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Time-dependent expression of heat shock proteins 70 and 90 in tissues of the anoxic western painted turtle

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Summary

Expression of the constitutive Hsp73, inducible Hsp72 and Hsp90 was investigated in brain, heart, liver and skeletal muscle of the anoxia-tolerant western painted turtle *Chrysemys picta bellii* in response to 2, 6, 12, 18, 24 and 30 h forced dives and following 1 h recovery from 12, 24 and 30 h forced dives at 17°C. During a dive, expression of all three Hsps examined remained at control levels for at least 12 h in all tissues examined except the liver, where Hsp72 showed a decrease at 12 h, reaching a significant threefold decrease by 24 h. Brain and liver Hsp73, 72 and 90 expression increased two- to threefold at 18, 24 and 30 h. Heart and muscle Hsp73 and heart Hsp90 expression remained at normoxic levels throughout the entire dive, while heart and muscle Hsp72 and muscle Hsp90 increased two- to fourfold at 24 and 30 h. Following reoxygenation, Hsp expression increased in all tissues examined. These data indicate that increased Hsp expression is not critical in the early adaptation to anoxic survival and that short-term anoxia is probably not a stress for species adapted to survive long periods without oxygen. However, the late upregulation of heat shock proteins during anoxia suggests that stress proteins play a role in promoting long-term anoxia tolerance.

Key words: stress protein, forced dive, recovery, western blot, *Chrysemys picta bellii*, western painted turtle.

Introduction

Most vertebrates are sensitive to oxygen deprivation, suffering irreparable cellular damage after minutes of anoxia. However, a few non-mammalian vertebrates have evolved the ability to survive anoxic conditions for days, weeks or even months, depending on the species and the ambient temperature. The western painted turtle *Chrysemys picta bellii* is the most anoxia-tolerant vertebrate known. It can survive 24 h of anoxia at 26°C and up to 6 months of anoxia at 3°C (Herbert and Jackson, 1985a,b). A key strategy of this adaptation is the maintenance of energy balance through a coordinated downregulation of ATP-producing and ATP-consuming pathways (Buck et al., 1993; Jackson, 1968).

One of the most energetically expensive processes – in terms of ATP – in the cell is protein biosynthesis. Consequently, its reduction represents a valid mechanism of saving energy during limited oxygen supply. Such a mechanism of anoxia adaptation has been demonstrated in *C. picta* and has been termed 'translational arrest' (Jackson, 2000). A 50% reduction in protein synthesis in heart and a 92% reduction in hepatocytes have been observed in response to anoxia (Bailey and Driedzic, 1996; Land et al., 1993). Interestingly, there was an upregulation in expression of five specific proteins in turtle hepatocytes under anoxia or heat shock but these proteins were not rigorously identified (Land and Hochachka, 1995). Heat shock proteins (Hsps) have been considered as possible candidates, since their expression is induced by various stresses such as hyperthermia, radiation, heavy metals, ischemia, anoxia and reoxygenation in anoxia-sensitive models (Airaksinen et al., 1998; Manzerra et al., 1997; Quraishi and Brown, 1995). Chang et al. (2000) provided the first evidence of increased Hsp70 expression in painted turtle myocardium subjected to a 12 h forced dive, but the antibody they used did not distinguish between the constitutive (Hsp73) and inducible (Hsp72) isoform. More recently, Scott et al. (2003) reported an increase in the expression of Hsp72 but not Hsp73 in brain, heart and skeletal muscle of western painted turtles force-dived for 24 h at 17°C.

Hsps are highly conserved proteins classified on the basis of their molecular mass and function. The 70 kDa family of Hsps is the best characterized and includes different isoforms. The constitutive (Hsp73) and inducible (Hsp72) isoforms of Hsp70 are both present in the unstressed cell, where they prevent premature folding of nascent polypeptides and assist translocation of other proteins to organelles. Following stress, the expression of Hsp73 is moderately upregulated whereas Hsp72 is highly induced (Lopez-Barneo et al., 2001; Manzerra et al., 1997). Under this condition they bind to damaged or misfolded polypeptides, either facilitating their repair or targeting irreparably damaged proteins for degradation by the ubiquitin/proteasome-dependent pathway (Lindquist and Craig, 1988). In contrast to the widely described 70 kDa family of stress proteins, the 90 kDa Hsp is less well characterized.

Under physiological conditions, Hsp90 specifically regulates the activity of other proteins, including steroid hormone receptors (Scheibel and Buchner, 1998), the dioxin or aryl hydrocarbon (Ah) receptor (Perdew, 1988), protein kinases (Pratt et al., 1993), calmodulin (Someren et al., 1999), actin (Miyata and Yahara, 1991) and tubulin (Garnier et al., 1998). Similarly to Hsp70, Hsp90 expression is induced in the stressed cell (Kawagoe et al., 2001; Quraishi and Brown, 1995), where it binds to partially unfolded proteins, holding them in a folding-competent state until other chaperones, such as Hsp70, are recruited to help restore the original structure of the protein.

Increased Hsp72 and Hsp90 expression in mammalian models correlates with increased protection from hypoxic/anoxic injury (for Hsp72, see Kawagoe et al., 2001; Kirino et al., 1991; Kitagawa et al., 1990; for Hsp90, see Nayeem et al., 1997). More specifically, neurons and cardiomyocytes overexpressing Hsp72 are more resistant to ischemia–reperfusion injury (Marber et al., 1995; Yenari et al., 1999), whereas Hsp72 antisense oligonucleotides increase cellular injury in mammalian cardiomyocytes (Nakano et al., 1997). Hsp90 levels increase threefold in cardiomyocytes preconditioned by mild heat shock, conveying protection from a subsequent anoxic/ischemic treatment (Nayeem et al., 1997). This evidence supports a protective role for Hsps in conditions of limiting oxygen supply. However, while a cytoprotective mechanism against such a stress is predictable in anoxia-sensitive species, it is unclear whether or not anoxia is a stressful event for anoxia-tolerant vertebrates such as the freshwater turtle.

Since this species undergoes ‘translational arrest’ during anoxia (Jackson, 2000), the specific upregulation of stress proteins in dived turtles suggests a protective role in long-term anoxic survival (Chang et al., 2000; Scott et al., 2003). However, it is not known whether turtle tissues induce Hsp expression early in a dive period or upregulation of Hsp expression occurs late in a dive as a part of a rescue mechanism triggered near the limit of survival.

The primary aim of this study, therefore, was to determine the time frame in which Hsp72 expression increases in *C. picta* during a forced dive. Since Hsp90 expression has also been shown to increase in response to hypoxia in mammalian brain (Kawagoe et al., 2001), changes in its time-course of expression during a forced dive are also determined.

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.

Male and female turtles *Chrysemys picta bellii* Schneider 1783, weighing between 250 g and 750 g, were purchased from Lemberger Co., Inc. (Oshkosh, WI, USA). Animals were housed in an indoor pond (2 m×4 m×1.5 m) equipped with basking platform, heating lamp and a flow-through dechlorinated freshwater system. The water temperature was maintained at approximately 17°C and the air temperature at 20°C. Turtles were given continuous access to food and kept on a 12 h:12 h light:dark photoperiod.

Submergence experiment

All animals were left out of the water and allowed to breathe for 1 h prior to the experiment. Groups of four turtles were placed in nine metal cages. Six cages containing four animals each were submerged for periods of 2, 6, 12, 18, 24, 30 h. To avoid aeration, all dived animals were removed from their cages underwater, neck-clamped and decapitated. Another three cages containing four animals each were first submerged for 12, 24 or 30 h and subsequently removed from the water and allowed to recover in air for 1 h. Animals were decapitated; a bone saw and a scalpel were used to separate carapace and plastron. The pericardium was then incised and a glass syringe used to collect 2 ml of blood from the aortic arc. A sample (200 μl) of blood was immediately used for determination of blood arterial P_{O_2} (Pa_{O_2}). The remaining blood was frozen in liquid nitrogen and later used for lactate and glucose analysis. Brain, heart, liver and pectoralis muscle were quickly dissected and frozen in liquid nitrogen.

Arterial blood oxygen measurement

Blood was collected *via* aortic puncture as described above. Blood Pa_{O_2} was determined using an oxygen meter (OM2000, Cameron Instruments, Yellow Springs, OH, USA) previously calibrated with distilled water equilibrated with room air (156 mmHg; 1 mmHg=98 kPa) or 100% nitrogen bubbled water (0 mmHg).

Deproteinization and neutralization of blood samples

Deproteinization of blood samples was performed by adding 50 μl of 70% perchloric acid to 450 μl of blood. The mixture was sonicated on ice for 30 s and subsequently centrifuged at 10 000 ***g*** for 10 min at 2°C. The supernatant (300 μl) was collected and a 150 μl volume of neutralizing buffer (KOH/Tris/KCl) was added to remove the perchlorate. The mixture was allowed to stand for 15 min on ice. The supernatant was collected, frozen in liquid nitrogen and stored at –20°C until used for lactate or glucose assay.

Lactate and glucose assay

Blood samples were analyzed for lactate and glucose content using a standard enzyme-coupled assay (Noll, 1974) and a modified enzymatic method (Keppler and Decker, 1974), respectively.

Tissue preparation and protein assay

Frozen tissues from control (N=5), 2, 6, 12, 24, 30 h dived (N=4 each) and 12, 24, 30 h dived and 1 h recovered (N=4 each) turtles were sonicated on ice in 50 mmol l^{-1} Tris, pH 7.4), 0.5 mmol l^{-1} 1,4-dithio-DL-threitol (DTT) and 0.5% Tween 20 at approximately 1 min intervals. The homogenates were

centrifuged at 10 000 ***g***, at 2°C for 10 min. The supernatant fraction was collected and used for protein analysis.

Protein concentrations were determined with a bicinchoninic acid kit (BCA protein assay kit; Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as a standard.

Western blot analysis

Western blot analysis was performed according to Scott et al. (2003). Samples were boiled for 5 min in loading buffer (1:1) containing sodium dodecyl sulfate (SDS; 2.5%) and 2-β-mercaptoethanol (10%). Following a 1 min centrifugation at 10 000 ***g***, equal amounts of protein (50 μg for Hsp72 and Hsp73 detection; 150 μg for Hsp90 detection) were loaded onto SDS-polyacrylamide gradient (4%–10%) gels and separated by electrophoresis at 110 V for 1.5 h. Prestained molecular mass markers (Invitrogen, Burlington, ON, Canada) were used to determine the migration of the proteins on the gel. In addition, 10 ng of pure Hsp73 (SPP-751; StressGen, Victoria, BC, Canada) or 10 ng of pure Hsp72 (NSP-555; StressGen) or 30 ng of pure Hsp90 (SPP-770; Stressgen) were used as internal controls. Proteins were transferred to nitrocellulose membranes (NitroBind, Westborough, MA, USA) by electroblotting using a Novex (San Diego, CA, USA) XCell II Mini-Cell and Blot Module unit set at 25 V or 100 mA for 2.5 h. Membranes were blocked overnight at 4°C in Tris buffer saline-Tween 20 (pH 7.5) (TBS-T; 20 mmol l^{-1} Tris, 500 mmol l^{-1} NaCl and 0.05% Tween 20) containing 5% non-fat dry skim milk. Following 2× 5 min washes with TBS-T, the blots were incubated for 4 h at room temperature with rabbit polyclonal antibody against Hsp72 (SPA-812; StressGen) diluted 1:5000 in TBS-T with 2% non-fat dry skim milk, or rat monoclonal antibody against Hsp73 (SPA-815; StressGen) diluted 1:3000 in TBS-T with 2% non-fat dry skim milk, or rat monoclonal antibody against Hsp90 (SPA-835; StressGen) diluted 1:200 in TBS-T with 2% non-fat dry skim milk. The primary mammalian antibodies against Hsp72 and Hsp73 used in this study are specific for the two turtle Hsp70 isoforms as demonstrated *via* two-dimensional gel electrophoretic analysis (Scott et al., 2003).

Following incubation with the primary antibodies, the blots were washed for 2× 5min with TBS-T and subsequently incubated for 1 h at room temperature with alkaline phosphatase (AP)-conjugated affinity-purified goat anti-rabbit (A3687; Sigma, Oakville, ON, Canada) or goat anti-rat (SAB-201; Stressgen) diluted 1:3000 in TBS-T with 2% non-fat dry skim milk. The blots were washed for 1× 5min with TBS-T and 1× 5min with TBS. Immunoreactivity was visualized by colorimetric reaction using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Invitrogen) as a chromagenic substrate for AP. Hsp73, Hsp72 and Hsp90 blots (samples and pure proteins) were incubated with NBT/BCIP for 7 min, 5 min and 5 min, respectively. The reaction was arrested by washing the blots with distilled water several times. Membranes were allowed to dry overnight.

To quantify the amount of Hsp73, Hsp72 and Hsp90 expressed in the tissues, standard curves were generated using pure Hsp73, Hsp72 and Hsp90 as standards. The regression equations for the Hsp73, Hsp72 and Hsp90 standard curves are $y=4.00x+11.13$, $r^2=0.95$; $y=3.50x+3.50$, $r^2=0.97$; and $y=0.42x+0.44$, $r^2=0.95$, respectively. Membranes were scanned with a Hewlett Packard DeskScan II scanner and bands were quantified with Lab Works 4.0 Image Analysis Software (Bioimaging System, GDS 8000, UVP Inc., Upland, CA, USA).

Statistical analysis

All data are expressed as mean ± S.E.M. Statistical analysis for P_{O_2} measurements was performed using an unpaired Student's *t*-test. Differences were considered statistically significant when $P<0.05$. Statistical analyses for lactate and glucose determinations as well as for dive and recovery data were performed using a one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls *post-hoc* test ($P<0.05$). Statistical analyses were performed using SigmaStat version 1.0 (Jandel Corporation, Chicago, IL, USA).

Results

Arterial blood oxygen measurement

To ensure that the dived turtles were anoxic, arterial blood oxygen (Pa_{O_2}) levels were measured under control conditions, after a 2, 6, 12, 18, 24 and 30 h forced dive and following 1 h of normoxic recovery after a 12, 24 and 30 h forced dive. Pa_{O_2} levels dropped significantly from 62.5±5.6 mmHg during normoxia to 2.7±0.8 mmHg after 2 h of forced dive and remained at a value indistinguishable from the zero oxygen, nitrogen-gassed water calibration point (0 to 1 mmHg) throughout the rest of the dive (not shown). Arterial blood oxygen levels returned to normoxic values following recovery.

Lactate and glucose measurement

To confirm the anoxic status of the animals during the dive, anaerobic metabolism was assessed by measuring the level of the glycolytic end-product lactate in the blood of control, dived and recovery turtles (Fig. 1). Lactate concentrations significantly increased from a control level of 2.7±0.4mmol l^{-1} to 56.6±3.6mmol l^{-1} after a 30 h dive. To establish whether the level of metabolic substrate was a limiting factor during the dive, blood glucose concentrations were determined under control conditions, after the dive and following recovery (Fig. 1). Glucose concentrations significantly increased from a control level of 2.3±0.5 mmol l^{-1} to 20.4±0.8 mmol l^{-1} after a 24 h dive and 11.5±2.8 mmol l^{-1} after a 30 h dive. Recovery for 1 h had no significant impact on lactate and glucose levels (data not shown).

Control experiment

To test the ability of Hsp expression to increase above baseline levels, painted turtles were exposed to a 40°C heat shock for 1 h followed by 1 h recovery at room temperature as previously described (Scott et al., 2003). The expression of Hsp73, 72 and 90 in turtle brain, heart, liver and skeletal

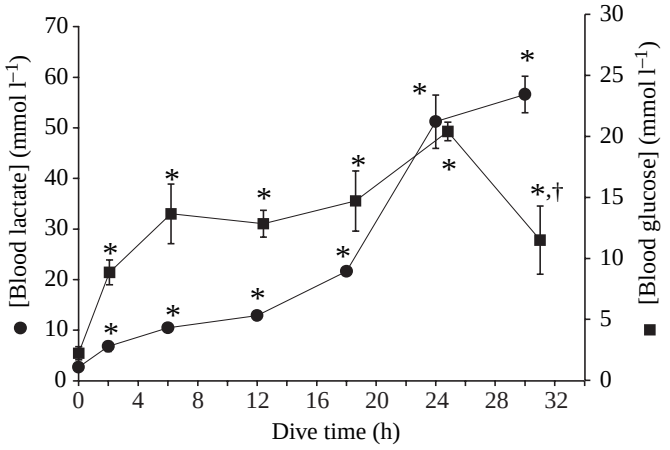

Fig. 1. Blood lactate and glucose levels in control, dived and recovery turtles. Blood lactate (circles) and glucose (squares) concentrations in control turtles and turtles force-dived for 2, 6, 12, 18, 24 and 30 h at 17°C. Values are means ± S.E.M. of 5 (control) and 4 (dive) replicates. *Statistical significance (P<0.05) from control animals. Note that symbols obscure error bars in some instances. †Statistical significance between 24 h and 30 h dived animals (P<0.05).

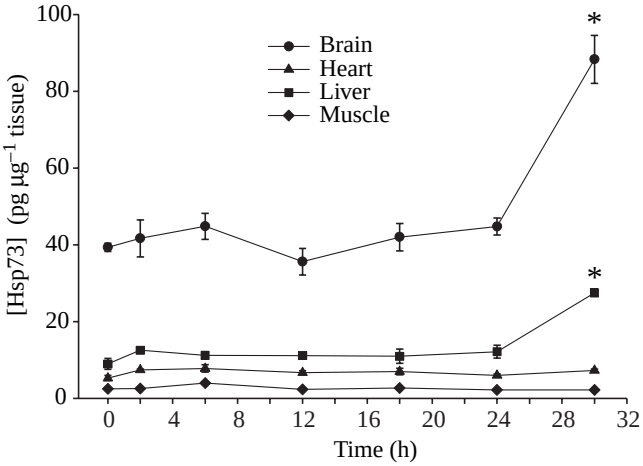

Fig. 2. Expression of Hsp73 following a forced dive. Expression of Hsp73 in tissues from turtles force-dived for 2, 6, 12, 18, 24 and 30 h. Values are means ± S.E.M. of 5 (control) and 4 (dive) replicates. *Statistical significance (P<0.05) from control animals. Note that symbols obscure error bars in some instances.

muscle followed the expected trend reported by Scott et al. (2003). Hsp72 expression increased in all tissues, Hsp73 expression increased only in heart and Hsp90 expression increased in brain and liver but not in heart and muscle (data not shown).

Hsp73, 72 and 90 expression following dive and recovery

To determine if the level of heat shock protein expression changes in turtles from the onset of anoxia through a 30 h dive and after normoxic recovery, the expression of Hsp73, 72 and 90 was determined by western blot analysis in brain, heart, liver and skeletal muscle of normoxic, anoxic and recovery turtles.

Brain

Hsp73 expression in brain increased significantly from a basal level of 39±1.1 pg μg^{-1} tissue to 88±6.6 pg μg^{-1} tissue after a 30 h dive (Fig. 2) and remained elevated following recovery (Fig. 3).

Similarly, brain Hsp72 expression increased from a control value of 12±1.9 pg μg^{-1} tissue to 23±1.2 pg μg^{-1} tissue after a 30 h dive (Fig. 4) and up to 36±2.1 pg μg^{-1} tissue following recovery (Fig. 5).

Basal Hsp90 expression in brain was 4.6±0.3 pg μg^{-1} tissue but significantly increased to 7.7±0.7 pg μg^{-1} tissue after the 18 h dive and to 15±0.6 pg μg^{-1} tissue after the 24 h dive (Fig. 6) and following recovery (Fig. 7).

Heart

Constitutive expression of Hsp73 in heart remained at control levels (5.3±0.7 pg μg^{-1} tissue) throughout the 30 h dive period (Fig. 2) and following recovery (Fig. 3).

Heart Hsp72 expression increased significantly from a control level of 19.9±2.9 pg μg^{-1} tissue to 32±1.8 pg μg^{-1} tissue following the 24 h dive and further increased to 70±1.9 pg μg^{-1} tissue following the 30 h dive (Fig. 4). The expression of Hsp72 in heart significantly increased throughout recovery (Fig. 5).

Similar to Hsp73, expression of Hsp90 in heart remained at control levels (2.2±0.1 pg μg^{-1} tissue) throughout the 30 h dive period (Fig. 6) but increased up to 8.2±0.4 pg μg^{-1} tissue following recovery (Fig. 7).

Liver

Basal expression of Hsp73 in liver increased from a control level of 9.0±1.4 pg μg^{-1} tissue to 27±0.6 pg μg^{-1} tissue following a 30 h dive (Fig. 2) and recovery (Fig. 3).

Hsp72 expression in liver followed a rather peculiar trend. It decreased from 26.3±2.7 pg μg^{-1} tissue under normoxic conditions to 10.8±0.6 pg μg^{-1} tissue following the 24 h dive but it significantly increased to 44±5.5 pg μg^{-1} tissue following a 30 h dive (Fig. 4) and up to 60±8.5 pg μg^{-1} tissue following 1 h recovery (Fig. 5).

Similar to the trend observed for brain Hsp90, basal expression of Hsp90 in liver (2.7±0.4 pg μg^{-1} tissue) increased to 4.3±0.2 pg μg^{-1} tissue after the 18 h dive and further increased to 5.6±0.7 pg μg^{-1} tissue after the 24 h and 30 h dive (Fig. 6). The expression of Hsp90 in the liver of recovery turtles was significantly higher than control levels (Fig. 7).

Pectoralis muscle

The expression of Hsp73 followed the same trend in skeletal muscle as in heart. It remained at normoxic level (2.5±0.1 pg μg^{-1} tissue) throughout the 30 h dive period and following recovery (Figs 2, 3).

Hsp72 expression followed the same trend in skeletal muscle

as in heart. Muscle Hsp72 expression remained at a control level of 9.3±0.7 pg μg^{-1} tissue throughout the 30 h dive period but increased up to 49±4.7 pg μg^{-1} tissue following the 30 h dive (Fig. 4) and up to 70±3.8 pg μg^{-1} tissue following 1 h recovery (Fig. 5).

The basal level of Hsp90 expression in skeletal muscle (1.1±0.2 pg μg^{-1} tissue) significantly increased to 2.6±0.4 pg μg^{-1} tissue following the 24 h and 30 h dives (Fig. 6) and increased to 6.1±1.6 pg μg^{-1} tissue following recovery (Fig. 7).

Discussion

More than three decades of research have shed light on the physiological mechanisms underlying anoxic survival in the naturally anoxia-tolerant freshwater turtle, *Chrysemys picta bellii* (Buck and Hochachka, 1993; Hochachka et al., 1996; Jackson, 1968; Lutz et al., 1984). However, the molecular mechanisms behind such an adaptation are less well known. Studies from anoxia-sensitive mammalian and non-mammalian species have taught us that stress proteins are rapidly upregulated as a result of low oxygen levels and seem to be involved in protection against hypoxia/anoxia and ischemia–reperfusion-induced cellular damage (Airaksinen et al., 1998; Kirino et al., 1991). However, little is known about the role stress proteins play in anoxia-tolerant species and it is unclear whether or not anoxia is even a stress for these animals.

The ability of anoxia-tolerant species to survive long periods without oxygen is partly dependent on 'translational arrest' (Jackson, 2000). Since protein biosynthesis is an expensive process in terms of ATP usage, its depression is essential to anoxic survival. In anoxic painted turtles, protein synthesis has been reported to be only 10% of the normoxic level in liver and 50% of the normoxic level in heart (Bailey and Driedzic, 1996; Land et al., 1993). In light of these findings, it is unclear whether or not Hsps would be produced during anoxia. Interestingly, two recent studies reported an increase in the expression of Hsps in tissues from the western painted turtle exposed to long-term anoxia (Chang et al., 2000; Scott et al., 2003). However, it is still unknown whether Hsp expression increases early, at the onset of anoxia, and remains at high levels throughout the anoxic period, or if it is only upregulated late during anoxia as part of a rescue mechanism triggered at the limit of survival. To understand whether or not increased Hsp expression is critical to anoxic survival, Hsp73, 72 and 90 expression was monitored in four tissues of the western painted turtle from the onset of anoxia throughout a 30 h forced dive and following reoxygenation.

The results of this study show that, with one exception, the expression of the Hsps examined in turtle brain, heart, liver and

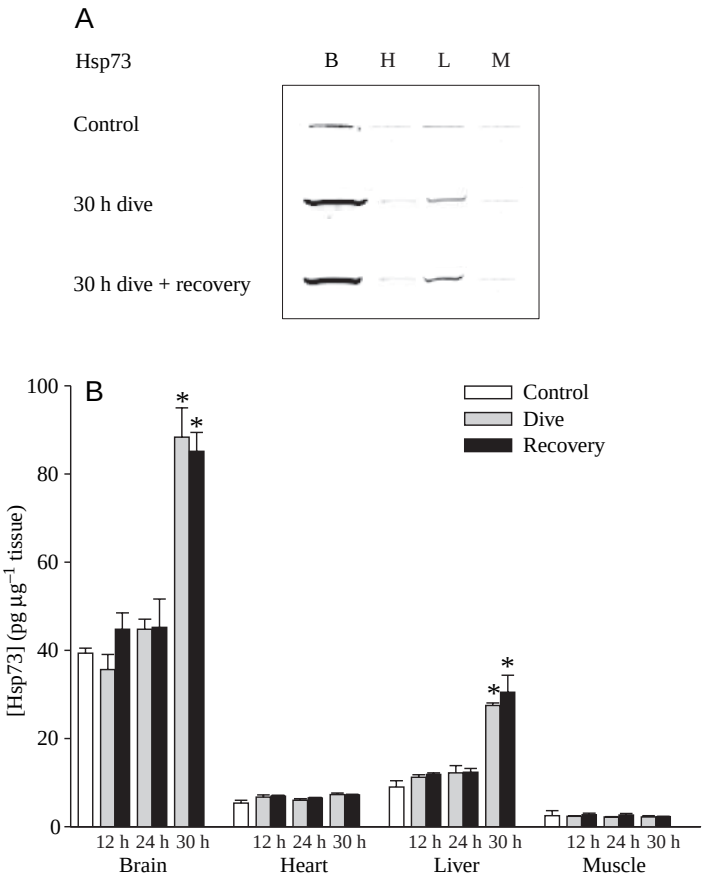

Fig. 3. Expression of Hsp73 following recovery from a forced dive. (A) Representative western blots of Hsp73 expression in turtle brain (B), heart (H), liver (L) and muscle (M) in normoxia (control), after a 30 h forced dive and following 1 h of recovery. (B) Quantitative analysis of Hsp73 expression in tissues from control turtles, turtles force-dived for 12, 24 and 30 h and turtles recovered for 1 h following a 12, 24 and 30 h dive. Values are means ± S.E.M. of 5 (control) and 4 (dive; recovery) replicates. *Statistical significance (P<0.05) from control animals; †statistical significance between dived animals and the respective recovery animals (P<0.05). Note that the error bars are obscured in some instances.

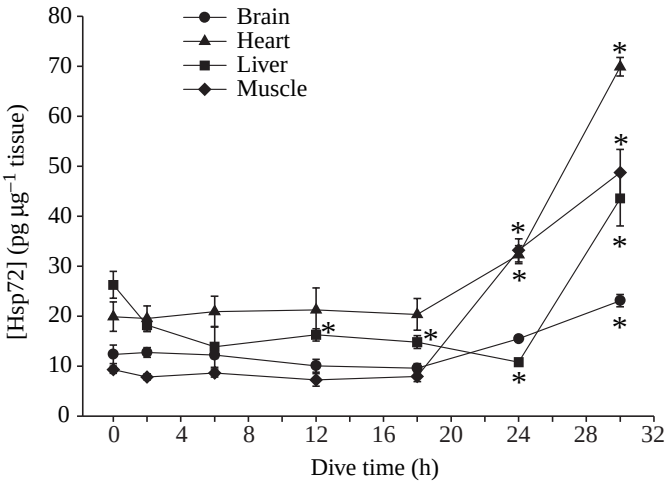

Fig. 4. Expression of Hsp72 following a forced dive. Expression of Hsp72 in tissues from turtles force-dived for 2, 6, 12, 18, 24 and 30 h. Values are means ± S.E.M. of 5 (control) and 4 (dive) replicates. *Statistical significance (P<0.05) from control animals. Note that error bars are obscured in some instances.

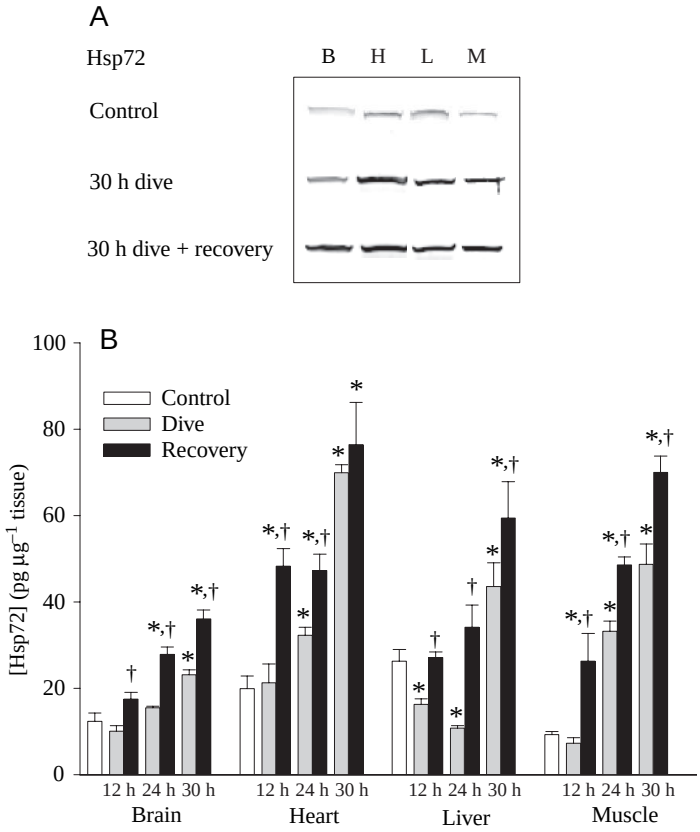

Fig. 5. Expression of Hsp72 following recovery from a forced dive. (A) Representative western blots of Hsp72 expression in turtle brain (B), heart (H), liver (L) and muscle (M) in normoxia (control), after a 30 h forced dive and following 1 h of recovery. (B) Quantitative analysis of Hsp72 expression in tissues from control turtles, turtles force-dived for 12, 24 and 30 h and turtles recovered for 1 h following a 12, 24 and 30 h dive. Values are means ± S.E.M. of 5 (control) and 4 (dive; recovery) replicates. *Statistical significance (P<0.05) from control animals; †statistical significance between dived animals and the respective recovery animals (P<0.05).

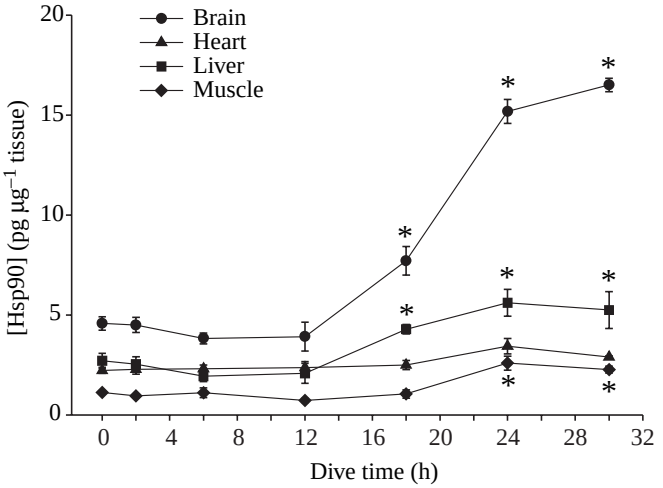

Fig. 6. Expression of Hsp90 following a forced dive. Expression of Hsp90 in tissues from turtles force-dived for 2, 6, 12, 18, 24 and 30 h. Values are means ± S.E.M. of 5 (control) and 4 (dive) replicates. *Statistical significance (P<0.05) from control animals. Note that symbols obscure error bars in some instances.

skeletal muscle did not change significantly from normoxic levels at the onset of anoxia but rapidly increased late during the dive. The exception was the liver, where basal Hsp72 expression decreased significantly throughout the dive before raising above normoxic values at the end of the anoxic period. As expected, expression of all Hsps was generally sustained or even increased upon reoxygenation. In addition, these results indicate a tissue-specific expression pattern during both anoxia and recovery.

Anaerobic status and substrate availability

As indicated by arterial blood P_{O_2} (Pa_{O_2}) levels, turtles force-dived for 30 h were anoxic throughout the dive and had returned to normoxic levels following 1 h recovery. These findings agree with previously reported values, where blood Pa_{O_2} levels dropped from normoxic values of 88 mmHg to 1.4 mmHg after a 6 h dive at 20°C (Crocker et al., 1999; Herbert and Jackson, 1985a).

Blood lactate levels were significantly higher than normoxic levels in turtles exposed to a 2 h dive and continued to increase throughout the longer dives, reaching 50 mmol l^{-1} after 30 h of submergence. This is a good indication that force-dived turtles were relying on anaerobic metabolism (glycolysis). The data are supported by previous studies where lactate levels in painted turtles increased from control levels of about 2 mmol l^{-1} to as high as 200 mmol (Chang et al., 2000; Crocker et al., 1999; Herbert and Jackson, 1985a; Ultsch and Jackson, 1982).

Together with an increase in the level of blood lactate during the dive, the present study also shows increases in blood glucose, the main metabolic substrate of anaerobic glycolysis. Blood glucose levels were significantly higher than normoxic levels in turtles exposed to a 2 h dive and continued to increase up to 20 mmol l^{-1} after a 24 h dive. These results are supported by studies where plasma glucose concentrations increased above normoxic levels to about 10 mmol l^{-1} and 16 mmol l^{-1} after 2 and 6 h anoxic dives at 22°C, respectively (Clark and Miller, 1973; Daw et al., 1967; Keiver and Hochachka, 1991; Keiver et al., 1992). Blood glucose levels reached a peak at 24 h in dived turtles but significantly decreased at 30 h. Although this is only one time point and a descending trend cannot be extrapolated, the same pattern has been observed previously (Daw et al., 1967).

Increased plasma glucose levels are probably the result of hepatic glycogenolysis; however, the importance of this is unclear. It could be essential to sustain glycolysis in the brain where the level of glycogen is low (1% w/w) compared to that of liver (15% w/w) (Clark and Miller, 1973; Sick et al., 1993).

Hsp70 and Hsp90 expression following dive and recovery

The expression of constitutive Hsp73, inducible Hsp72 and

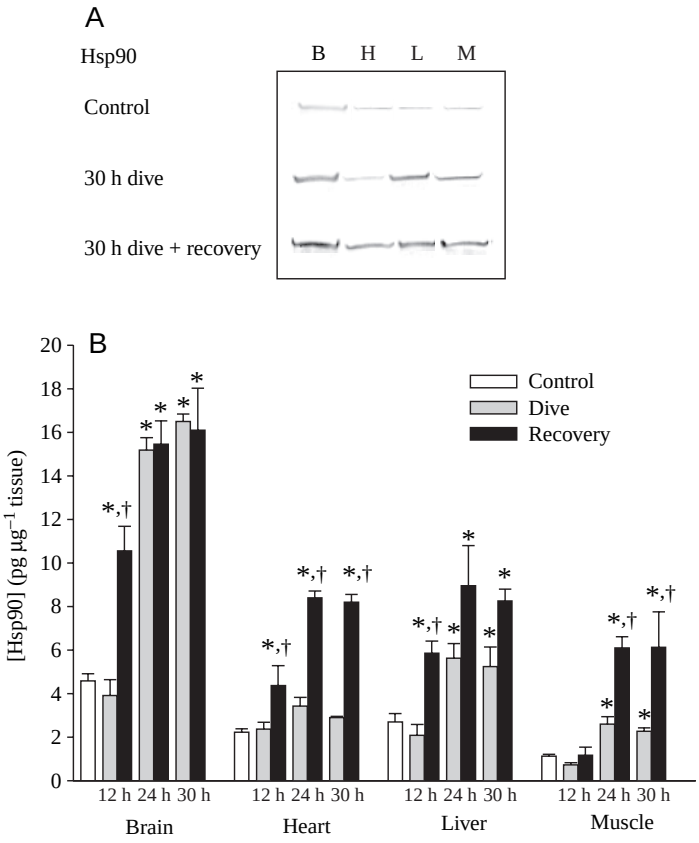

Fig. 7. Expression of Hsp90 following recovery from a forced dive. (A) Representative western blots of Hsp90 expression in turtle brain (B), heart (H), liver (L) and muscle (M) in normoxia (control), after 30 h dive and following 1 h of recovery. (B) Quantitative analysis of Hsp90 expression in tissues from control turtles, turtles force-dived for 12, 24 and 30 h and turtles recovered for 1 h following a 12, 24 and 30 h dive. Values are means ± S.E.M. of 5 (control) and 4 (dive; recovery) replicates. *Statistical significance (P<0.05) from control animals; †statistical significance between dived animals and the respective recovery animals (P<0.05). Note that error bars are obscured in some instances.

Hsp90 in *C. picta* brain, heart, liver and skeletal muscle did not increase significantly from normoxic levels during the first 12 h of a forced dive. One exception to this trend was liver Hsp72, where expression followed a curious pattern. During the dive it showed an early drop from normoxic values, reaching a significant threefold decrease by 24 h. Interestingly, forced-dive expression of Hsp73 and 72 in brain and liver increased two- to three-fold above normoxic levels at 30 h. The same forced-dive-mediated increase was detected for brain and liver Hsp90 but it occurred earlier, at 18 h. While the expression of heart and muscle Hsp73 as well as heart Hsp90 remained at normoxic levels throughout the entire dive, muscle Hsp90 as well as heart and muscle Hsp72 increased two- to fourfold at the 24 h and 30 h dive times.

Lack of early induction of Hsp73, 72 and 90 in anoxic painted turtle tissues suggests that increased expression of these Hsps is not critical in this species' adaptation to tolerate anoxia. It is unlikely that lack of early Hsp induction results from an increased basal level of Hsp expression in anoxia-tolerant species compared to anoxia-sensitive species, since it has been demonstrated that normoxic levels of heart Hsp72/73 were not significantly different in the highly anoxia-tolerant painted turtle, the relatively less anoxia-tolerant softshell turtle and the anoxia-sensitive rat and rabbit (Chang et al., 2000). Given the lower metabolic rate, lower ion channel densities and lower enzyme levels of an ectotherm such as *C. picta* compared to those of an endothermic mammal, the lack of a difference in Hsp72/73 expression actually suggests that Hsp levels are somewhat elevated in *C. picta*. This may represent an important survival strategy for anoxia-tolerant species, although our results indicate that further increases in Hsp expression are also important.

The only previous study on the effect of short-term anoxia on the expression of Hsps in anoxia-tolerant species is that of Clegg et al. (2000), who monitored changes in the expression level of combined Hsp72/73 and Hsp90 in encysted embryos of the brine shrimp *Artemia franciscana* exposed to 38 days and 2.6 years of anoxia. Since this crustacean is able to survive almost 7 years of anoxia, a 2.6 year period could be considered a relatively short-term anoxia. They reported no changes from normoxic levels in the expression of these Hsps during either anoxic period. Their findings support the results from this study.

A surprising result of the present study was the decrease in the expression of liver Hsp72 observed during short-term anoxia. Although not confirmed by this study, such a pattern has been observed in our laboratory for painted turtle liver Hsp73 expression following heat shock and a 24 h forced dive (Scott et al., 2003). The reduced levels of basal Hsp73 expression could result from reduced levels of protein synthesis, especially in the liver. Protein synthesis decreases by 92% in *C. picta* hepatocytes during anoxia (Land et al., 1995). This has also been demonstrated in the anoxia-tolerant crucian carp *Carassius carassius* where, following a 48 h anoxic exposure, protein synthesis in liver decreased by 95% of normoxic levels (Smith et al., 1996).

While the expression of Hsps (with the exception of liver Hsp72) remained at normoxic levels for most of the dive, a rapid increase was observed late in the dive protocol. These findings are supported by a previous study where the combined expression of Hsp72/73 was measured in anoxia-tolerant painted turtles and the less anoxia-tolerant softshell turtle heart exposed to a 12 h anoxic dive and recovery at 22°C (Chang et al., 2000). While the level of normoxic Hsp72/73 did not differ significantly between the two species, it increased in painted turtle heart from basal levels of 2.8 mg g^{-1} to 3.9 mg g^{-1} following the dive but decreased in softshell turtle heart from basal level of 2.4 mg g^{-1} to 1.3 mg g^{-1} following the dive. We recently extended this study (Scott et al., 2003) to brain, liver and skeletal muscle tissues, distinguishing between the constitutive (Hsp73) and the inducible (Hsp72) isoform of Hsp70. Western painted turtles exposed to a 24 h forced dive and 1 h recovery at 17°C exhibited a pattern of Hsp72 and

Hsp73 expression that was tissue-specific under both normoxic and anoxic conditions. Following the dive, expression of Hsp73 remained at normoxic levels in all tissues but the liver, where it decreased significantly from 70 pg μg^{-1} tissue to 46 pg μg^{-1} tissue. In contrast, Hsp72 expression increased in all tissues but the liver, where a downward trend was observed. With the exception of liver Hsp73, these results support the findings from the present study. The level of expression of liver Hsp73 previously measured was generally higher than that observed in this study (Scott et al., 2003). Additionally, the previously observed dive-induced decrease in liver Hsp73 expression was not detected in this study.

While the expression of Hsp72 and Hsp73 has been investigated in the anoxia-tolerant freshwater turtle (Chang et al., 2000; Scott et al., 2003), this is the first time that the expression of Hsp90 has been monitored in this species. The pattern of Hsp90 expression in turtle tissues is similar to that of Hsp73 and Hsp72; it remains at control levels early during anoxia and increases above normoxic levels during long-term anoxia. However, the upregulation of Hsp90 expression occurs earlier in the dive than Hsp73 or Hsp72. As mentioned in the introduction, Hsp90 works in concert with other Hsps by binding to partially unfolded proteins in preparation for the binding of other Hsps (Hsp72) to complete the refolding process. Thus, the early increase in Hsp90 expression that we observed may occur for this purpose.

One well-documented change in turtles exposed to anoxia is a decrease in extracellular and intracellular pH (Clark and Miller, 1973; Herbert and Jackson, 1985a,b; Jackson and Heisler, 1983; Ultsch and Jackson, 1982; Wasser et al., 1991). Blood pH of normoxic turtles is 7.8; following 2 h submergence in aerated water at 20°C it decreases by 0.4 units, by 0.6 units at 6 h and by 0.7 units at 12 h (Herbert and Jackson, 1985a,b). Intracellular pH also decreases: after 6 h submergence at 20°C, brain intracellular pH decreases from 7.5 to 6.9, heart pH from 7.2 to 7.0, liver pH from 7.5 to 6.9 and skeletal muscle pH from 7.2 to 6.8. Ultsch and Jackson (1982) monitored changes in arterial pH in turtles submerged at 3°C. The last pH value recorded before death ranged from 6.7 to 7.0 and they suggest that this could represent the lower limit to survival. Although changes in pH have not been monitored in turtles force-dived at 17°C, it is possible to extend the findings from previous work to this study and assume that turtles force-dived at 17°C experienced acidosis by 12 h of anoxia. Furthermore, lactate, an indicator of cellular acidosis, increased significantly at this temperature and time frame.

High concentrations of hydrogen ions interact with proteins and disrupt non-covalent bonds that hold proteins in a proper folded state. Interestingly, changes in intracellular pH can trigger Hsp induction (Narasimhan et al., 1996; Nishimura et al., 1989). Indeed, exposing cultured rat astrocytes to acidic medium (pH 5.5) for 3 h induced the expression of a protein belonging to the Hsp70 (68 kDa) family of stress proteins (Nishimura et al., 1989). Also, brief exposure of cultured cortical rat astrocytes to acid (pH 5.2 for 40 min) markedly induced Hsp70 mRNA and protein expression (Narasimhan et al., 1996). In addition, they showed that heat pretreatment enhanced astrocyte survival against acidosis. More recently, the rate of survival of crucian carp exposed directly to acidic water (pH 4.5) for 2 h was compared with the rate of survival of carp pretreated with a 2 h period of heat shock at 33°C before a longer (24 h) acidic shock (Martin et al., 1998). Preconditioning increased acidosis resistance in pretreated fish. In addition, the expression of Hsp70 in nervous tissues (brain and spinal cord) from pretreated carp increased by 40% of control levels.

Consistent with our previous findings from *C. picta* (Scott et al., 2003) as well as from other non-mammalian (Airaksinen et al., 1998) and mammalian species (Krueger et al., 1999), this study shows a tissue-specific pattern in the expression of the Hsps examined. Remarkable is the relatively high level of expression of Hsp73 in the brain of normoxic turtles with respect to heart, liver and muscle basal Hsp73 levels. As previously suggested (Manzerra et al., 1997), high levels of Hsp73 could reflect the involvement of this stress protein in axonal transport of neuronal proteins and synaptic vesicle recycling. Hsp73 is also thought to interact with PDZ-like domains, in the organization of postsynaptic structures and clustering of neurotransmitter receptors and ion channels (Feng and Gierasch, 1998). In addition, observation of stress-induced nuclear relocalization of Hsp73 has suggested a possible role as a nuclear shuttle for various proteins (Hayashi et al., 1991).

Perspectives

Hochachka et al. (1996) proposed that the response of hypoxia-tolerant systems to oxygen lack occurs in two phases: ‘defense’ and ‘rescue’. The first line of ‘defense’ against hypoxia includes a coordinated suppression of ATP-consuming and ATP-producing pathways to reach a new steady state level of ATP, even when energy turnover is suppressed by a factor of almost tenfold (Buck and Hochachka, 1993). While ATP demands of ion pumping are downregulated by generalized ‘channel arrest’ in cells (Hochachka, 1986) and by ‘spike arrest’ in neurons (Sick et al., 1993), ATP demands of protein synthesis are downregulated by ‘translational arrest’ (Jackson, 2000). In hypoxia-sensitive cells ‘translational arrest’ seems to be irreversible. In contrast, if the period of oxygen lack is extended, hypoxia-tolerant systems activate ‘rescue’ mechanisms by preferentially regulating the expression of several proteins. The theory of a molecular ‘rescue’ phase was proposed based on the observation that, under conditions of prolonged O_2 lack, the expression of five proteins in painted turtle hepatocytes was preferentially upregulated whereas the expression of four proteins was preferentially downregulated (Land and Hochachka, 1995).

In this study, lack of early induction of Hsp expression followed by a late upregulation of stress proteins during anoxia strongly supports the theory of ‘defense’ and ‘rescue’ phases in the adaptation to anoxic survival. Two time points are essential in determining the chronological development of this adaptation. First, the transition from normoxia to anoxia must be detected by oxygen sensing mechanism(s) and the message

delivered by signal transduction pathways that will eventually trigger the 'defense' phase. Second, the transition from short-term anoxia to long-term anoxia must be detected to induce the 'rescue' phase. Although various oxygen sensing mechanisms have been proposed (reviewed in Lopez-Barneo et al., 2001), the theory of a transition from short- to long-term anoxia is new and the sensors upstream from the 'rescue' phase are still unknown.

The definition of short- and long-term anoxia is relative to the length of time a given species can survive without oxygen and is generally temperature-dependent. The freshwater turtle *Chrysemys picta bellii* has been shown to fully recover from anoxia after 12 h at 20°C, 3 days at 15°C, 10 days at 10°C and 90 days at 3°C (Herbert and Jackson, 1985a,b). However, experiments that monitored survival time in this species reported that painted turtles can live for approximately 24 h at 26° and 155 days at 1.5°C (Musacchia, 1959). In the present study, painted turtles were force-dived for 30 h at 17°C. Although no report has described the survival time of turtles at this temperature, 30 h of anoxia is probably close to their limit of survival at 17°C. We propose that changing pH is the challenge that signals the transition from the short-term to the long-term anoxia.

In summary, we show no early induction of Hsp73, 72 and 90 expression in four tissues from force-dived western painted turtles, suggesting that increased Hsp expression is not critical in the early adaptation to anoxic survival and that short-term anoxia is probably not a stress for species adapted to survive long periods without oxygen. However, a rapid increase in the expression of all stress proteins examined occurs late during the forced dive, suggesting that increased expression of stress proteins could be part of a rescue mechanism triggered at the limit of survival.

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