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Tissue-specific expression of inducible and constitutive Hsp70 isoforms in the western painted turtle

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Summary

Expression of Hsp73 and Hsp72 in four tissues of the naturally anoxia-tolerant western painted turtle (*Chrysemys picta*) was investigated in response to a 24 h forced dive and following 1 h recovery. Of the tissues examined, brain and liver displayed approximately threefold and sevenfold higher basal Hsp73 expression than heart and skeletal muscle. Basal Hsp72 expression was relatively low in all tissues examined. After the 24 h forced dive and 1 h recovery, Hsp73 expression did not differ significantly from basal expression with the exception of liver, where expression decreased significantly after 1 h recovery. Hsp72 expression was unchanged in liver following a 24 h dive; however, it increased twofold in brain and threefold in heart and skeletal muscle. Dive-induced Hsp72 expression was found to correlate inversely with basal Hsp73 expression. Following 1 h recovery, Hsp72 expression was significantly elevated in all tissues above levels in dived animals. These data indicate a tissue-specific pattern of Hsp73 and Hsp72 expression in the western painted turtle during both unstressed and stressed conditions.

Key words: stress proteins, forced dive, recovery, heat shock, western blot, *Chrysemys picta belli*, western painted turtle.

Introduction

Although vertebrates are generally very sensitive to oxygen deprivation, there are a few non-mammalian vertebrates known to survive anoxic conditions for extended periods of time; the length of survival depends on the species and the ambient temperature. One such anoxia-tolerant vertebrate is the western painted turtle (*Chrysemys picta belli*), which is adapted to survive extended periods of oxygen deprivation. It can fully recover from a 24 h anoxic dive at 25°C, as well as more than 3 months submergence at 3°C (Jackson and Ultsch, 1982). The turtle's main survival strategy is a large suppression of metabolic rate, such that the anoxic cell is functioning at approximately 10% of the aerobic capacity (Herbert and Jackson, 1985; Jackson and Ultsch, 1982; Lutz et al., 1984). This is achieved by a coordinated reduction in key energy-utilizing cellular processes, including protein synthesis. While a general downregulation of protein synthesis occurs during anoxia, a number of studies have shown the upregulation of specific proteins (Carpenter and Hofmann, 2000; Hochachka et al., 1996; Land and Hochachka, 1995). In turtle hepatocytes subjected to anoxia or heat shock, the expression of five types of proteins was shown to increase (Land and Hochachka, 1995). More specifically, Chang and colleagues (2000) have shown the induction of heat-shock proteins (Hsp73/Hsp72) in painted turtle myocardium in response to a 12 h forced dive but were unable to distinguish between the constitutive and inducible forms of this protein.

The expression of heat-shock proteins (Hsps) is known to increase in response to various stresses, including hyperthermia, oxygen limitation (ischemia), radiation, heavy metals, anoxia and reoxygenation (Lindquist and Craig, 1988). Many heat-shock proteins are considered ATP-dependent molecular chaperones and thus recognize unfolded, misfolded and aggregated proteins (Welch, 1992; Xu and Lindquist, 1993). As molecular chaperones, Hsps share an ability to modulate the folding and unfolding of other proteins and to facilitate the assembly and disassembly of multisubunit complexes (Lindquist and Craig, 1988).

The 70 kDa family of stress proteins is highly conserved, and several isoforms exist (Creagh et al., 2000; Lindquist and Craig, 1988). In mammals, a constitutive isoform of Hsp70 (Hsp73) is expressed and demonstrates moderate stress inducibility under certain conditions (Manzerra et al., 1997). Under non-stress conditions, Hsp73 proteins are thought to act as chaperones for other cellular proteins by binding to nascent polypeptides and preventing premature folding, as well as translocating proteins into organelles. By contrast, the stress-inducible isoform of Hsp70 (Hsp72) is generally not expressed in unstressed cells; however, upon exposure to stressful conditions, Hsp72 is highly inducible. During conditions of stress, both Hsp72 and Hsp73 are thought to bind to damaged and misfolded polypeptides and to facilitate repair (Lindquist and Craig, 1988).

As protein synthesis is a costly process in terms of energetics, requiring the hydrolysis of four ATP equivalents

per peptide bond and one additional ATP for amino acid transport, the upregulation of specific heat-shock proteins in energy-compromised anoxic tissues indicates that Hsps may play a role in promoting anoxia tolerance. Indeed, increased expression of Hsp72 in mammalian brain and heart does correlate with increased protection from hypoxic/anoxic injury (Marber et al., 1995; Yenari et al., 1999). However, it is unclear whether Hsp72 expression is responsible for the protective effect or a phenomenon of it. More recently, the protective role of Hsp70 has been tested directly in cells made to overexpress Hsp72 and in cells where expression is blocked with antisense oligonucleotides (Marber et al., 1995; Yenari et al., 1999). In these instances, Hsp72 overexpression in heart and neuronal tissue was clearly protective during ischemia, and blocking Hsp72 expression during hypoxic stress in mammalian cardiomyocytes with an Hsp72 antisense oligonucleotide leads to increased cellular injury (Nakano et al., 1997). In addition, transgenic mice overexpressing Hsp70 in their myocardium are more resistant to ischemia reperfusion injury (Marber et al., 1995). Therefore, increased Hsp72 expression during anoxia or ischemia is more than just strongly correlative; increased Hsp72 expression promotes anoxia tolerance. However, whether increased Hsp72 expression in a non-mammalian anoxia-tolerant vertebrate, such as the freshwater turtle, is important for long-term anoxic survival remains unknown.

The main focus of the present study is to investigate the tissue-specific expression of Hsp73 and Hsp72 in response to heat stress and force dive-induced anoxia in the western painted turtle. In an animal adapted for long-term breath-hold diving, it is unclear whether anoxia is stressful to the animal. Thus, the purpose of this study is threefold: first, to expose the western painted turtle to a hyperthermic stress to determine the magnitude of Hsp73 and Hsp72 expression in brain, heart, liver and skeletal muscle; second, to examine Hsp73 and Hsp72 expression in these four tissues after a 24 h forced dive and 1 h recovery; and third, to quantify the relative expression of Hsp73 and Hsp72 in these tissues.

Materials and methods

Animals

This study was approved by the University of Toronto Animal Care Committee and conforms to relevant guidelines for the care of experimental animals.

Western painted turtles, *Chrysemys picta belli* Schneider 1783, weighing between 590 g and 760 g were commercially obtained from Lemberger Co., Inc. (Oshkosh, WI, USA). Animals were housed in an indoor pond (2 m×4 m×1.5 m) with a sloping floor; one end contained water approximately 0.5 m deep, while the other end had a shallow, rocky basking platform with a heating lamp. The aquarium was equipped with a flow-through dechlorinated freshwater system, with water temperature maintained at approximately 17°C. Turtles were given continuous access to food and kept at a room temperature of 20°C on a 12 h:12 h light:dark cycle.

Experimental setup

Hyperthermic experiment

A Plexiglas container of dimensions 0.5 m×0.5 m×0.25 m (with a Plexiglas cover) was half filled with water heated to a temperature of 35°C. Four turtles were placed in the heated pool for 1 h, after which time the container was drained and refilled with room-temperature water (20°C). The turtles remained in the room-temperature bath for 5 h, at which point turtles were killed by decapitation. Brain, heart, liver and skeletal muscle tissue were rapidly dissected and immediately frozen in liquid nitrogen.

Submergence experiment

Three empty metal cages were placed in the indoor pond for several days to allow turtles to acclimate to their presence. Water temperature and oxygen partial pressure (P_{O_2}) were 17°C and 5865.2 Pa, respectively. Three turtles were placed in each cage, then gradually submerged and left undisturbed for 24 h. Following this period, five animals had their necks clamped underwater (to prevent the possibility of aeration) and were killed by decapitation. The remaining four turtles were brought to the surface and allowed access to air for 1 h, after which time they were sacrificed. Four other turtles, which were not caged and had free access to air, were sacrificed as controls following the same 24 h period. Animals were cut open with a bone saw and scalpel, and the brain, heart, liver and skeletal muscle were dissected in less than 3 min, wrapped in aluminum foil and frozen in liquid nitrogen. Aortic blood was collected *via* a glass syringe for P_{O_2} analysis. Additional aortic blood was quickly collected and frozen in liquid nitrogen to be used for lactate analysis.

Experimental protocol

To determine the degree of anoxia in the dived turtles, blood P_{O_2} and lactate levels were measured.

Blood P_{O_2} *measurement*

1 ml of aortic blood was collected *via* a glass syringe for P_{O_2} analysis using an oxygen meter (OM2000, Cameron Instruments, Port Aransas, TX, USA) previously calibrated with nitrogen-bubbled distilled water.

Deproteinization of blood samples

To deproteinize the blood samples, 450 µl of blood was mixed with 50 µl of ice-cold 70% perchloric acid and sonicated for 30 s. The mixture was then centrifuged at 10 000 ***g*** for 10 min at 2°C. A 100 µl volume of neutralizing buffer (3 mol l^{-1} KOH, 0.4 mol l^{-1} Tris, 0.3 mol l^{-1} KCl) was added to the supernatant and centrifuged at 10 000 ***g*** for 10 min at 2°C. The mixture was allowed to stand for 15 min on ice, at which time the supernatant was removed and stored at –20°C until used for lactate assay.

Lactate assay

Samples were analyzed for lactate content using a standard enzyme-coupled assay (Noll, 1974).

Two-dimensional gel analysis

Isoelectric focusing (IEF) followed the technique of O'Farrell (1975) with the modifications described by Rodenhiser et al. (1985). Glass tubes (15 cm×4 mm) were rinsed with double-distilled water, dried and one end covered with parafilm. An acrylamide solution consisting of 5.5 g urea, 1.98 ml H_2O, 2 ml 10% (octylphenoxy)polyethoxyethanol (IGEPAL), 300 µl ampholines pH 5.0–8.0, 200 µl ampholines pH 3.5–10, 1.33 ml 40% acrylamide, 11 µl 10% ammonium persulfate and 13 µl N,N,N,N′-tetraethylethylenediamine (TEMED) was quickly poured to a height of 10 cm and carefully overlaid with H_2O. After the gels had polymerized and the parafilm was removed, gels were pre-run at 200 V for 15 min, 300 V for 30 min and 400 V for 1 h. The upper buffer consisted of 50 mmol l^{-1} NaOH, and the lower buffer consisted of 138 mmol l^{-1} phosphoric acid. After the pre-run, the upper buffer was replaced with fresh 50 mmol l^{-1} NaOH, and samples suspended in 5 µl 10% IPEGAL, 3 µl ampholines pH 5.0–8.0, 2 µl ampholines pH 3.5–10, 5 µl β-mercaptoethanol and made up to 100 µl with 9 mol l^{-1} urea were placed in the tubes. Electrophoresis consisted of 14 h at 400 V and 4 h at 800 V. Following IEF separation, gels were removed from the tubes and either frozen at –20°C or equilibrated for 1 h in 20% glycerol, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) in a total volume of 100 ml 80 mmol l^{-1} Tris (pH 6.8). IEF gels were placed over a 16 cm×12 cm 5–15% SDS–polyacrylamide gradient gel and separated in the second dimension (SDS-PAGE) as described below.

Isolation of protein and western blot analysis

For protein extraction, tissues were sonicated on ice in 50 mmol l^{-1} Tris (pH 7.4), 0.5 mmol l^{-1} EDTA, 1.25 mmol l^{-1} 1,4-dithio-DL-threitol (DTT) and 0.5% Tween 20 in approximately 1 min intervals until the tissue was completely homogenized. The homogenate was then centrifuged at 10,000 ***g*** for 10 min, and the resulting supernatant fraction transferred to a fresh tube. Protein concentrations were determined using a bicinchoninic acid kit (BCA protein assay kit, Pierce, Rockford, IL, USA) and bovine serum albumin (BSA) standards. Western blot analysis was performed according to Locke and Tanguay (1996). Equal amounts of protein (50 µg, determined with the BCA kit; samples were diluted with loading buffer) were loaded into each well of an SDS–polyacrylamide (10%) gel and separated electrophoretically. Prestained molecular mass markers (Invitrogen, Burlington, ON, Canada) were used to estimate the positions of various proteins on the gel. Proteins were electro-blotted onto nitrocellulose membrane (NitroBind, Westborough, MA, USA) using a Novex (San Diego, CA, USA) mini trans-blot electrophoretic transfer unit set at 25 V or 100 mA for 2.5 h. Membranes were blocked overnight at 4°C in 5% non-fat dry skim milk in Tris buffer saline–Tween 20 (TBS-T; 20 mmol l^{-1} Tris, pH 7.5, 500 mmol l^{-1} NaCl and 0.05% Tween 20). The blots were then incubated for 4 h with rabbit polyclonal antibody against Hsp72, diluted 1:5000 with TBS-T (SPA-812; StressGen, Victoria, BC, Canada) or rat monoclonal antibody against Hsp73 diluted 1:3000 with TBS-T (SPA-815; StressGen) and washed for 2×5 min with TBS-T. Following the washes, blots were reacted for 1 h with affinity-purified goat anti-rabbit (A3687; Sigma, Oakville, ON, USA) or anti-rat conjugated with alkaline phosphatase diluted 1:3000 with TBS-T (SAB-201; Stressgen), washed for 1×5 min with TBS-T and subsequently for 1×5 min with TBS. Immunoreactivity was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) western blotting detection reagents (Invitrogen). Hsp73 blots (samples and standards) were incubated for 10 min with NBT/BCIP, and Hsp72 blots were incubated for 5 min with NBT/BCIP. All blots were performed in this manner. To quantify the amount of Hsp73 and Hsp72 expressed in the tissues, standard curves were generated using commercially obtained pure Hsp72 and Hsp73 standards (SPP-755 and SPP-751, respectively; Stressgen). The regression equations for the Hsp73 and Hsp72 standard curves are $y=134.97x-60.09$, with an r^2 value of 0.99, and $y=1922.57x+895.38$, with an r^2 value of 0.99, respectively. In addition, as an internal control, 10 ng of either Hsp72 or Hsp73 protein standard was loaded onto each experimental gel. Membranes were dried overnight, scanned using a Hewlett Packard ScanJet scanner, and quantification of bands was performed using Kodak 1D 2.0 Image Analysis Software (Kodak Scientific Imaging Systems, New Haven, CT, USA).

Statistical analysis

All data are expressed as means with corresponding S.E.M. The statistical analysis software program SigmaStat version 1.0 (SPSS, Chicago, IL, USA) was used to perform the one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* test ($P=0.05$). In Fig. 4, mean values are plotted rather than individually paired values, as it is not possible to obtain control Hsp73 data and dived Hsp72 data from the same animal.

Results

Specificity of mammalian antibodies

To determine whether mammalian antibodies to Hsp73 and Hsp72 react with turtle isoforms, two-dimensional gel electrophoresis (IEF/SDS-PAGE) followed by western blotting was performed using liver tissue (Fig. 1). Proteins from liver probed with only Hsp72 antibody demonstrated a single spot (Fig. 1A). Proteins from liver probed with only Hsp73 antibody also demonstrated only a single spot (Fig. 1B); however, the location of the spot was slightly more acidic. When proteins from liver were probed with both Hsp72 and Hsp73 antibodies, two spots appeared (Fig. 1C). No other discrete spots were observed on the blots.

Hsp73 and Hsp72 expression following heat shock

In mammals, the induction of stress proteins occurs upon exposure to a variety of stressors, including heat shock. Therefore, as a positive control for Hsp73 and Hsp72

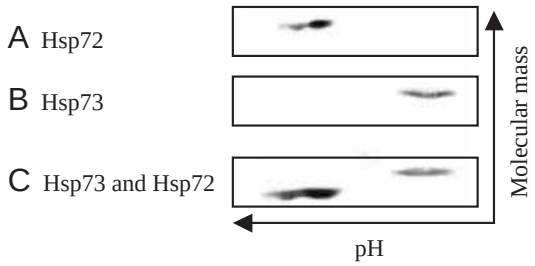

Fig. 1. Portions of representative two-dimensional [isoelectric focusing (IEF) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)] blots showing the specificity of antibodies for Hsp72 and Hsp73 in turtle liver (200 µg protein). Blots were reacted with antibodies specific to Hsp72 (A), Hsp73 (B) or both Hsp73 and Hsp72 (C). Arrows indicate increasing molecular mass and pH (pH range of original blot approximately 7.4 to 4.0).

expression, the effect of a 35°C heat shock on the expression of Hsp73 and Hsp72 was examined in turtle brain, heart, liver and skeletal muscle.

After western blot analysis, quantification by densitometry showed that Hsp73 expression remained unchanged in all tissues examined, except in liver where a significant reduction in Hsp73 was observed (Fig. 2A). By contrast, Hsp72 expression was significantly increased (P<0.05, N=4) in all tissues examined (Fig. 2B).

Metabolic status

To determine whether turtles were relying on anaerobic metabolism during the dive, blood lactate levels were measured in control animals, in animals dived for 24 h and in 1 h recovery animals. Blood lactate concentrations were 1.74±0.34 mmol l^{-1} (N=4) in controls, increased significantly to 53.14±7.60 mmol l^{-1} (N=5) after 24 h of submergence (P<0.05) and remained significantly raised after a recovery period of 1 h (53.74±3.32 mmol l^{-1}; N=4, P<0.05).

To confirm the anoxic status and to ascertain whether animals were obtaining oxygen *via* extrapulmonary respiratory routes, arterial blood oxygen (Pa_{O_2}) levels were measured under control conditions, after the 24 h dive and after the 1 h normoxic recovery. Pa_{O_2} levels dropped significantly from 2492.7±4.0 Pa (N=4) in controls to 29.3±21.3 Pa (N=5) after the 24 h dive (P<0.05) but recovered to 7991.3±485.2 Pa (N=4, P<0.05) after the 1 h recovery.

Hsp73 and Hsp72 expression and quantitative analysis after dive and recovery

Using mammalian antibodies specific for Hsp73 and Hsp72, Hsp70 isoform expression was determined by western blot analysis (Fig. 3A) and quantified by densitometry (Fig. 3B) in selected tissues of normoxic control animals (N=4) and animals following a 24 h dive (N=5) and 1 h recovery (N=4).

Brain

After the 24 h dive and 1 h recovery, basal Hsp73 expression in brain (32.50±3.80 pg µg tissue^{-1}) remained unchanged from control values (Fig. 3Bi). By contrast, Hsp72 expression increased significantly from 7.20±0.70 pg µg tissue^{-1} in controls to 14.50±1.00 pg µg tissue^{-1} after the 24 h dive and further increased to 30.50±3.50 pg µg tissue^{-1} after 1 h normoxic recovery (P<0.05; Fig. 3Bi).

Heart

Similar to the results for brain, heart Hsp73 expression (control 13.30±0.20 pg µg tissue^{-1}; Fig. 3Bii) did not change following the 24 h dive and 1 h recovery. Hsp72 expression was detectable in heart (7.20±0.70 pg µg tissue^{-1}) and increased significantly following the 24 h dive to 32.00± 4.40 pg µg tissue^{-1} (P<0.05). Following 1 h recovery, Hsp72 expression increased further to 37.80±4.00 pg µg tissue^{-1} (P<0.05; Fig. 3Bii).

Liver

The constitutive expression of Hsp73 in liver (73.20±2.10 pg µg tissue^{-1}) was the highest of all the tissues examined (Fig. 3Biii). After the 24 h dive, Hsp73 expression decreased to 45.70±6.10 pg µg tissue^{-1} and further decreased following 1 h recovery to 17.20±3.90 pg µg tissue^{-1} (P<0.05).

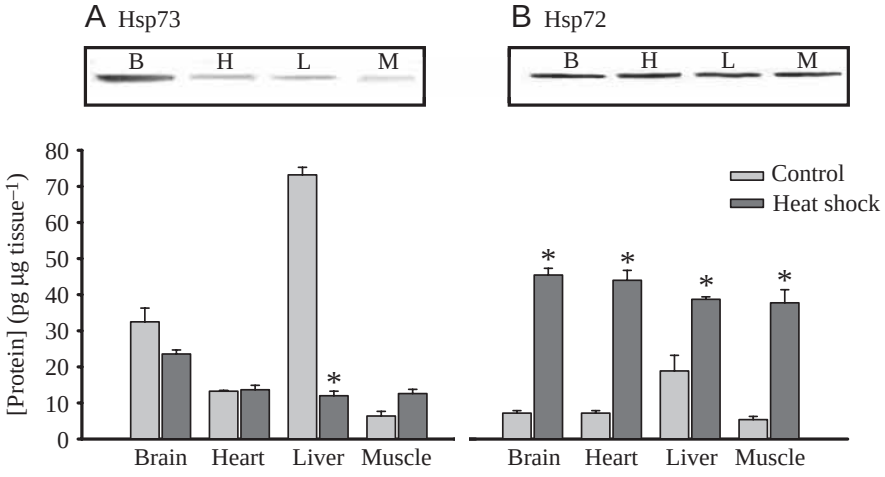

Fig. 2. Expression of Hsp73 and Hsp72 following heat shock. (A) and (B) show representative one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) western blots and the results of densitometric analyses of Hsp73 and Hsp72 expression, respectively, in turtle brain (B), heart (H), liver (L) and skeletal muscle (M) following heat shock. Values represent means ± S.E.M. of four replicate experiments and are expressed as absolute concentrations calculated from standard curves. Statistical significance (P<0.05) compared with the control is indicated by asterisks. Control western blots are shown in Fig. 3.

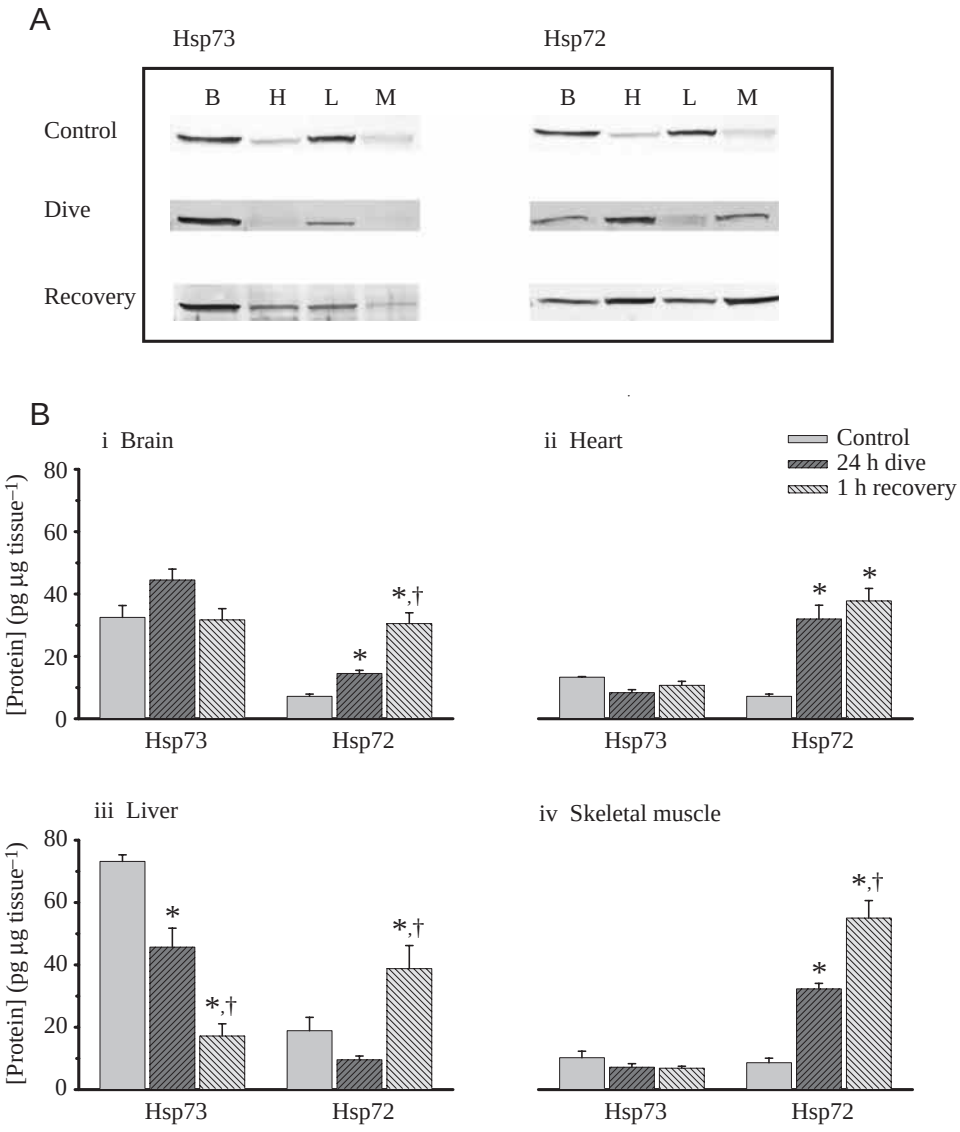

Fig. 3. Expression of Hsp73 and Hsp72 in response to 24 h dive and 1 h recovery. (A) Representative western blots of Hsp73 and Hsp72 expression in turtle brain (B), heart (H), liver (L) and skeletal muscle (M) following dive and recovery. (B) Results of densitometric analyses of Hsp73 and Hsp72 expression. Blots from control, anoxic and recovery animals were scanned, and the net intensity of each band was determined using the Kodak 1D imaging program. Values for control, dived and recovery experiments represent means ± S.E.M. of four, five and four replicates, respectively, and are expressed as absolute concentrations calculated from standard curves. Statistical significance compared with the control is indicated by asterisks, and comparison between 24 h dived and 1 h recovery tissue is denoted by daggers (P<0.05).

By contrast, basal Hsp72 expression (Fig. 3Biii) was only 18.90±4.30 pg μg tissue^{-1} and remained unchanged after a 24 h dive (9.60±1.20 pg μg tissue^{-1}). However, after 1 h recovery, Hsp72 expression was significantly increased to 38.80±7.40 pg μg tissue^{-1} (P<0.05).

Skeletal muscle

Hsp73 expression in skeletal muscle tissue (Fig. 3Biv) was relatively low in controls (6.40±1.30 pg μg tissue^{-1}) and was not significantly different from values obtained after the 24 h dive (4.50±0.70 pg μg tissue^{-1}) or 1 h recovery (4.30±0.40 pg μg tissue^{-1}). Basal Hsp72 expression (Fig. 3Biv) was also relatively low (5.40±0.90 pg μg tissue^{-1}). However, after the 24 h dive, Hsp72 expression increased significantly to 20.20±1.10 pg μg tissue^{-1} (P<0.05). An additional increase to 34.40±3.50 pg μg tissue^{-1} was observed after 1 h recovery (P<0.05).

Taken together, these results show that the expression of Hsp72, but not Hsp73, is increased in all tissues examined, except liver, after a 24 h dive and increased in all tissues following a 1 h recovery.

Discussion

While the physiological mechanisms underlying the remarkable anoxia tolerance of the freshwater turtle *Chrysemys picta belli* have been widely investigated (Bickler et al., 2000; Buck et al., 1993; Herbert and Jackson, 1985; Hochachka et al., 1996; Lutz et al., 1984; Ultsch et al., 1999), the molecular mechanisms are less well understood. In particular, the role that stress proteins may play in anoxia tolerance has received little attention. Hsp70 has been investigated in a number of mammalian species exposed to lowered oxygen levels and appears to be involved in protection against hypoxia- and reoxygenation-induced cellular damage (Donnelly et al., 1992; Hutter et al., 1994; Nakano et al., 1997). In the western painted turtle, an anoxia-tolerant reptile, we show that the expression of the inducible isoform of the Hsp70 family (Hsp72) increases in response to a 24 h forced dive, a 1 h recovery and a 1 h 35°C heat shock in all but one of the four turtle tissues examined. The exception was liver, which showed no change in Hsp72 expression after the 24 h dive, but, after 1 h recovery, levels of Hsp72 doubled in comparison to pre-anoxia levels. These results indicate both a tissue-specific and a stress-specific pattern of expression for both Hsp73 and Hsp72 isoforms. Furthermore, by employing two-dimensional gel electrophoretic analysis (Fig. 1), we demonstrate that the mammalian antibodies used in this study

are specific for the two turtle Hsp70 isoforms. The Hsp73 isoform appears slightly more acidic than the Hsp72 isoform in agreement with comparable mammalian studies (Locke et al., 1990).

Hsp70 expression following heat shock

Exposing tissues to elevated temperatures has been shown to induce heat-shock proteins in almost all organisms examined to date (Morimoto et al., 1997). In the present study, it was shown that turtles are no exception, as an ambient temperature increase from 17°C to 35°C induced Hsp72 expression in all four tissues of the western painted turtle examined (Fig. 2). Brain, heart and muscle exhibited an approximately sixfold increase and liver a twofold increase in Hsp72 levels after heat shock, while Hsp73 expression was unchanged in all tissues except liver, which showed a sixfold decrease (Fig. 2). Similar increases in Hsp70 expression have been shown in non-mammalian species, including the rainbow trout (*Oncorhynchus mykiss*; Airaksinen et al., 1998), the African clawed toad (*Xenopus laevis*; Phang et al., 1999) and the toad-headed agamid lizard (*Phrynocephalus interscapularis*; Ulmasov et al., 1999). Airaksinen et al. (1998) demonstrated that a temperature increase from 18°C to 26°C induced the synthesis of 67 kDa and 69 kDa proteins (members of the Hsp70 family of stress proteins) in cultured *O. mykiss* hepatocytes, gill epithelial cells and gonadal fibroblasts. In cultured *X. laevis* kidney epithelial cells, Phang et al. (1999) showed that a temperature increase from 22°C to 35°C induced the synthesis of Hsp70 family members. Additionally, Ulmasov et al. (1999) demonstrated that induction of Hsp70 family members occurs at 39°C and proceeds up to 47–50°C in *P. interscapularis*. In mammalian species, heat shock also results in induction of Hsp70 proteins (Bechtold et al., 2000).

Metabolic status

In the present study, turtles that had dived for 24 h were shown to be relying on anaerobic metabolism (glycolysis), as indicated by increased lactate levels. Blood lactate levels of this magnitude (53 mmol l^{-1}) are associated with severe anoxia in the turtle (Jackson et al., 1996). These data are supported by previous studies showing lactate levels in the painted turtle increasing from low control levels (<2 mmol l^{-1}) to 25 mmol l^{-1} after a 12 h dive in anoxic water at 22°C (Chang et al., 2000) and to 14 mmol l^{-1} after a 6 h dive in normoxic water at 20°C (Crocker et al., 1999).

In addition to lactate, blood P_{O_2} levels indicate that turtles were anoxic during the 24 h dive but had returned to normoxic levels after 1 h of recovery. Similar results were obtained by Crocker et al. (1999), where blood P_{O_2} was observed to drop from control levels of 11,730.4 to 186.6 Pa after a 6 h dive at 20°C. In addition, Herbert and Jackson (1985) demonstrated that by 1.5 h of submergence, blood P_{O_2} was minimal, O_2 stores were essentially exhausted and all turtles were relying primarily on anaerobic metabolism.

In the present study, blood oxygen levels of control animals were relatively low (2492.7±533.2 Pa). A possible reason for the low P_{O_2} level observed in control animals may be that turtles were voluntarily diving prior to capture and sacrifice. As turtles respire intermittently, and resting blood P_{O_2} levels can vary greatly, another explanation could be that the animals were sacrificed when blood P_{O_2} was naturally low. This observation is supported by Ultsch et al. (1999), where turtles that had access to air had variable blood P_{O_2} levels, ranging from approximately 666.5 to 11,997.0 Pa.

Hsp73 and Hsp72 expression after dive and recovery

The pattern of Hsp73 and Hsp72 expression in response to a 24 h forced dive and 1 h recovery showed tissue- and stress-specificity, an observation that is common for stress protein and mRNA expression in both mammalian and non-mammalian species (Airaksinen et al., 1998; DiDomenico et al., 1982; Manzerra et al., 1997; Rodenhiser et al., 1985). In the present study, Hsp73 expression was relatively high in brain and liver under control conditions as well as after the 24 h dive and recovery (Fig. 3Bi,iii) as compared with heart and skeletal muscle (Fig. 3Bii,iv). In contrast to Hsp73 expression, Hsp72 expression was very low in all tissues under control conditions (Fig. 3B). Hsp72 expression in liver was also very low after a 24 h dive (Fig. 3Biii), and, in brain, Hsp72 expression was induced twofold (Fig. 3Bi). By comparison, heart and skeletal muscle Hsp72 expression was induced approximately fourfold after the 24 h dive (Fig. 3Bii,iv). Furthermore, all tissues showed a significant increase in Hsp72 expression after the 1 h recovery period. These results correlate well with studies examining stress protein expression in various species where tissue- and stress-specific responses have been demonstrated (Airaksinen et al., 1998; DiDomenico et al., 1982; Manzerra et al., 1997; Rodenhiser et al., 1985).

Correlation between Hsp73 and Hsp72 expression

Interestingly, a strong correlation was observed between the expression of the two Hsp70 isoforms (Fig. 4). There is a strong correlation (r^2=0.82) between basal Hsp73 and dive-induced Hsp72 expression. High levels of Hsp73 expression, such as in brain and liver, correlated with low levels of dive-induced Hsp72 expression. Alternatively, low basal levels of Hsp73 expression, such as in heart and skeletal muscle, correlated with high levels of dive-induced Hsp72 expression. Liver displayed high Hsp73 expression under control conditions, with no induction of Hsp72 after a 24 h dive (Fig. 3iii). Brain showed intermediate Hsp73 expression under control conditions with a twofold induction of Hsp72 during the 24 h dive (Fig. 3i). By contrast, heart and skeletal muscle expressed low basal Hsp73 expression and, following a 24 h dive, exhibited an approximately fourfold increase in Hsp72 expression (Fig. 3ii,iv). Previous studies have shown similar patterns of expression correlating a high constitutive Hsp73 expression to low inducible Hsp72 expression and *vice versa*. Manzerra et al. (1997) demonstrated that rabbit cerebrum showed high expression of constitutive Hsp73 and correspondingly low expression of inducible Hsp72 upon hyperthermic conditions. Manzerra and colleagues (1997) also

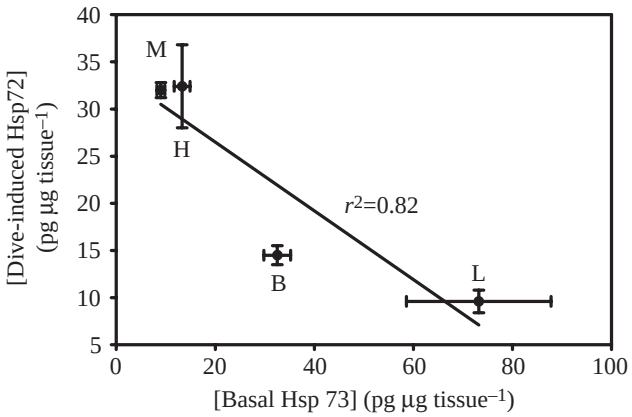

Fig. 4. Correlation between Hsp73 and Hsp72 expression after the 24 h dive. Mean values for basal Hsp73 expression in brain (B), heart (H), liver (L) and skeletal muscle (M) are plotted relative to the dive-induced Hsp72 expression in each tissue from Fig. 3. Linear regression of the data reveals a strong regression coefficient or r^2 value of 0.82 and the equation y=–0.36x+33.8. These data are the mean ± S.E.M. of 16 basal Hsp73 and 20 dive-induced Hsp72 expression measurements; mean values are presented because it is not possible to obtain both measurements from the same animal.

showed that other tissues, such as liver, heart and muscle, had an opposite expression pattern, with low Hsp73 expression and a large induction of Hsp72 expression when stressed. Carpenter and Hofmann (2000) demonstrated a similar result, showing a higher constitutive Hsp70 expression in brain tissue as compared with white muscle tissue in several different notothenid fish species. Several other studies also showed tissue-specific differences in Hsp72 induction following hyperthermia; however, these studies did not examine the constitutive levels of Hsp73 and basal levels of Hsp72 in relation to the induction response (Flanagan et al., 1995; Hotchkiss et al., 1993; Lu and Das, 1993). In addition, it was shown that when Hsp73 was directly injected into *Xenopus* oocytes prior to an increase in temperature that would normally result in Hsp72 induction, an attenuation of the stress response was observed (Mifflin and Cohen, 1994). Generally, when levels of Hsp73 or Hsp72 are elevated, the stress response appears to be diminished (DiDomenico et al., 1982; Mosser et al., 1993). Taken together, these studies demonstrate that the amount of pre-existing Hsp73 and/or Hsp72 can influence the inducible expression of Hsp72 upon exposure to stressful conditions.

The tissue-specific expression of Hsp73 is probably attributable to differences in tissue protein synthetic rates. In studies where protein synthesis was measured in rat and fish liver, brain, heart and skeletal muscle, the highest rates were found in liver (rat, Garlick et al., 1975; fish, Smith et al., 1996). The liver protein synthesis rate was 2–3-fold higher than those measured in heart, muscle and brain, which all had similar rates. Protein synthesis in turtle hepatocytes consumes 36% of the total cellular ATP turnover (Land et al., 1993), which is a much greater proportion than the 2–3% measured in heart, brain and muscle (measurements from fish, Smith et al., 1996). As Hsp73 is an important chaperone protein, it follows that tissues with high protein synthetic rates, such as liver, would have higher basal levels of Hsp73. An exception to this reasoning is found in brain, where the rate of protein synthesis is similar to the rate in heart and muscle but basal Hsp73 expression is much higher. As Manzerra et al. (1997) point out, high Hsp73 levels detected in mammalian brain are probably a result of its involvement in axonal transport of neuronal proteins and as a clathrin-uncoating ATPase in vesicle-mediated cycling events. These additional functions probably account for the high level of Hsp73 expression we detect in turtle brain.

One curious result that we cannot explain is the decrease in liver Hsp73 expression following heat shock and the 24 h forced dive. To our knowledge, this is the first time that this has been reported. We have no reason to believe that our control Hsp73 data are artifactually high. This value is a mean of measurements collected from four separate animals, and the S.E.M. is very small. Furthermore, Hsp73 expression decreased throughout the control, 24 h dive and recovery protocol, suggesting a trend. Anoxia-tolerant animals, such as the western painted turtle, reduce metabolism dramatically when faced with anoxia. One cell function that decreases is protein synthesis, which in liver decreases by 92% (Land et al., 1993). We propose that reduced levels of protein synthesis result in reduced levels of basal Hsp73 expression. This is consistent with changes in protein synthesis observed in crucian carp (*Carassius carassius*) tissues following a 48 h anoxic exposure (Smith et al., 1996); protein synthesis was found to decrease 95% in liver, 53% in heart, 54% in muscle, and no change in brain. As mentioned above, protein synthesis is such a small proportion of total energy metabolism in heart, muscle and brain that one would not expect large changes in basal Hsp73 levels resulting from decreased synthetic rates. Even though protein synthetic rates are relatively low in brain, the lack of an anoxia-mediated decrease supports our finding that basal Hsp73 levels do not change in anoxic turtle brain.

As discussed above, hyperthermia is well established as a cellular stress that results in protein denaturation; however, oxidative stress such as anoxic exposure and reoxygenation after anoxia can also lead to the denaturation of proteins. During hypoxia and subsequent reoxygenation, changes in the cellular redox status occur, altering the ratio of oxidized and reduced forms of glutathione. This, in turn, can alter the redox state of cysteine residues on various cellular proteins, resulting in partial loss of tertiary structure or denaturation (Piacentini and Karliner, 1999). Although the exact nature of the trigger for Hsp70 induction is not established, it is thought that under unstressed conditions Hsp70 members are complexed with heat shock factor (HSF) monomers. An accumulation of denatured protein displaces Hsp70, which allows HSF monomers to form a trimer, resulting in the activation of HSF and the subsequent transcription of Hsp70 (Morimoto et al., 1997). Although Hsps have not been extensively studied in the

western painted turtle, it is presumed that the same mechanism of Hsp70 induction may occur.

We have shown that Hsp73 and Hsp72 expression in turtle tissues does not follow a simple model of low basal Hsp73 and/or Hsp72 expression and induction of Hsp72 upon anoxic stress but rather three complex tissue-specific strategies. Firstly, we conclude that liver maintains a very high basal level of Hsp73 expression and minimal, if any, induction of Hsp72 upon anoxic stress. High Hsp73 levels may be a result of the high normoxic protein synthetic rates in liver and the concomitant need for increased chaperone levels. Secondly, heart and skeletal muscle display an alternative strategy, maintaining low levels of Hsp73 expression and undergoing a strong induction of Hsp72 under anoxic stress. Lastly, brain maintains intermediate basal levels of Hsp73 and undergoes a moderate induction of Hsp72 upon anoxic exposure.

A further examination of the precise molecular mechanisms underlying the anoxia tolerance of the western painted turtle and the possible role that stress proteins play in this tolerance is no doubt required. As protein synthesis is a costly process in terms of energetics, the upregulation of heat-shock proteins in anoxic tissues suggests that they may play a role in promoting anoxia tolerance.

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