CPA during anoxia

Under normoxic conditions, neither 1μM CPA nor 1μM DPCPX elicited an HSP70 response as detected by Northern blot analysis (Fig. 16). The band intensity was similar to control (1 h anoxia). Cells that were pretreated with 100 nM or 1μM DPCPX showed a significant decrease in the levels of expression of HSP70 mRNA (see Fig. 24). These results were confirmed by Western blot analysis (Fig. 17). Cells pre-treated with 1μM CPA and subjected to anoxia showed no significant change in the expression of HSP70. This expression was similar to the one observed in anoxic 1 h treatment. This indicated that CPA had no effect on the expression of HSP70 during anoxia. This was in contrast to cells that were pretreated with 1μM DPCPX, which showed a decrease in the expression of HSP70.

(ii) The effect of DPCPX and CPA during heat shock

To investigate whether the observed effect of DPCPX on the expression of HSP70 is repeated under other stress conditions, e.g., heat shock, hepatocytes were pretreated

with 1 μM DPCPX and 1 μM CPA and exposed to heat shock at 40°C. Proteins were subsequently analyzed using Western analysis. Neither DPCPX nor CPA affected the observed expression of HSP70 during heat shock (see Fig. 18).

Fig. 3. Evaluating cell viability using ATP concentrations: ATP concentrations of normoxic, anoxic, DPCPX + anoxic treated hepatocytes. Data points represent means ± S.E.M of 3 hepatocyte preparations. Hepatocytes were stored for no longer than 24 h before experiment.

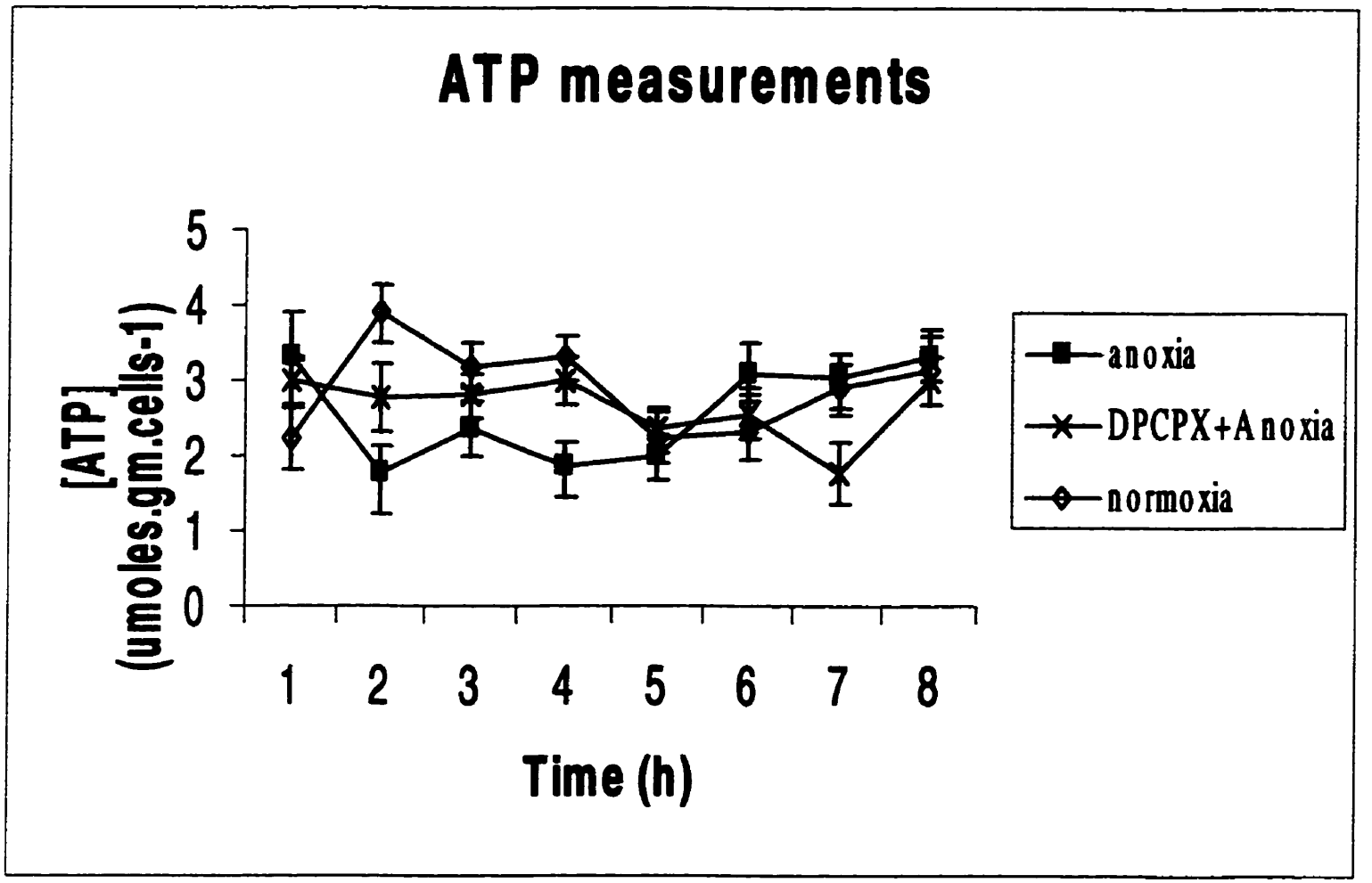

ATP measurements
[ATP] (umoles.gm.cells-1)
0
1
2
3
4
5
1
2
3
4
5
6
7
8
Time (h)
anoxia
DPCPX+Anoxia
normoxia

Fig. 4. Evaluating cell viability using Trypan blue staining: Trypan blue exclusion of 8 h normoxic, anoxic, and DPCPX treated hepatocytes (25°C). Values are means ± S.E.M; Percentage values are given as total stained cells/total cells (n =5).

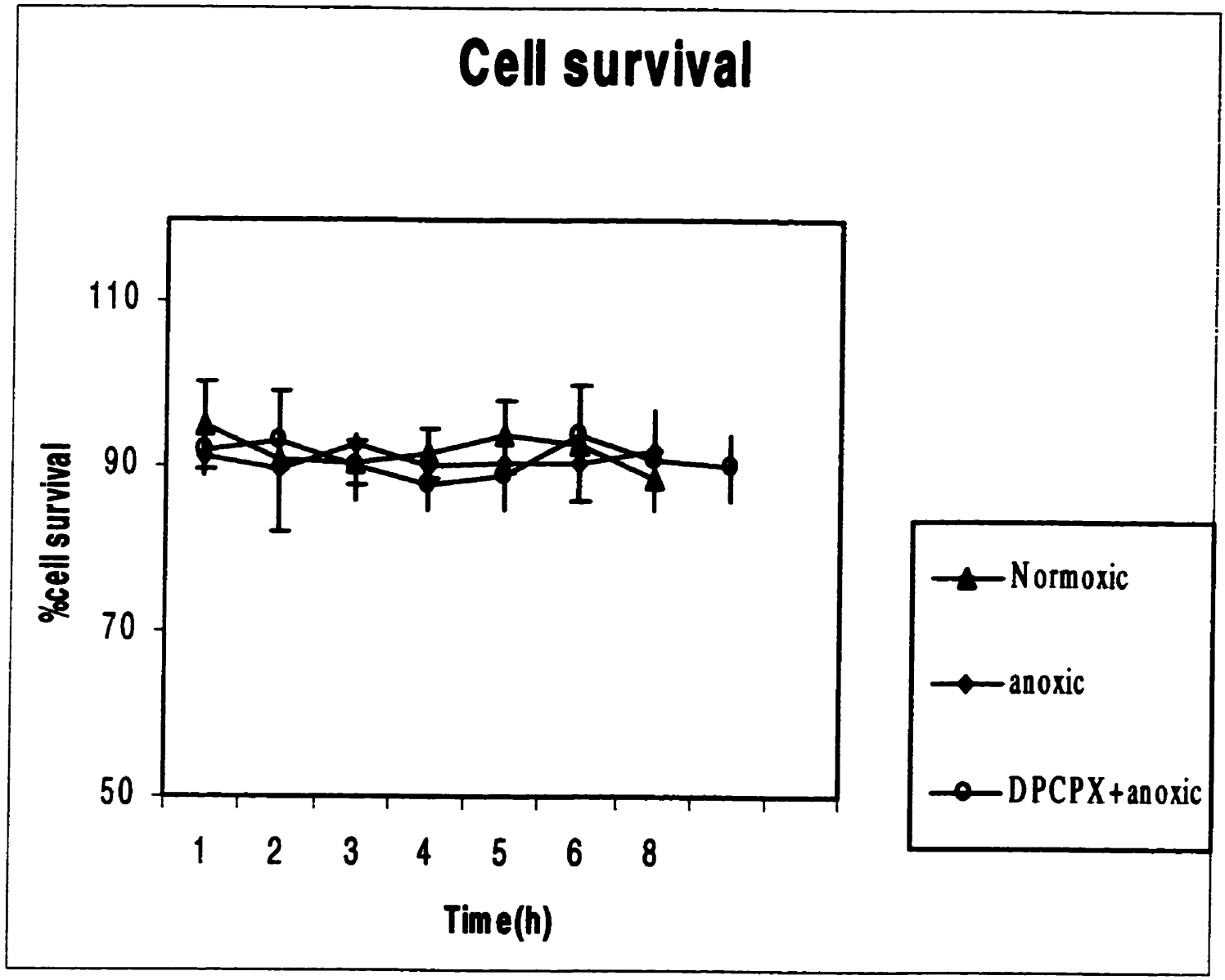

Cell survival
110
90
70
50
%cell survival
1
2
3
4
5
6
8
Time(h)
Normoxic
anoxic
DPCPX+anoxic

Fig. 5. Assessment of cell viability following heat shock using Trypan blue staining. Values are means ± SE; Number of independent preparation in parentheses. Percentage values are given as total stained cells/total cells (n = 5). Percent cell survival at 55°C was significantly different from the rest of the groups ($p<0.05$). Statistical significance are indicated by an asterisk (*).

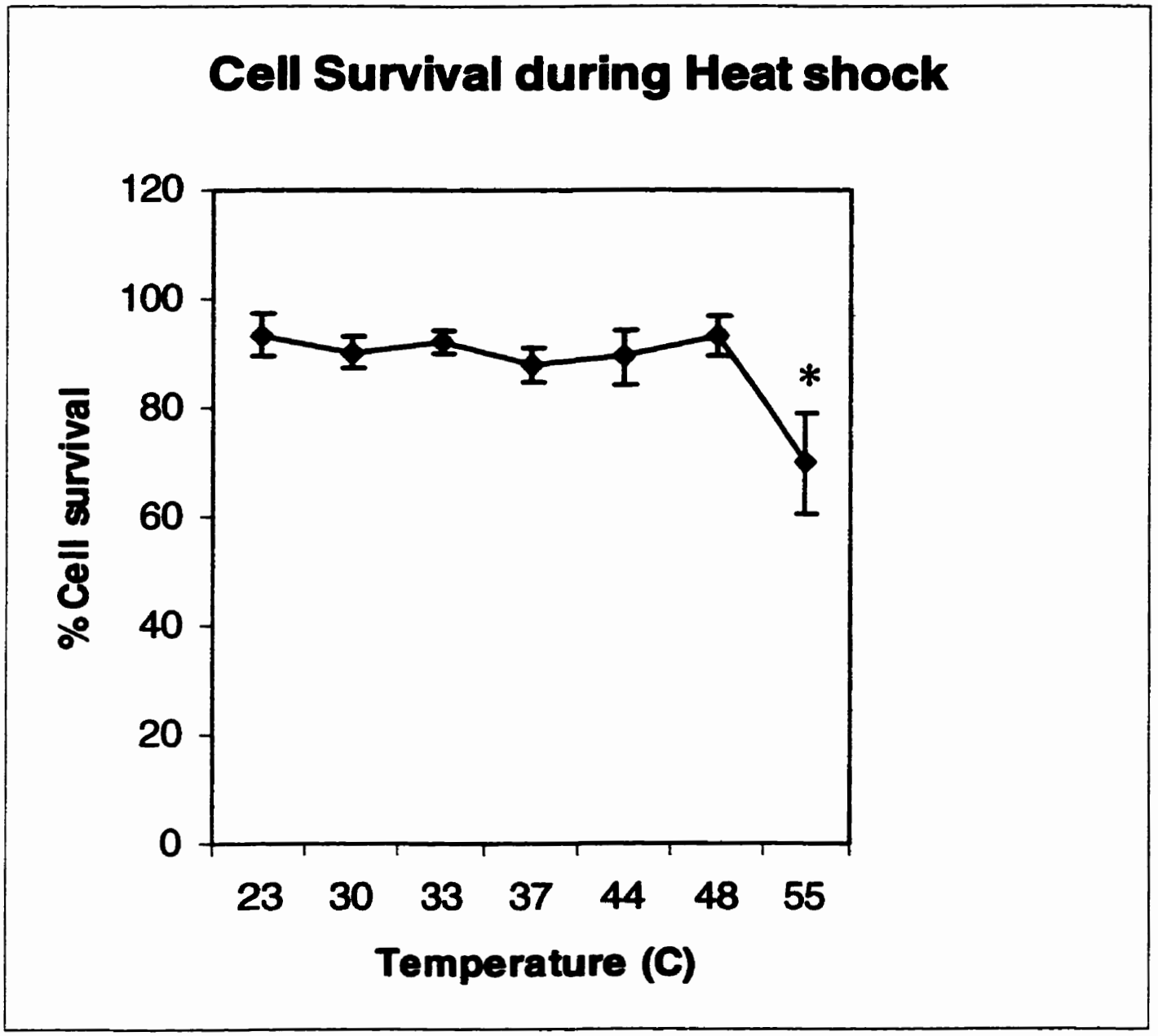
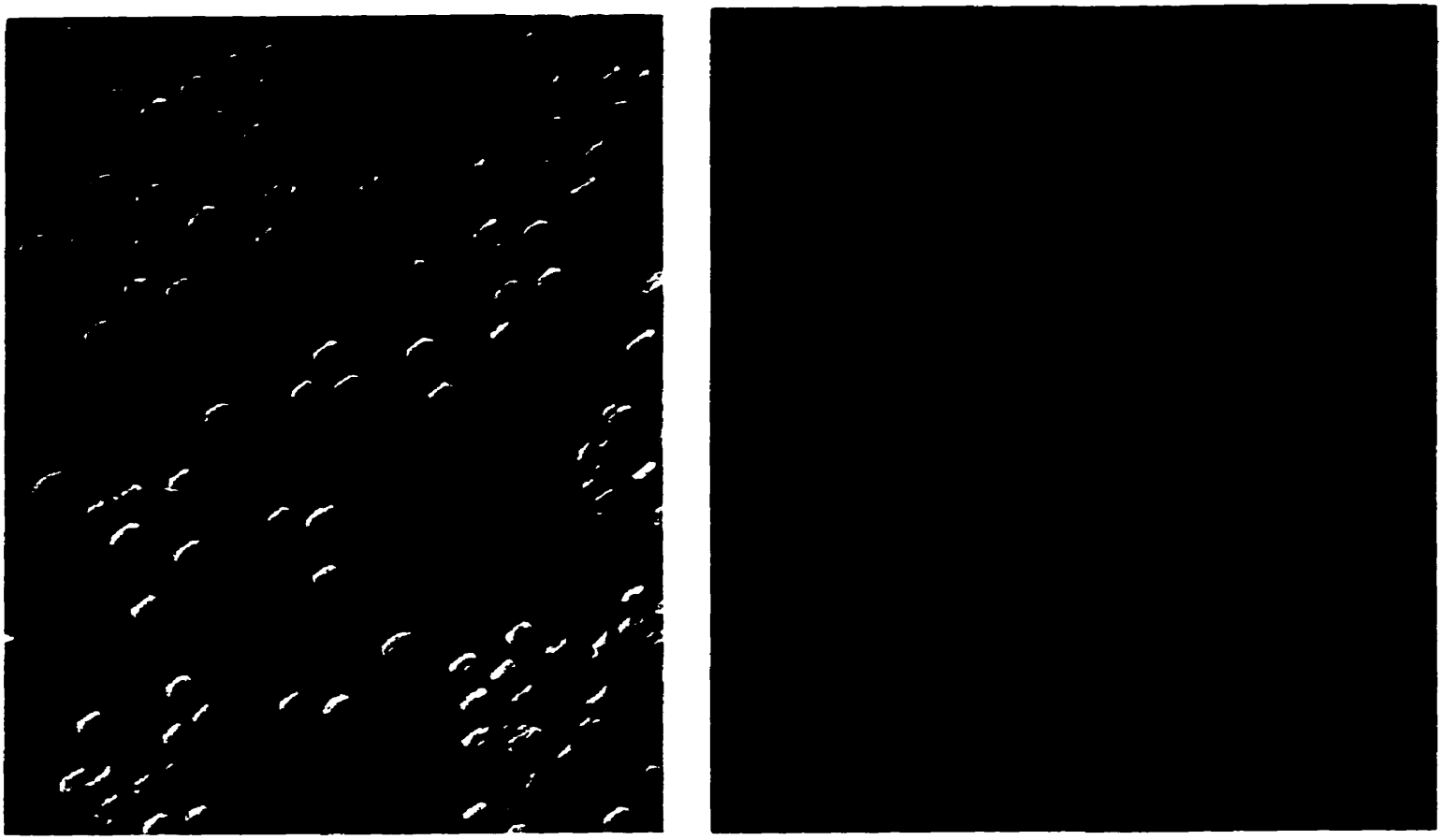

Cell Survival during Heat shock
120
100
80
60
40
20
0
% Cell survival
*
23
30
33
37
44
48
55
Temperature (C)

Fig. 6. Assessment of cell viability using Propidium Iodide (PI): (A); PI labeling of isolated hepatocytes (see text). (B); PI labeling of hepatocytes exposed to 1 h anoxia. (C); PI labeling of hepatocytes exposed to heat shock at 50°C. (D); PI labeling of hepatocytes exposed to heat shock at 55°C.

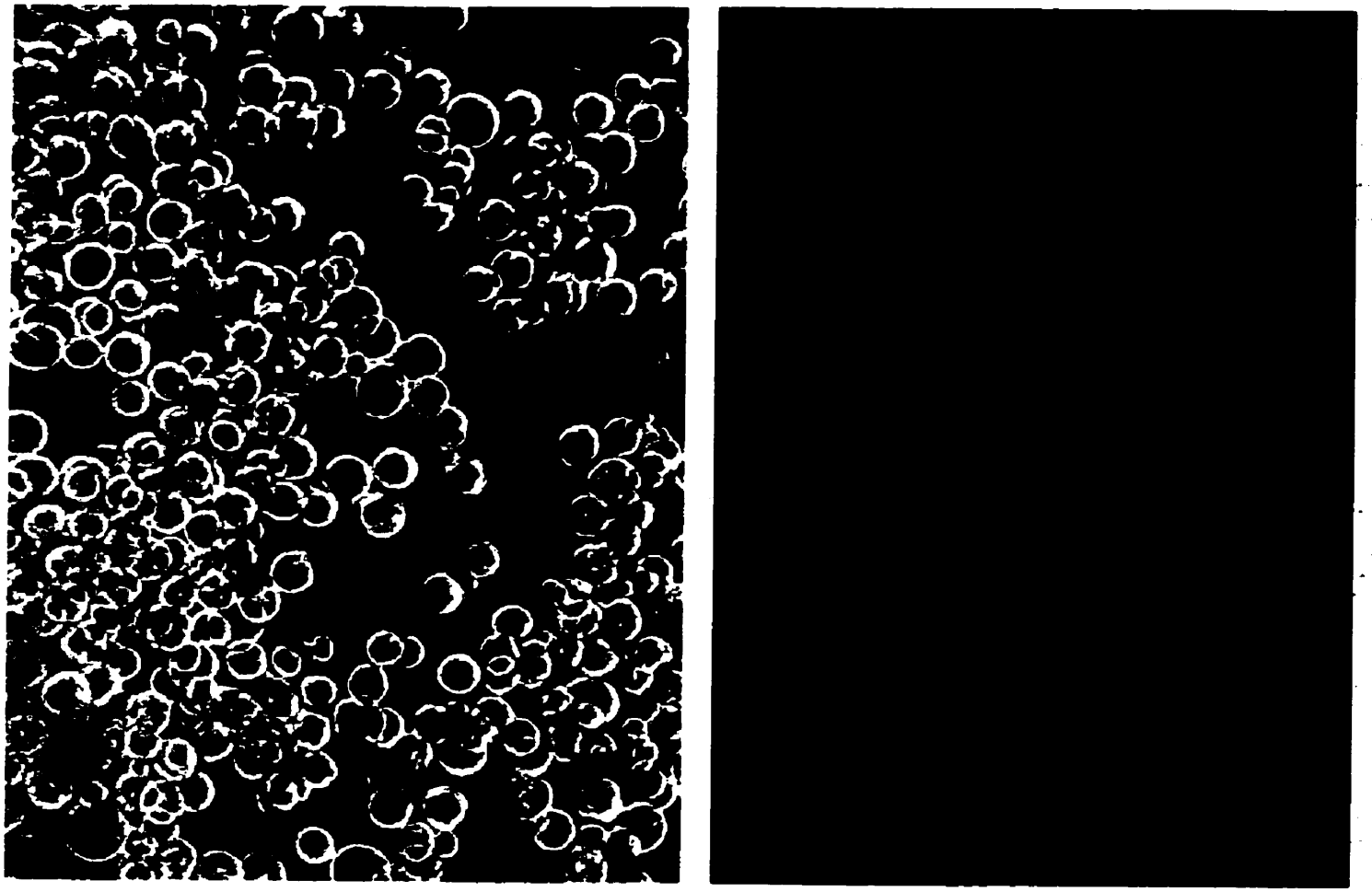
Phase **Fluorescent**



A

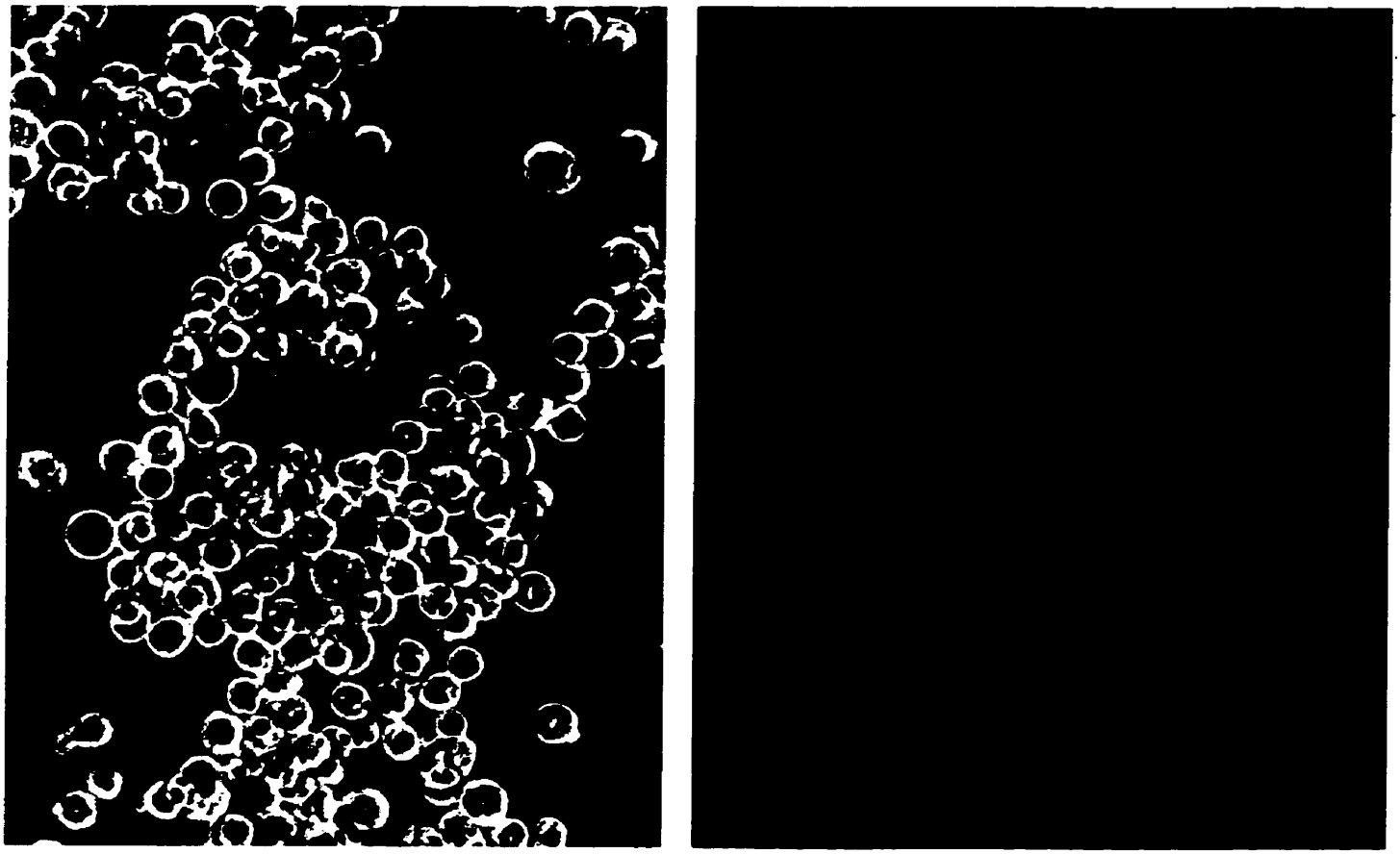
Phase

Fluorescent



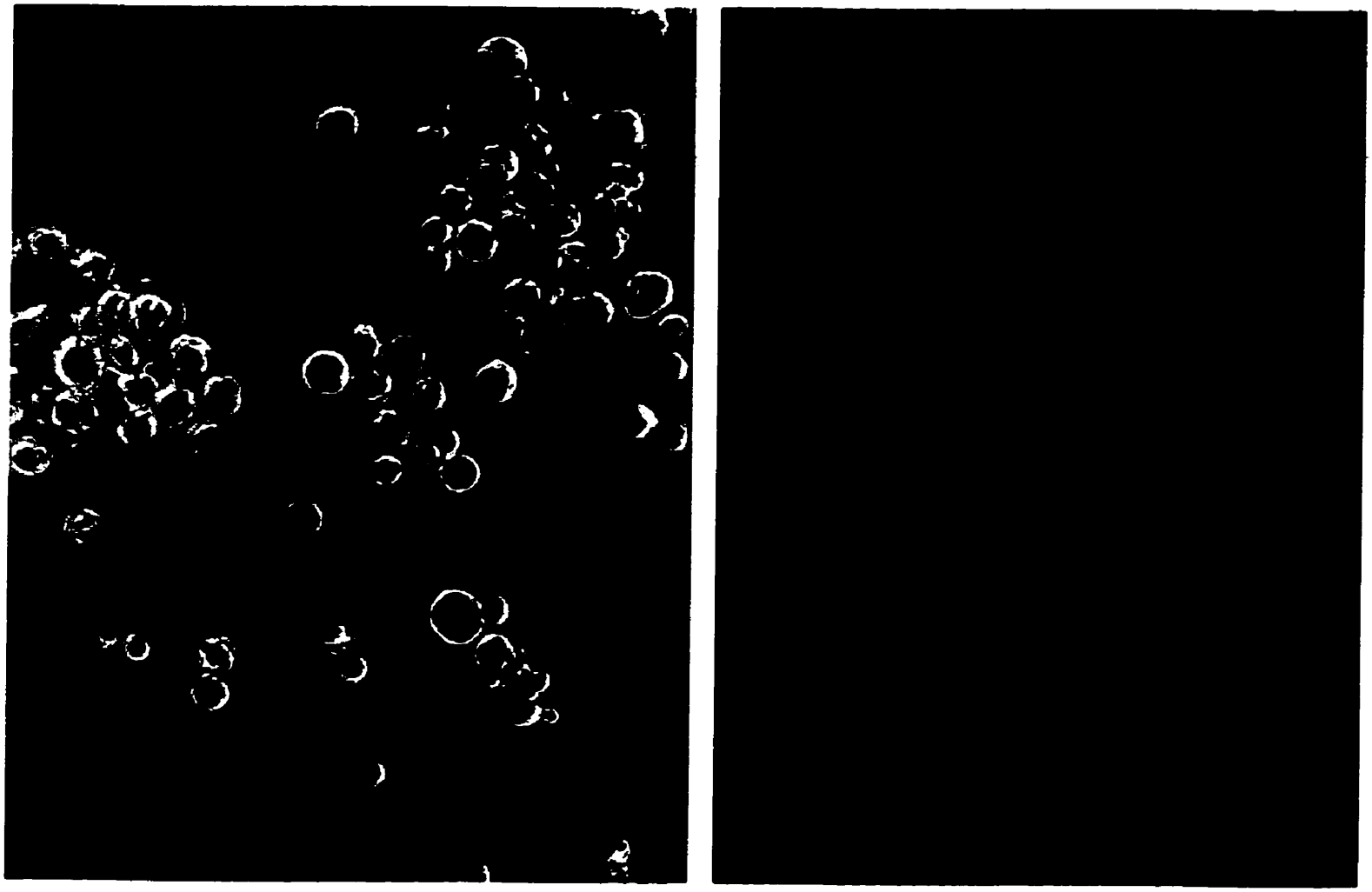
B

Phase **Fluorescent**



C

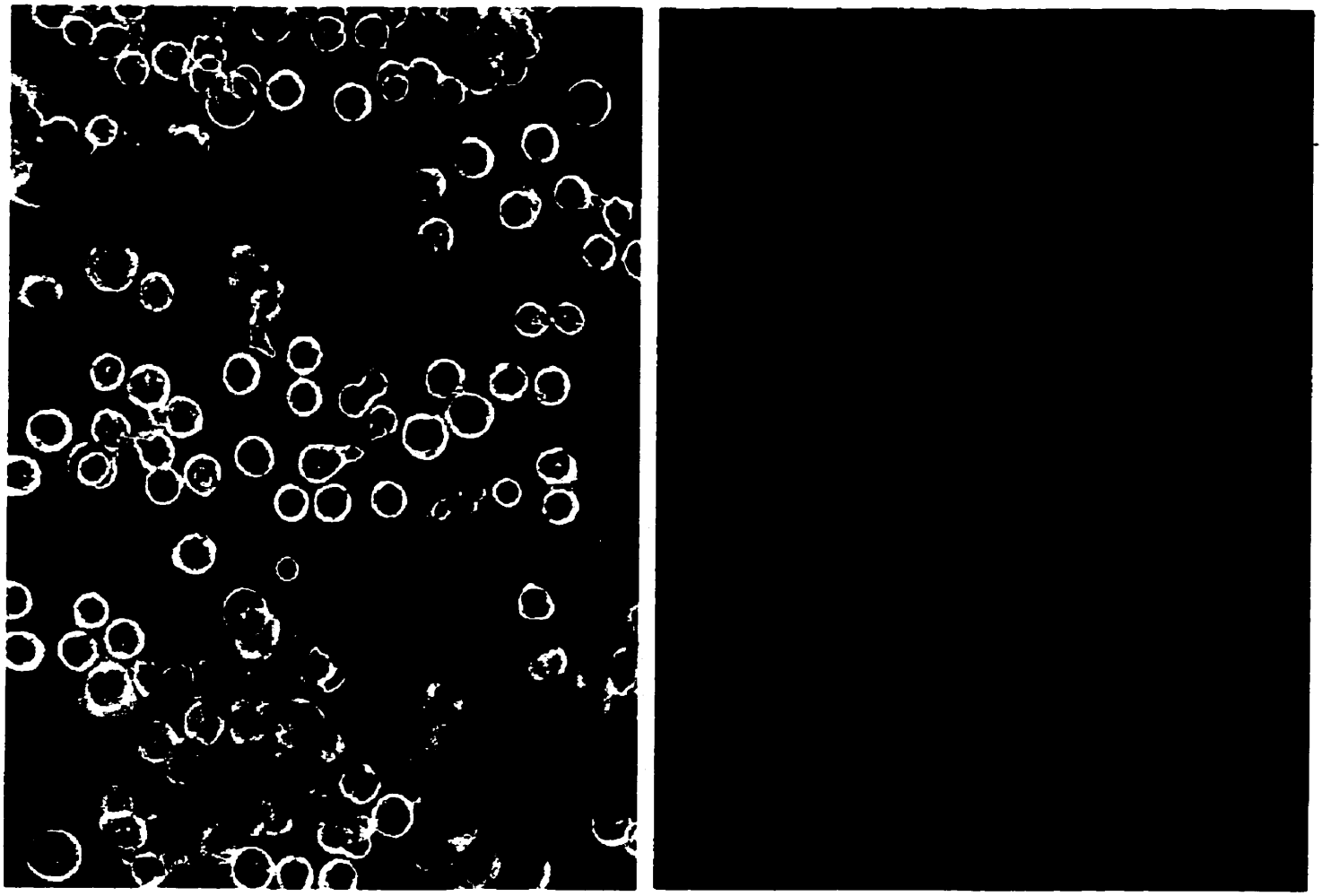
Phase **Fluorescent**



D

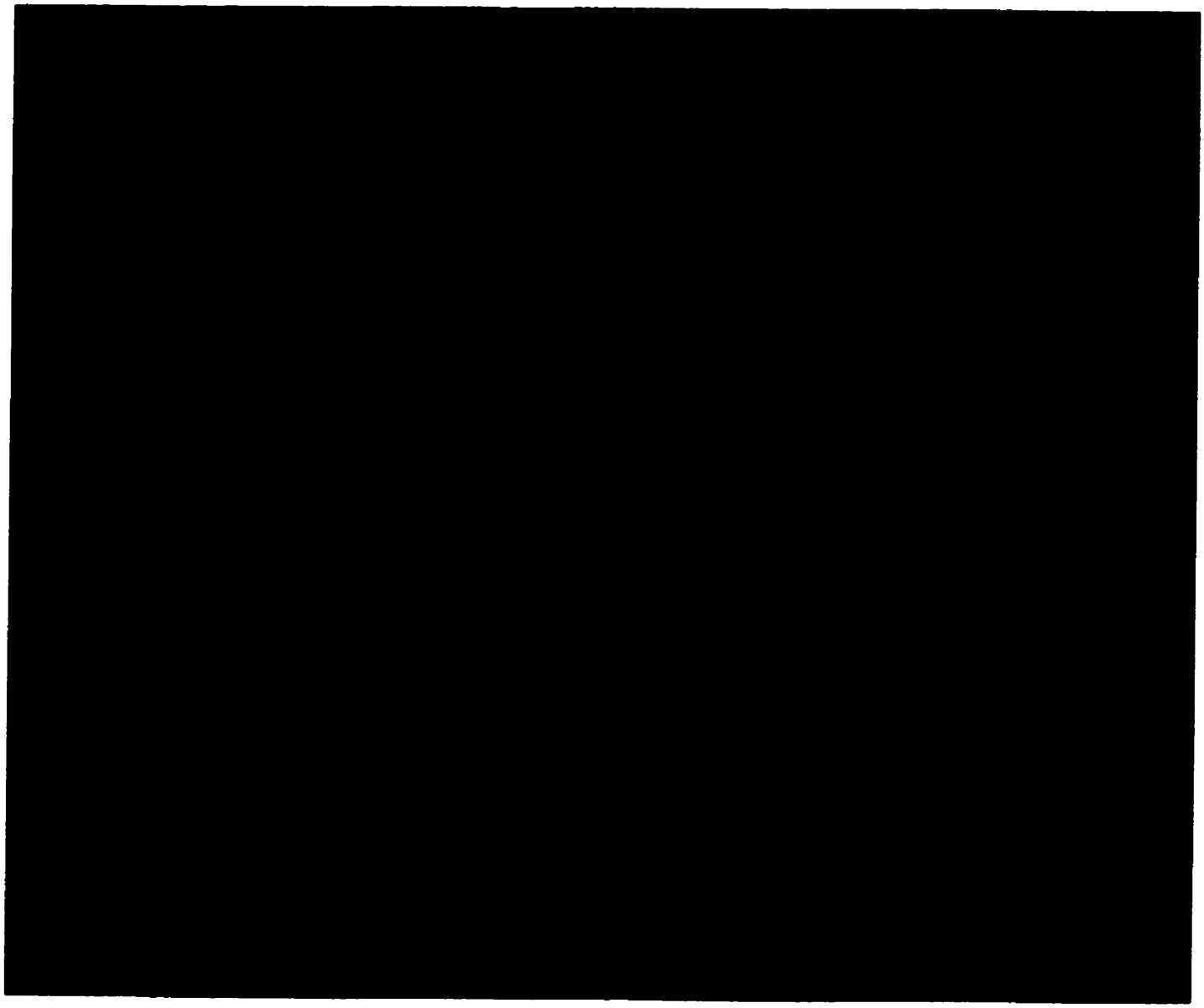
Fig. 7. Evaluation of cellular functionality using the Ca^{++} indicator Fluo-3 AM. Flou-3 AM loading was examined following isolation (A); double labelling with PI and Flou-3 AM were used to confirm high cell viability (B).

Phase **Fluo-3 AM**



A

Fluorescent



B

Fig. 8. Evaluating Fluo-3 AM signal using the Ca^{++} ionophore A23187. Hepatocytes loaded with Fluo-3 AM were incubated with the Ca^{++} ionophore A23187, and the increase in fluorescence was monitored over a 50 seconds interval.

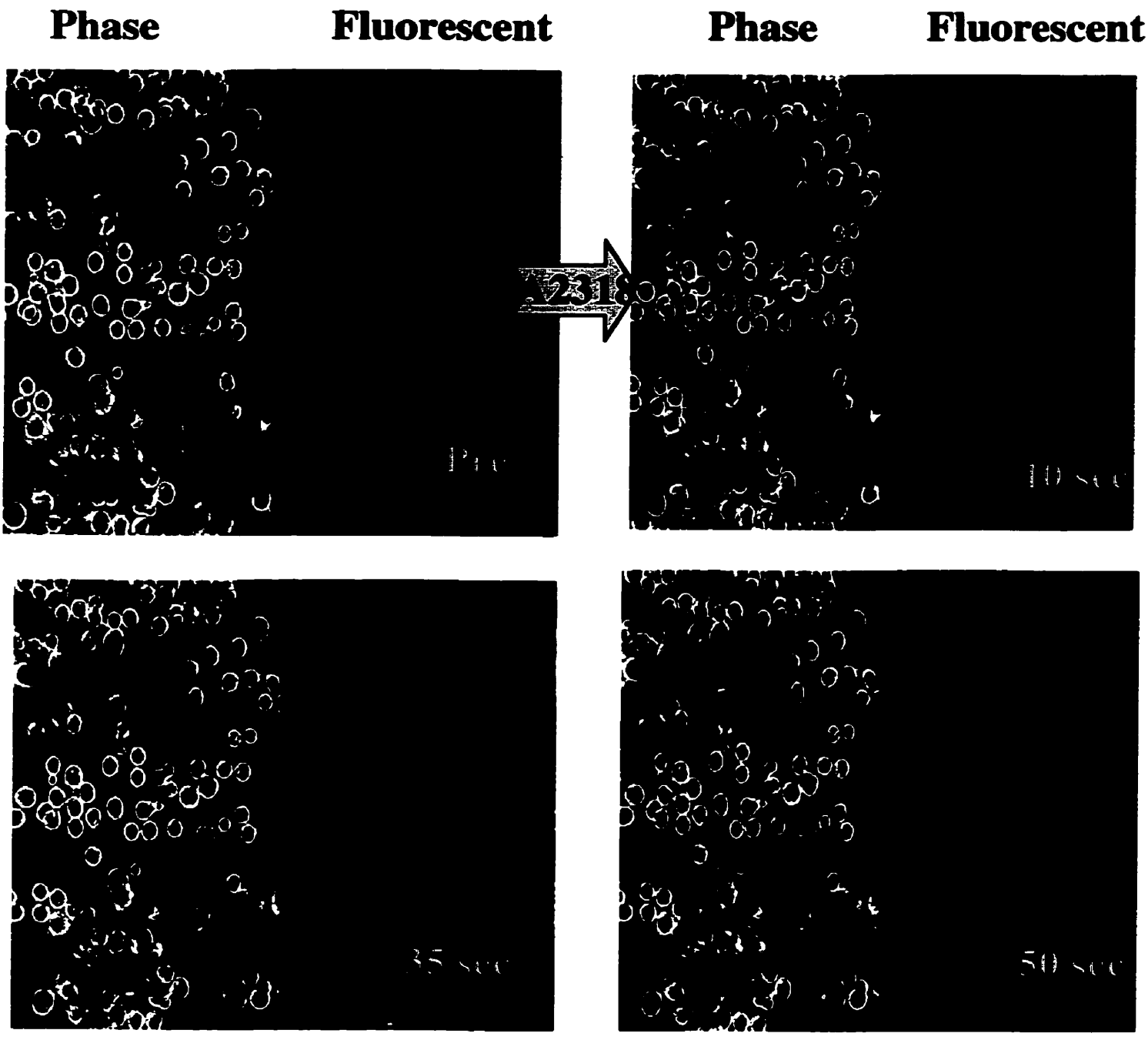

Phase
Fluorescent
Phase
Fluorescent
231

Fig. 9. Expression of HSP70 in response to isolation and incubation at 4°C, 24 h. Representative Western blot analysis of HSP70 in turtle liver tissue, freshly isolated hepatocytes, and overnight incubation of cells at 4°C. Total protein was extracted from hepatocytes was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with HSP70-specific antibody *(top)* and HSC70-specific antibody *(bottom)*. Lane 1: Liver tissue, Lane 2:freshly isolated hepatocytes and lane 3: hepatocytes incubated at 4°C for 24 h.

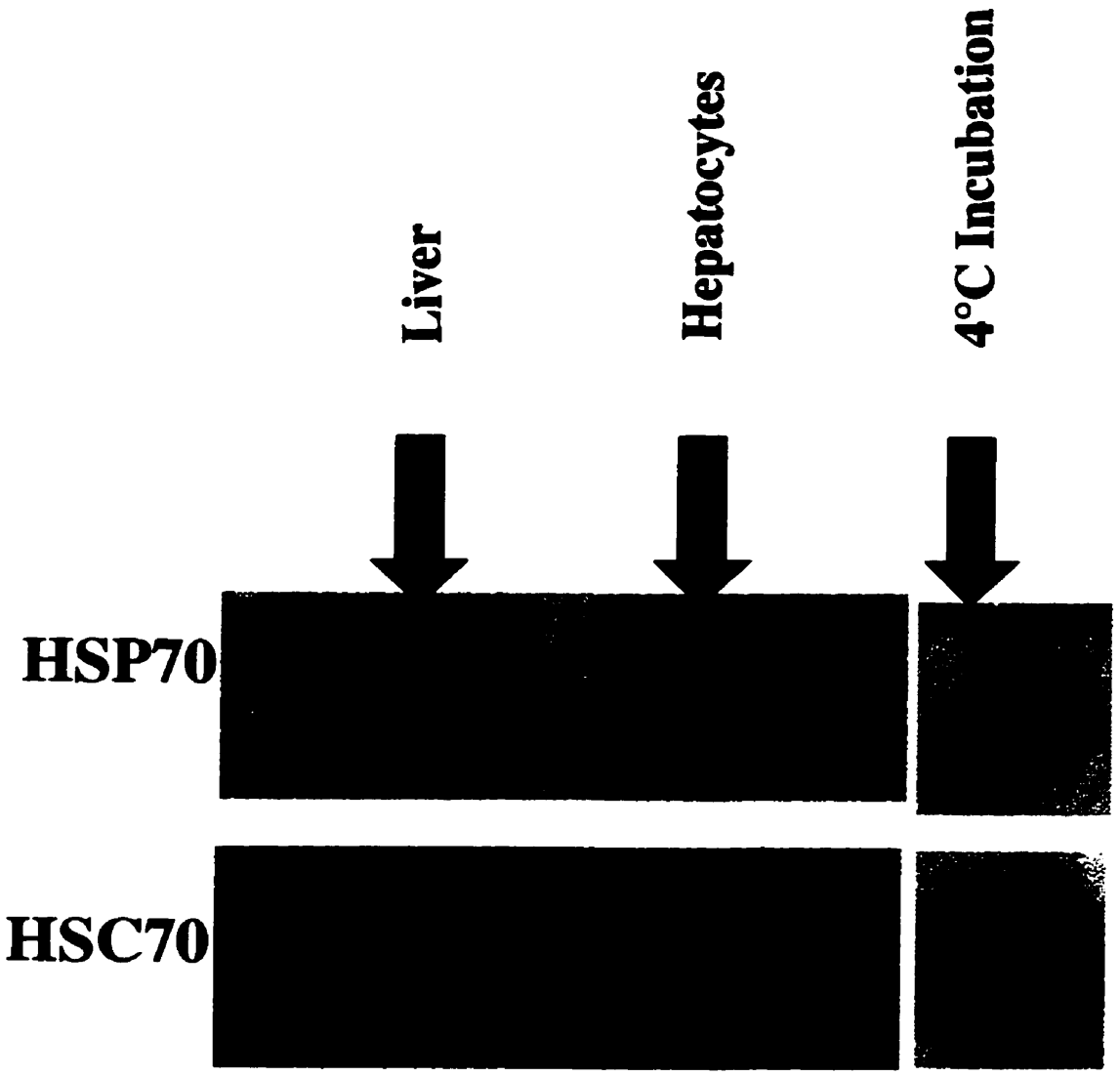

Liver
Hepatocytes
4°C Incubation
HSP70
HSC70

Fig. 10. Expression of HS70 mRNA following heat shock. Representative Northern blot analysis of HSP70 mRNA in turtle hepatocytes following heat shock. Hepatocytes were maintained at either control temperature (23°C) or heat shocked at 30°C, 33°C, 37°C, 40°C, 44°C or 48°C for 1 h (n=2). Total RNA isolated from the hepatocytes was separated by 1.5% of agarose gel, transferred to a NYTRAN-plus membrane and probed with mammalian HSP70 cDNA. An equal amount of RNA (10 ug) was loaded in each lane

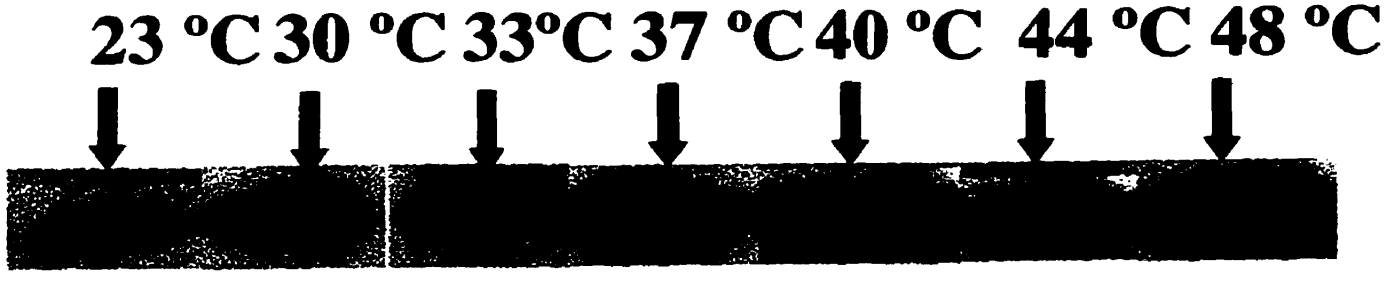

23 °C
30 °C
33°C
37 °C
40 °C
44 °C
48 °C

Fig. 11. Expression of HSP70 following heat shock. Representative Western blot analysis of HSP70 in turtle hepatocytes following heat shock (28°C, 33°C, 37°C, and 45°C). Total protein was extracted from hepatocytes was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with HSP70-specific antibody (n=2). An equal amount of protein (7 ug) was loaded in each lane.

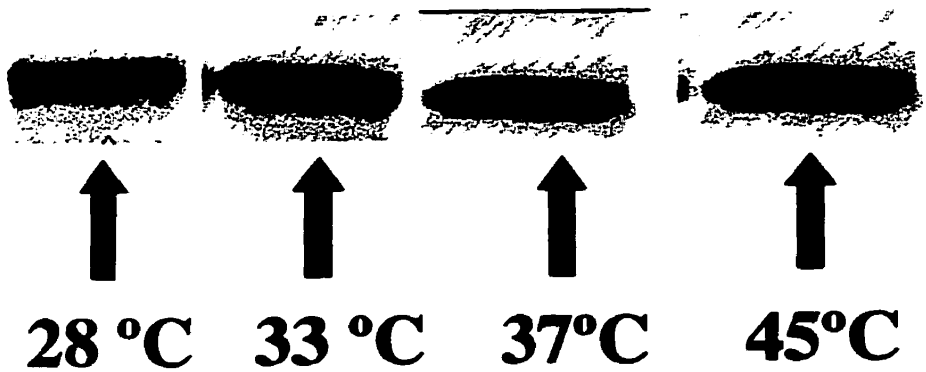

28 °C
33 °C
37°C
45°C

Fig. 12. Expression of HSP70 following exposure to Cadmium Chloride and Hydrogen Peroxide. Representative Northern blot analysis of Cadmium Chloride on the expression of HSP70 mRNA *(Top)* (n=2). An equal amount of RNA (10 ug) was loaded in each lane. Western blot analysis of Hydrogen Peroxide on the expression of HSP70 (n=1) *(bottom)*. An equal amount of protein (7 ug) was loaded in each lane.

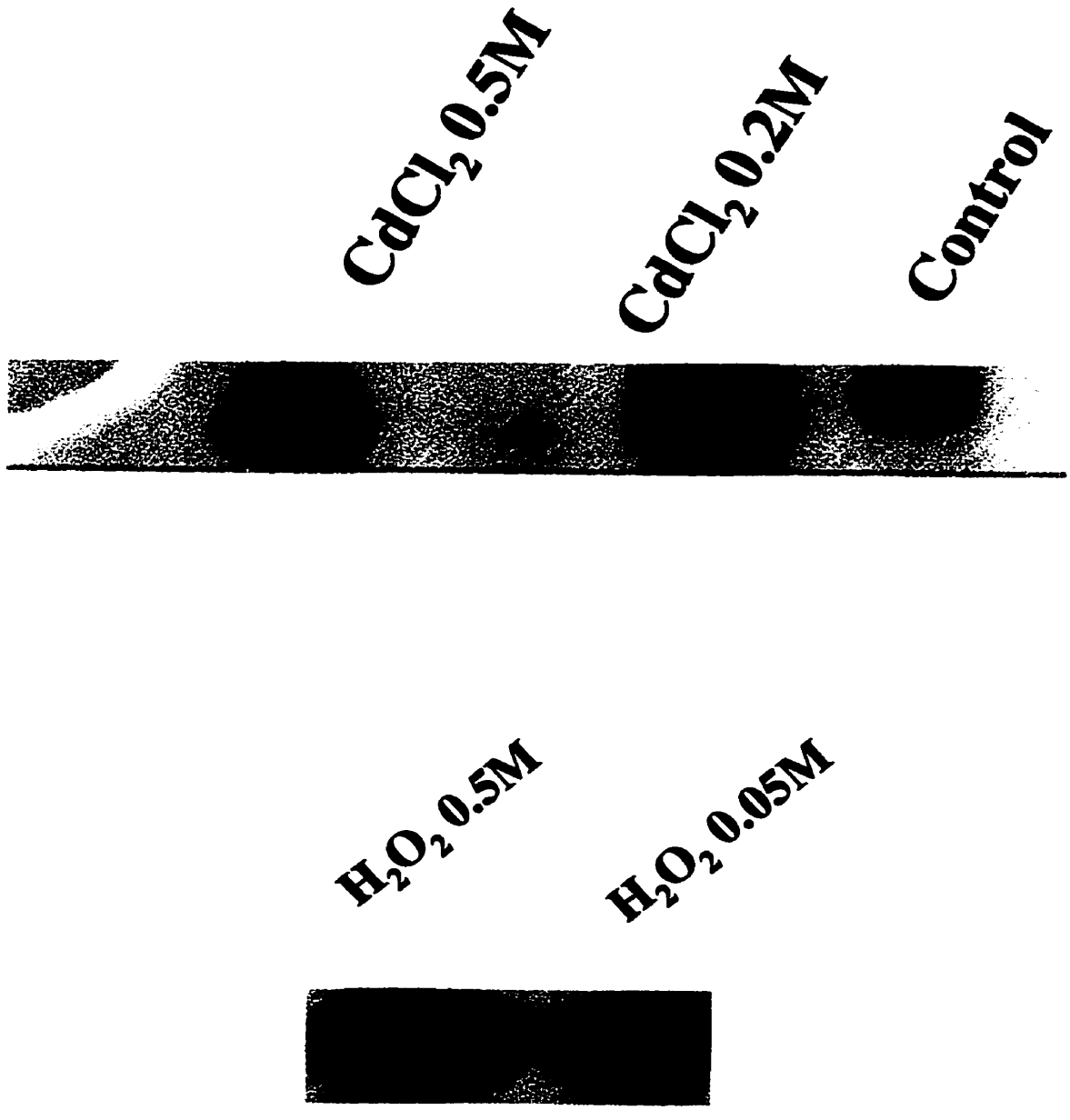

$CdCl_2$ 0.5M
$CdCl_2$ 0.2M
Control
H_2O_2 0.5M
H_2O_2 0.05M

Fig. 13. Expression of HSP70 mRNA following anoxia. Representative Northern blot analysis of HSP70 mRNA in turtle hepatocytes following anoxia. Hepatocytes were exposed to anoxic conditions for 1, 2, 3, 4, 6, and 8 h anoxia (95% N_2-5%CO_2). Total RNA isolated from the hepatocytes was separated by 1.5% of agarose gel, transferred to a NYTRAN-plus membrane and probed with mammalian HSP70 cDNA. Total RNA was quantified using spectrophotmetry and equal amount of RNA (10 ug) was loaded in each lane. Blots were normalized to α-Tubulin (n=3).

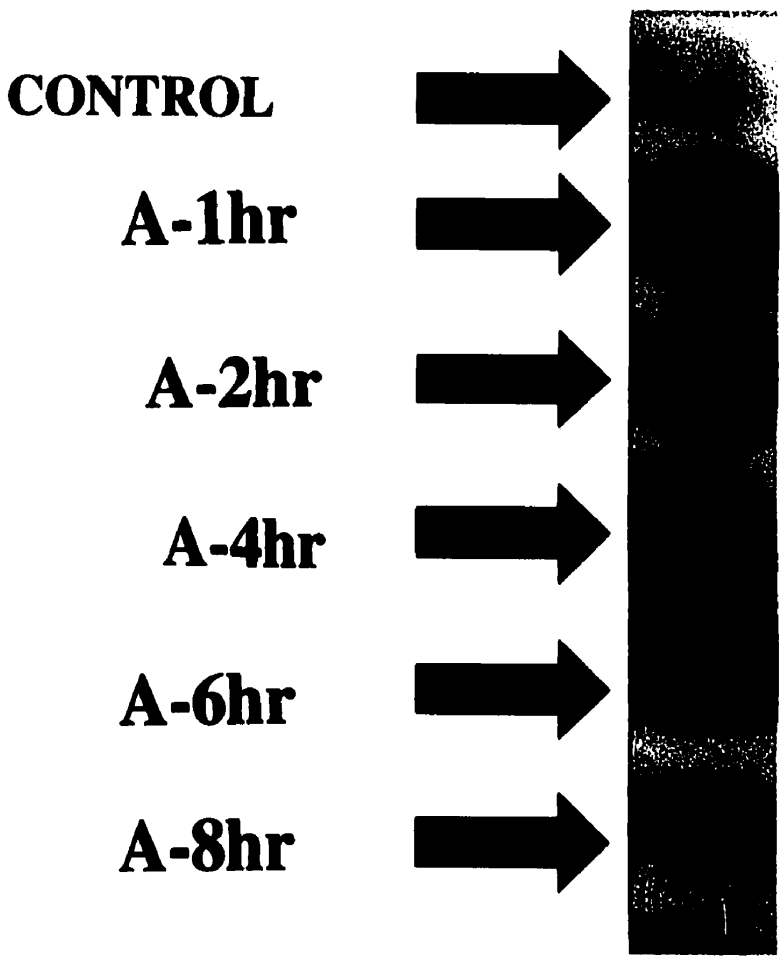

CONTROL
A-1hr
A-2hr
A-4hr
A-6hr
A-8hr

Fig. 14. Expression of HSP70 following anoxia (at 4°C and 23°C). Representative Western blot analysis of HSP70 in turtle hepatocytes following anoxia for 1 h at 4°C and for 1, 2, 3, 4, and 6 h at 23°C. Total protein was extracted from hepatocytes was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with HSP70-specific antibody (n=2). An equal amount of protein (7 ug) was loaded in each lane.

4°c
A- 1hr
A-1hr
A-2hr
A-3hr
A-4hr
A-6hr

Fig. 15. Examining co-expression of HSP70 and HSC70 in turtle hepatocytes following anoxia (95% N_2-5%CO_2). The expressions of HSP70 and HSC70 in hepatocytes following exposure to anoxia for 1, 2, 3, 4, and 6 h were examined by Western blot analyses (n ≥2). Total protein was extracted from hepatocytes was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with HSP70-specific antibody (top) or HSC70-specific antibody (Bottom). An equal amount of protein (7 ug) was loaded in each lane.

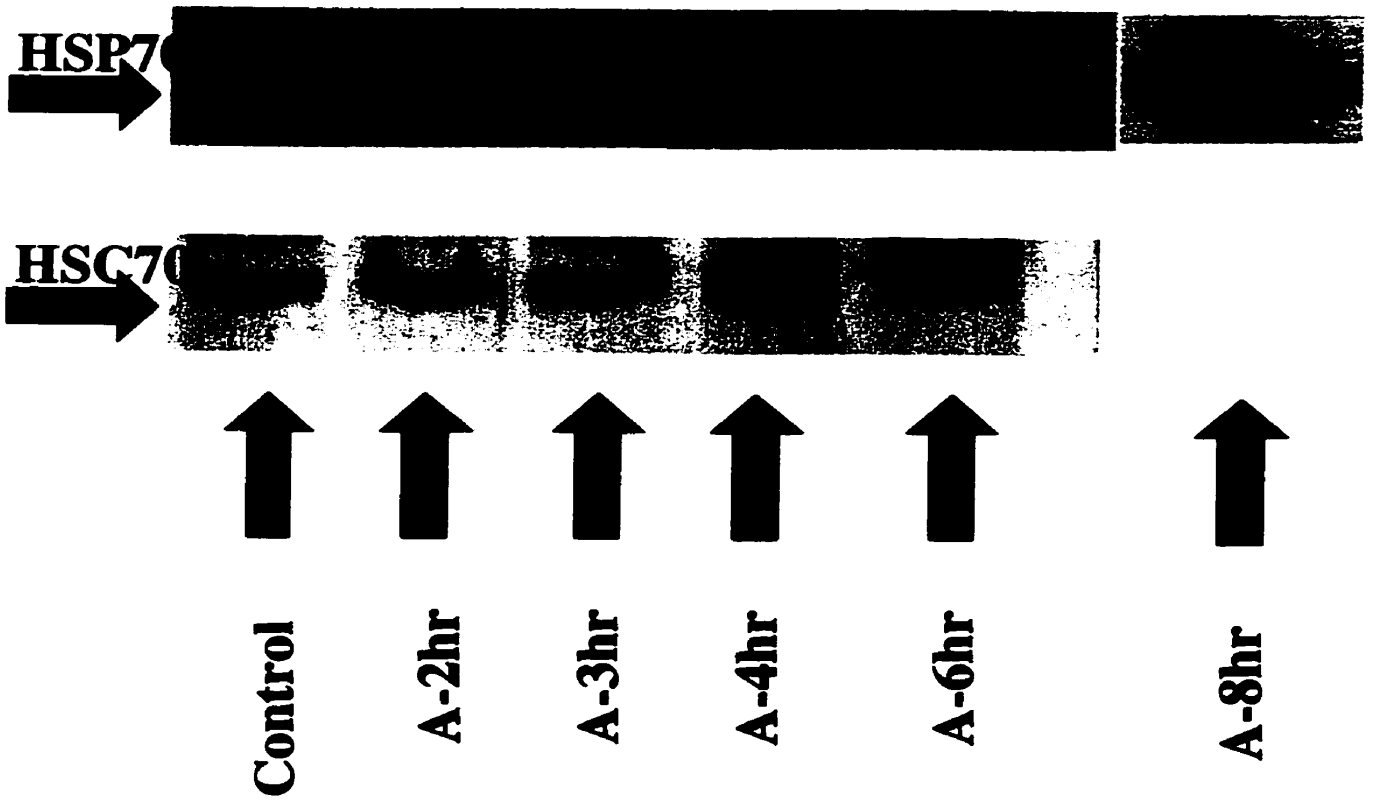

HSP7
HSC7
Control
A-2hr
A-3hr
A-4hr
A-6hr
A-8hr

Fig. 16. Effect of CPA and DPCPX on the expression of HSP70 mRNA following anoxia. Hepatocytes were pretreated with either CPA or DPCPX and exposed to anoxic for 1 h (a) or normoxia for 1 h (b). Total RNA isolated from the hepatocytes was separated by 1.5% of agarose gel, transferred to a NYTRAN-plus membrane and probed with mammalian HSP70 cDNA. An equal amount of RNA (10 ug) was loaded in each lane.

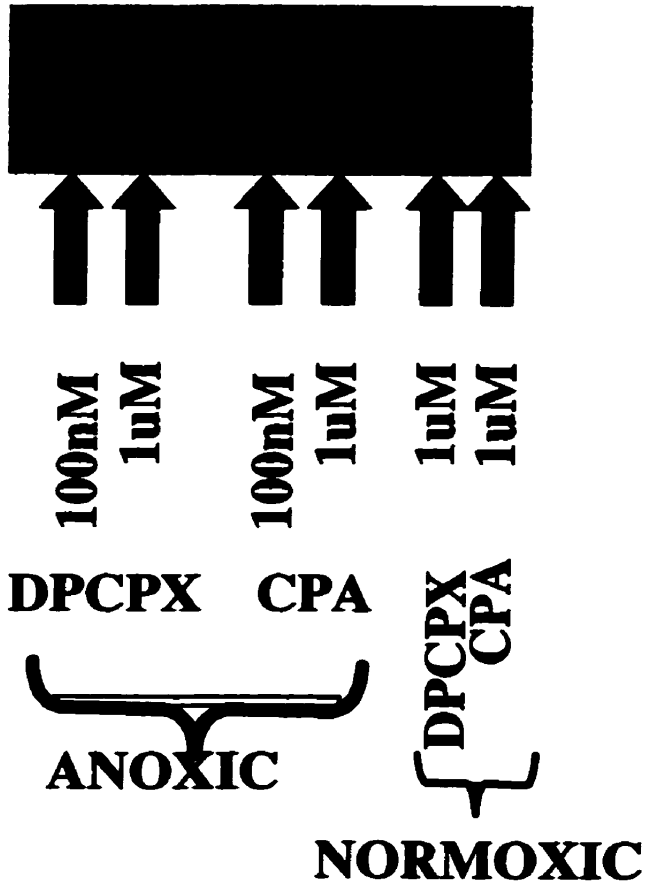
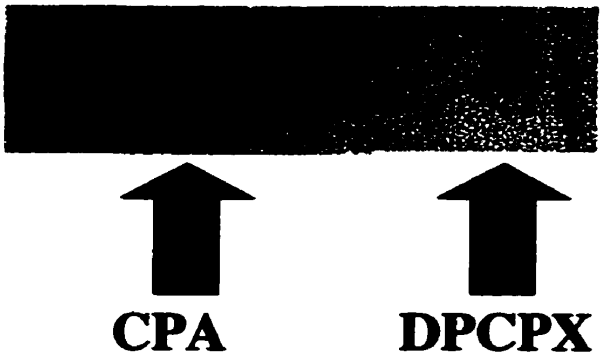
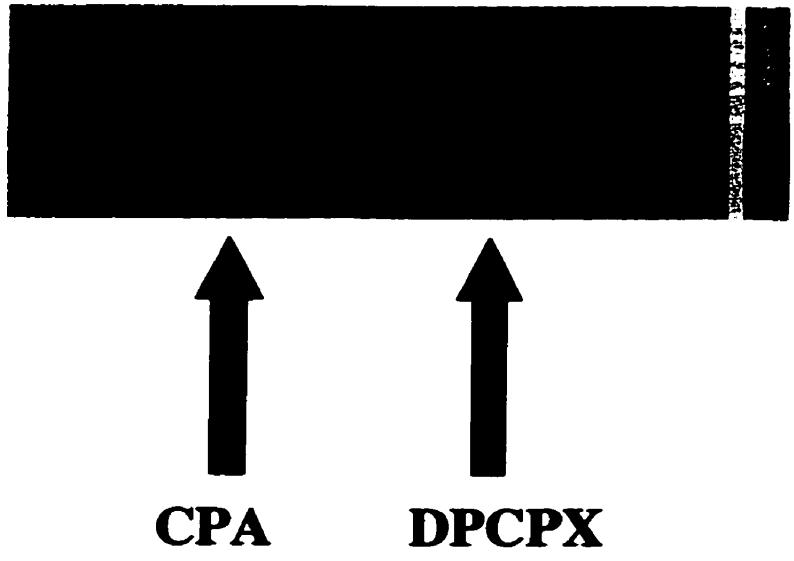

100nM
1uM
100nM
1uM
1uM
1uM
DPCPX
CPA
DPCPX
CPA
ANOXIC
NORMOXIC

Fig. 17. Expression of HSP70 in response to CPA and DPCPX. Using Western blot analyses, the effect of CPA and DPCPX on the expression of HSP70 was examined following anoxia. Hepatocytes were pretreated with either 1 μM CPA or 1 μM DPCPX and exposed to anoxic 1 h. Total protein was extracted from hepatocytes was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with HSP70-specific antibody. Each lane was loaded with 7 ug of protein.



ANOXIA

Fig. 18. Effect of CPA and DPCPX on the expression of HSP70 following heat shock. Hepatocytes were pretreated with either 1 μM CPA or 1 μM DPCPX and exposed to heat shock (40°C, 1 h). Total protein extracted from hepatocytes was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with HSP70-specific antibody. Each lane was loaded with 7 ug of protein.



Heat shock

Table 1: Typical spectrophotometric analyses of total RNA: Calculations were done according to Sambrook *et al.*, 1989.

	RATIO	ABSOR.	PURITY		RNA
same day	1.87	1.207	93%		18ug
next day	1.855	1.987	92%		20ug
N T=0	1.807	1.079	90%		16ug
A T=1hr	1.832	1.178	91%		18ug
N T=2	1.841	1.902	92%		29ug
A T=2hr	1.838	0.765	91%		12ug
N T=4hr	1.831	0.58	90%		8.8ug
N T=6hr	1.864	0.146	93%		2ug
A T=4hr	1.831	0.655	91%		9.8ug
A T=6hr	1.854	0.654	92%		9.8ug

Fig. 20. Quantification of anoxia-induced accumulation of HSP70 mRNA in turtle hepatocytes. Autoradiograms from several different experiments were scanned and the background values were subtracted from the values obtained for each lane. The values were then expressed as a percentage of the maximum mRNA accumulation (Y-axis). The asterisk indicates a significant difference ($P<0.05$) from anoxic 1 h. All values are expressed as mean ± S.E.M.

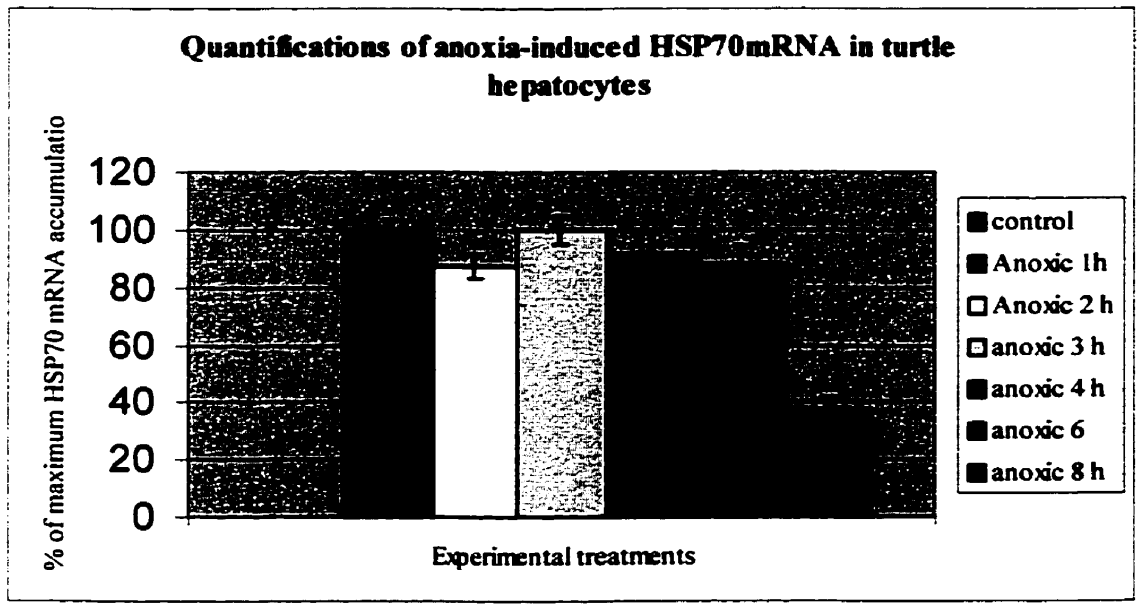
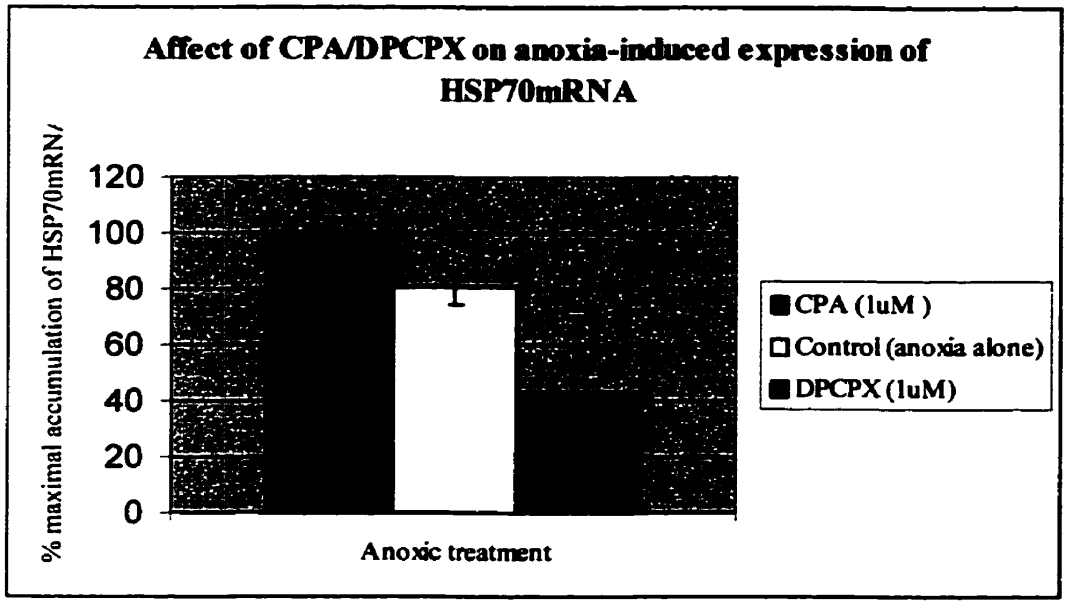

Quantifications of anoxia-induced HSP70mRNA in turtle hepatocytes
% of maximum HSP70 mRNA accumulatio
120
100
80
60
40
20
0
Experimental treatments
control
Anoxic 1h
Anoxic 2 h
anoxic 3 h
anoxic 4 h
anoxic 6
anoxic 8 h

Fig. 21. Quantification of HSP70 mRNA transcription in turtle hepatocytes in the presence of A1-receptor agonist/antagonist during anoxia.

Autoradiograms from several different experiments were scanned and the background values were subtracted from the values obtained for each lane. The values were then expressed as a percentage of the maximum mRNA accumulation (Y-axis). The asterisk indicates a significant difference ($P<0.05$) from anoxic 1 h. All values are expressed as mean ± S.E.M.


Affect of CPA/DPCPX on anoxia-induced expression of HSP70mRNA
% maximal accumulation of HSP70mRN
120
100
80
60
40
20
0
Anoxic treatment
CPA (1uM)
Control (anoxia alone)
DPCPX (1uM)

CHAPTER 4 DISCUSSION

Although the physiological responses to long-term anoxia have been well studied in the turtle species *Chrysemys picta belli* (Buck *et al.*, 1993b; Land *et al.*, 1993; Buck *et al.*, 1993a), the molecular components underlying this tolerance are still under investigation. A number of mechanisms have been suggested based on findings from other models. Two candidates that have recently been considered as potential role players in inducing such anoxia tolerance are the stress proteins and the endogenous cellular modulator adenosine. In various models, stress proteins have been shown to be produced during anoxia and other stressful conditions (Hammond *et al.*, 1982; Sciandra *et al.*, 1984; Tuijl *et al.*, 1991; Myrmel *et al.*, 1994; Mestril *et al.*, 1994; Itoh *et al.*, 1995). Adenosine has also been found to increase extracellularly during anoxia, and to provide multidimensional central and peripheral protective effects (Barber and Butcher, 1988; Nilson *et al.*, 1992; Rudolphi *et al.*, 1992b; Aden *et al.*, 1994; Lutz and Kabler, 1997; Sweeney, 1997; Buck and Bickler, 1998). Despite these findings, the direct correlation between these two factors has not yet been conclusively investigated.

The results presented in this study show that HSP70 is induced in turtle hepatocytes in response to anoxia and other environmental stresses. In addition, they demonstrate for the first time the modulatory role of the adenosine A1 receptor in blocking the anoxia induced production of HSP70.

In these experiments, it was found that anoxia alone did not affect cell viability, as examined by ATP concentrations, Trypan blue staining, propidium iodide labeling, and Flou-3 AM loading of hepatocytes (Fig. 3, 4, 5, 6, and 7). This was in accord with the study done by Buck *et al* (1993)a, where turtle hepatocytes survived 10 h anoxia at 25°C

(Buck *et al.*, 1993b). Nevertheless, it was in contrast to reports from rat hepatocytes, which when exposed to anoxia at 37°C showed a 70% decrease in their intracellular adenylates in less than 10 min with more than 75% cell loss at 3 h (Jones *et al.*, 1989).

4.1 HSP70 induction during anoxia

While expression of HSP70 was not detected in turtle hepatocytes during normoxic incubation, exposure to 1, 2, 3, 4, and 6 h anoxia induced its expression at both the mRNA and protein levels (Fig.13, 14, and 15). This expression did not persist as the production of both HSP70 mRNA and protein declined markedly at 8 h (Fig. 13 and Fig. 14), cell viability was not affected. The explanation for such decrease in HSP70 during long term anoxia in turtles is unresolved. Although the production of HSP70 may be cytoprotective, it is an energetically expensive process (Hightower and McCready, 1994). Considering that remarkable suppression of energetically expensive cellular process is key to long term anoxic survival in the turtle, the selective production of HSP70 suggests that it may play a vital role in promoting anoxia tolerance in turtles. As such, once tolerance is reached/triggered (e.g., at 6 h), its production is suppressed.

In this study, the presence of the constitutive form of HSP70-HSC70, was also investigated. HSC70 was present during both normoxic and anoxic conditions (Fig. 15). Previous studies have shown that under normoxic conditions HSC70 chaperones cellular proteins to facilitate their translocation through membrane pores or across the nuclear membrane, and that it interacts with clathrins in vesicle recycling (Gething and Sambrook, 1992; Welch, 1992; DeLuca-Flaherty *et al.*, 1990). Additionally, under stressful conditions such as anoxia, HSC70 is found to actively participate in protein maturation, proteolysis, protein transport etc. (Lindquist and Craig, 1988). Being the

main component of the chaperone mechanism in eukaryotes, the presence of HSC70 during anoxia indicates that it is vital for cellular activities under both normal conditions, as well as stressful conditions in turtle hepatocytes.

4.2 Effect of Cadmium Chloride on HSP70

In this project, the effects of Cadmium Chloride, Hydrogen Peroxide, and heat shock on the expression of HSP70 in turtle hepatocytes were examined as a positive control. Cadmium is widely known as an environmental pollutant and is a potent poison for cells and organisms (Seidman, 1986; Foulkes and McMullen, 1986; Morselt, 1991). This heavy metal can cause damage to various organs and tissues, especially the liver where the Cadmium is stored (Goering *et al.*, 1995). Several mammalian studies have shown that upon exposure to Cadmium, expression of HSP70 gene is triggered, resulting in an increase in the synthesis of HSP70 protein (Goering *et al.*, 1995; Wiegant *et al.*, 1994). Recently, Cadmium toxicity has been studied in *Aedes albopictus* c6/36 cells (Braeckman *et al.*, 1999). At toxic concentrations of $CdCl_2$ (>33 μM) hepatocyte cultures have shown a surprising resistance, which was not seen at low metal concentrations. This was postulated to be the result of activation of a defense mechanism (Braeckman *et al.*, 1999), which was coincident with an induction of HSP70. While the mechanism of Cadmium-induced HSP70 expression has not been studied in turtles, in other models it has been shown that Cadmium reacts with vicinal thiol groups and substitutes Zinc in proteins and thus generates denatured proteins (Vallee and Ulmer, 1972; Jungmann *et al.*, 1993). The occurrence of abnormal proteins is recognized as the signal for inducing HSP70, and likely the mechanism responsible for HSP70 induction in turtle hepatocytes.

4.3 Effect of Hydrogen Peroxide on HSP70

For many cells, exposure to oxygen radicals is a common occurrence. While anti-oxidant defenses are constitutively expressed, adaptive responses are induced when the amount of environmental oxidants exceeds a threshold level, thereby becoming a threat to cell integrity (Minghetti and Gennis, 1988; Katoh *et al.*, 1991). While the exact mechanism by which oxygen radicals induce HSP70 is still unclear it has been found that H_2O_2 interferes and disturbs the integrity of structural proteins (Becker *et al.*, 1990).

4.4 Hyperthermia and HSP70

HSP70 can also be induced when cells are exposed to higher than their physiological temperatures. In these experiments, exposure of turtle hepatocytes to temperatures between 28°C and 50°C lead to induction of HSP70 at both mRNA and protein levels (Fig. 10 and Fig. 11). This type of molecular response to heat shock is also observed in other organisms. For instance, when the temperature of cultured rainbow trout (*Oncorhynchus mykiss*) hepatocyte were increased from 18°C to 26°C, a pronounced accumulation of HSP70 mRNA was observed (Airaksinen *et al.*, 1998). In another study, DiDomenico and colleagues (1982) showed that when the temperature of *Drosophila* cells shifted from 25°C to 37°C, they rapidly re-directed protein syntheses from a complex pattern, characteristic of normal growth, to a simple pattern of heat shock proteins. On return to 25°C, however, syntheses of normal proteins were gradually reactivated and that of HSP70 repressed (DiDomenico *et al.*, 1982). Furthermore, sub-tidal seaweed (*Plocammium cartilagineum*) from Antarctic waters exhibited a heat shock response similar to that of other organisms at 5-10°C above their physiological temperatures. In this study, expression of HSP70 in turtle hepatocytes was detectable at

temperatures higher than 28°C (Fig. 11). Such a scenario, where a specific temperature above which HSP70 is induced is also seen in other models. For example, in *Xenopus* heart, it has been shown that the induction of HSP70 starts at 26°C, with the highest amount of HSP70 detected at 28°C (Ali *et al.*, 1997).

In these experiments, anoxia was shown to induce the expression of HSP70 at room temperature (23°C). Nevertheless, it was critical to examine if this expression persisted under physiological over-wintering conditions, i.e., anoxia at 4°C. Under such conditions, HSP70 was detected both at the mRNA and protein levels. Few mammalian studies have investigated the effect of cold temperatures on HSP70 expression (under normoxic conditions). A study by Matz and co-workers (1995) showed that storing mouse brown adipose tissue at a cold temperature increased the production of HSP70 (Matz *et al.*, 1995). Since blocking the adrenergic receptor antagonized such HSP70 protein induction, they concluded that the nor-epinephrine, released in response to cold, induced the production of HSP70. In turtles, it has been shown that plasma levels of catecholamines increase during anoxia at cold temperatures (Keiver *et al.*, 1992; Wasser and Jackson, 1991), and returns to basal levels upon recovery. However, the direct involvement of catecholamines in the production of HSP70 during such condition, i.e., anoxia and cold, has not yet been found.

4.5 How do Animals Survive Elevated Temperatures?

An important benefit of a "stress response" is the protection induced against a later, more intense stressful insult (Parsell *et al.*, 1993). This phenomenon is referred to as "stress-induced tolerance" or "preconditioning". In many organisms, a correlation has

been shown between the tolerance induced by increasing temperatures, i.e., preconditioning, and the production of HSP70. Hashmi and colleagues (1998) showed that over expression of the gene for HSP70 by a non-toxic heat treatment was thermo-protective against a subsequent lethal heat treatment (Hashmi *et al.*, 1998). Similarly, in mammalian studies it has been shown that transgenic mice over expressing the HSP70 gene were resistance to the adverse effects of lethal heat or ischemia (Marber *et al.*, 1995). This type of heat induced tolerance additionally helps the organism survive various other stresses. For instance, a non-lethal heat shock protects human gastric cells from sepsis-induced injury (Villar *et al.*, 1994), and the rabbit heart from ischemic-reperfusion injury (Stojadinovic *et al.*, 1997).

4.6 Second messengers in the induction of HSP70

The cascade of events leading to the induction of HSP70 are not well understood; however, some studies have shown that Ca^{++}, cyclic AMP (cAMP), Inositol 1,4,5-trisphosphate (IP_3), and protein kinase C (PKC) and adenosine may contribute to such induction. Since these components are all intermediates of receptor based signaling it is reasonable to speculate that induction of HSP70 can also be receptor mediated.

Calcium has been shown to play an important role in the induction of HSP70. Studies have shown that increasing the intracellular Ca^{++} by a Ca^{++} ionophore induced HSP70 expression (Ding *et al.*, 1996), while addition of Ca^{++} chelators such as EGTA greatly attenuated the production of HSP70.

It has also been shown that heat shock increases intracellular cAMP in several models (Kampa and Frascella, 1977; Kiang *et al.*, 1991) which results in the induction of HSP70. Furthermore, inhibitors of protein kinase C, such as 1-(5-isoquinolinylsulfonyl)-

2-methylpiperazine have been shown to have an inhibitory effect on the expression of HSP70 (Yamamoto *et al.*, 1994).

Inositol 1,4,5-triphosphate (IP_3) is also an important intermediate in the regulation of HSP70. Kiang and Tsokos (1988) showed that treatment of human epidermoid cells with pertussis toxin, cholera toxin, or forskolin, increased IP_3 which lead to an increase in HSP70 mRNA and protein synthesis. Furthermore, U-73122 an IP_3 inhibitor decreased the heat-induced expression of HSP70 (Kiang and Tsokos, 1998). In studying cardiac adaptation to repeated stresses, Meerzon and colleagues (1996) found that maximal activation of the IP_3-DAG regulatory circuit was accompanied by an increase in HSP70 induction which coincided with the increased resistance to cardiac post-ischemic reperfusion injuries (Meerson *et al.*, 1996).

These studies show that while the induction of HSP70 may involve several mediators (e.g., cAMP, Ca^{++}, IP_3-DAG, and several protein kinases), whether these pathways are receptor mediated is still unclear.

4.7 Adenosine and HSP70

The induction of HSP70 has been shown to provide protection during anoxia/ ischemia. Since the increase in adenosine release and its A1 receptor mediated actions have also been shown to be protective during anoxia, in these experiments it was examined whether adenosine A1 receptor agonist/antagonist modulate the induction of HSP70 during anoxia.

(i) Effect of DPCPX and CPA during anoxia

During 1 h of anoxia, pre-incubation of hepatocytes with CPA showed HSP70 mRNA and protein expression was not significantly different than the anoxic control

groups (Fig. 16 and Fig. 17). However, when hepatocytes were pre-incubated with the A1 receptor blocker, DPCPX, and exposed to anoxia, expression of HSP70 mRNA or protein was blocked. This observation suggests that the expression of HSP70 is mediated through A1 receptors during anoxia.

(ii) Effect of DPCPX and CPA during heat shock

While heat shock, at 40°C, induced the expression of HSP70 in hepatocytes (Fig. 17), pre-incubation with DPCPX did not effect this expression. This suggests that, unlike in anoxia, the triggering mechanism for the induction of HSP70 in response to heat shock may not to be mediated through adenosine A1 receptors. Pre-incubation with CPA led to similar HSP70 induction as seen in the controls.

A study by Heurteaux et al., (1995) showed that ischemic preconditioning could be induced either by the activation of A1 receptors (by CPA) or by the presence of HSP70. They reported that CPA alone decreased the observed levels of HSP70 mRNA during ischemic preconditioning (Heurteaux *et al.*, 1995). This was contradictory to our results. Furthermore, they did not examine the effect of DPCPX on HSP70, and therefore it did not provide any conclusive evidence.

4.8 Importance of HSP70 in anoxia tolerant turtles

While HSP70 has been shown to play a significant role in the mechanism of preconditioning in various mammalian models, the extent of such contribution in the anoxia tolerant turtles has not been fully determined. Our study showed that blocking the production of HSP70 during anoxia did not compromise cell survival, at least for the short period measured after anoxia. Nevertheless, it may be that a delayed cell death was triggered in the absence of HSP70, which could not be measured as our experiments did

not allow for long term monitoring of cell survival. The fact that during stressful conditions, e.g., anoxia, most protein syntheses are down regulated, the persistence of HSP70 production suggests a vital role for this protein in the observed tolerance.

4.9 Proposed mechanism of HSP70 induction during anoxia and heat shock

Thus far, these results indicate that while both anoxia and heat shock lead to the production of HSP70, each stimulus may trigger its induction differently. More specifically, the observation that DPCPX blocked the induction of HSP70 only during anoxia, and not during heat shock, demonstrates that the induction of HSP70 during anoxia is A1R mediated.

Furthermore, these results suggest that heat induced expression of HSP70, which did not appear to be receptor mediated, may have an intracellular triggering system stimulating its production. This is supported by studies where cells presented with an intracellular stimuli, e.g., denatured proteins, showed increased induction of HSP70 (Ananthan *et al.*, 1988; Mifflin and Cohen, 1994).

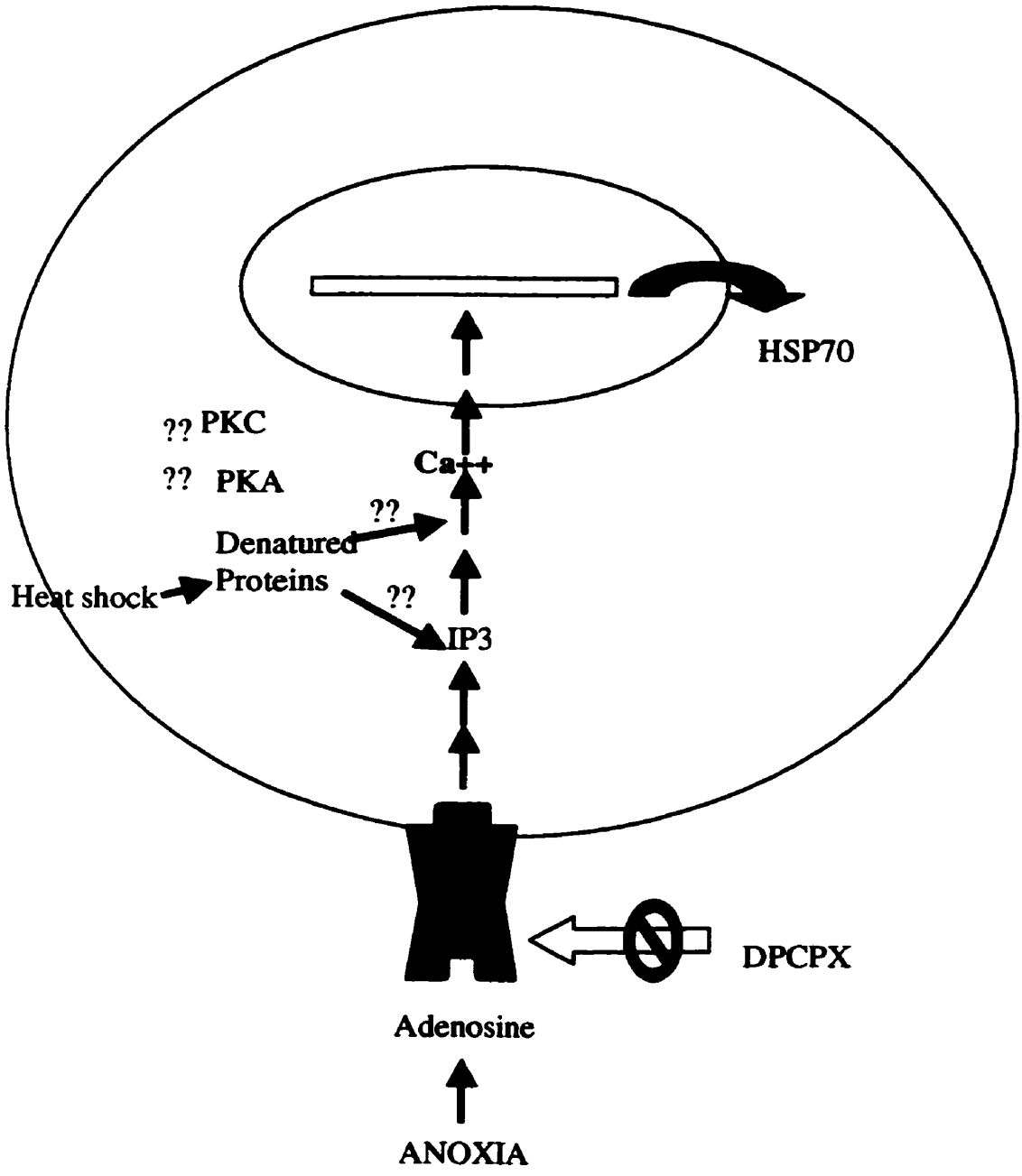
While anoxia and heat shock may initiate the production of HSP70 differently, the question arises on whether the later steps of these inductions are mediated through one common pathway. For instance, A1R activation, during anoxia, has been reported to increase intracellular levels of IP_3 (Collis and Hourani, 1993). This increase in IP_3 is also observed in response to heat shock (Kiang and McClain, 1993). Interestingly, such elevation of IP_3 has been correlated with the production of HSP70 (Kiang *et al.*, 1994). Therefore, while anoxia and heat shock may work independently to increase the levels of intracellular IP_3, the events downstream of this point may not be much different (see

Model 1). The confirmation of the IP_3 as the beginning of a common pathway, however, requires further examination.

In summary, this report shows that in turtle hepatocytes HSP70 is induced in response to anoxia. This study further indicates that the levels of HSP70 decline after the first few hours of anoxia. Taken together these findings suggest that the initial presence of HSP70 may be critical in promoting the long-term anoxia tolerance in turtles.

The induction of HSP70 involves a complex cascade of events. In this report, it has been demonstrated that the production of HSP70 may be triggered by different stimuli. Nevertheless, whether these different routes of HSP70 production will join later to form a common pathway needs further investigation.

Fig. 22. Model 1: Proposed mechanism of stress induced increase in HSP70 in turtle hepatocytes.


HSP70
?? PKC
?? PKA
Ca++
??
Denatured
Proteins
Heat shock
??
IP3
DPCPX
Adenosine
ANOXIA

CHAPTER 5 REFERENCES

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