Expression of heat shock proteins in turtle and mammal hearts: relationship to anoxia tolerance

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Chang, J., A. A. Knowlton, and J. S. Wasser. Expression of heat shock proteins in turtle and mammal hearts: relationship to anoxia tolerance. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R209–R214, 2000.—Heat shock proteins (HSPs) may play a cardioprotective role during hypoxia or ischemia. We hypothesized that cardiac tissue from hypoxia-tolerant animals might have high levels of specific HSPs. We measured myocardial HSP 60 and HSP 72/73 in painted and softshell turtles during normoxia and anoxia (12 h) and after recovery (12 or 24 h). We also measured myocardial HSPs in normoxic rats and rabbits. During normoxia, hearts from the most highly anoxia-tolerant species, the painted turtle, expressed the highest levels of HSP 60 (22.6 ± 2.0 mg/g total protein) followed by softshells (11.5 ± 0.8 mg/g), rabbits (6.8 ± 0.9 mg/g), and rats (4.5 ± 0.5 mg/g). HSP 72/73 levels, however, were not significantly different. HSP 60 levels in hearts from both painted and softshell turtles did not deviate significantly from control values after either 12 h of anoxia or 12 or 24 h of recovery. The pattern of changes observed in HSP 72/73 was quite different in the two turtle species. In painted turtles anoxia induced a significant increase in myocardial HSP 72/73 (from 2.8 ± 0.1 mg/g normoxic to 3.9 ± 0.2 mg/g anoxic, P < 0.05). By 12 h of recovery, HSP 72/73 had returned to control levels (2.7 ± 0.1 mg/g) and remained there through 24 h (2.6 ± 0.2 mg/g). In softshell turtles, HSP 72/73 decreased significantly after 12 h of anoxia (from 2.4 ± 0.4 mg/g normoxic to 1.3 ± 0.2 mg/g anoxic, P < 0.05). HSP 72/73 levels were still slightly below control after 12 h of recovery (2.1 ± 0.1 mg/g) and then rose to significantly above control after 24 h of recovery (4.1 ± 0.7 mg/g, P < 0.05). We also conclude that anoxia-tolerant and anoxia-sensitive turtles exhibit different patterns of myocardial HSP changes during anoxia and recovery. Whether these changes correlate with their relative degrees of anoxia tolerance remains to be determined.

Mammalian myocardium is highly sensitive to hypoxia or ischemia. During these stresses, a rapid depletion of high-energy phosphate compounds occurs, along with intracellular acidosis and a decrease in cardiac function (24). Although there are interspecific differences in cardiac anoxia and ischemia tolerance among mammal species, all mammals are incapable of surviving prolonged bouts of these stresses under physiological conditions. For example, irreversible injury begins to occur when the circumflex artery is occluded for more than 15 min in canine heart (23). One sees a similar time course for irreversible injury to occur in isolated globally ischemic rat heart (2). In contrast, however, cardiac muscle of freshwater turtles is extremely tolerant to anoxia and ischemia (6, 13, 15, 20, 21, 41–43). Turtles can maintain cardiac function for 12 wk of submergence in oxygen-free water at 3°C and recover to control levels of contraction rate and pressure generation after air breathing (15). Even at higher temperatures, the ability of turtles to withstand anoxia is impressive (15). Different turtle species have also been shown to have widely varying degrees of anoxia tolerance, at least in vivo (40). Softshell turtles, the relatively hypoxia-sensitive species, can survive only one-sixth as long as painted turtles, the most hypoxia-tolerant species, at 10°C (40).

We now know that organisms respond to the imposition of sublethal stresses by synthesizing heat shock or stress proteins (HSPs). Animals pretreated with a sublethal stress show enhanced resistance against subsequent stress, perhaps due to this stress-related increase in HSPs (29, 39, 46). There are a variety of families of HSPs present in both eukaryotic and prokaryotic cells, and they function both as molecular chaperones and by protecting proteins from denaturation. HSPs can even renature denatured proteins (25, 38, 44).

Both hypoxia and ischemia can induce the expression of HSPs or HSP mRNA in cardiac tissue (10, 11, 26, 30, 33). Elevated levels of HSP 72 can protect myocardium from hypoxic or ischemic injury and enhance the recovery of postischemic function (8, 17, 31, 36). HSP 60 is a mitochondrial heat shock protein and a critical factor in stabilizing mitochondrial oxidative enzymes and biogenesis (1, 7).

Our goal was to address the following questions: 1) do HSPs play a role in the intrinsic anoxia tolerance exhibited by turtles? 2) is there differential expression of the various types of HSP in hypoxia-sensitive mammals and hypoxia-tolerant turtles? and 3) if so, does this difference play a part in the metabolic adaptation that permits turtles to survive prolonged anoxia? We therefore measured myocardial HSP 72/73 and HSP 60 in painted and softshell turtles during normoxia and...
anoxia and after recovery. We also measured myocardial HSPs in normoxic rats and rabbits. We hypothesized that cardiac tissue from hypoxia-tolerant animals (e.g., turtles) will have high levels of specific HSPs either under normoxic conditions (constitutive HSPs) or after anoxia or recovery stresses (inducible HSPs). This superexpression of HSP may contribute to the turtles’ anoxia tolerance. Our data supported this hypothesis for HSP60 but not for HSP72/73.

**MATERIALS AND METHODS**

**Animals**

Commercially obtained western painted turtles, Chrysemys picta bellii (body wt 350–640 g, mean = 542 ± 63 g, n = 24), and softshell turtles, Trionyx spinifer (body wt 350–1800 g, mean = 982 ± 465 g, n = 18), of either sex, were used in these experiments. We maintained the painted turtles in large, fiberglass tanks with running water and a basking platform with a heating lamp. Softshell turtles were held in a fiberglass tank with running water and a 5- to 10-cm layer of sand for burrowing. Both species were fed live goldfish and moist cat food ad libitum and kept at a room temperature of ~22°C on an approximate 12:12-h light-dark cycle. We obtained male Sprague-Dawley rats (mean body wt = 313 ± 9 g, n = 6) and male New Zealand white rabbits (mean body wt = 3.05 ± 0.18 kg, n = 6) from commercial suppliers and maintained them on standard chows and water ad libitum. None of the animals were fasted prior to an experiment.

**Experimental Protocols**

Normoxia (turtles and mammals). Our experimental turtle chamber was an 80-liter fiberglass tank fitted with a custom-designed plastic insert that allowed us to isolate the turtles from one another and allowed either access to air (normoxia experiments) or forced submergence (anoxia experiments). We placed painted (n = 9) and softshell turtles (n = 7) into this chamber under normoxic conditions (room temperature, 22°C) and killed them by decapitation after 12, 24, or 36 h. We collected blood from the severed neck vessels into a heparinized glass beaker, centrifuged it to separate plasma, and deproteinated a portion (0.1 ml) in 0.2 ml 0.6 N perchloric acid. Deproteinated plasma was centrifuged and frozen for subsequent analysis of lactate. We removed the plastron (lower shell) and rapidly extracted the hearts. Hearts were perfused via the aorta with cold saline to remove blood and trimmed of their atria and great vessels, and the ventricles were then freeze-damped with liquid nitrogen-cooled aluminum clamps. Both rats and rabbits were killed by intraperitoneal injection of pentobarbital sodium (40 mg/kg body wt). The hearts were rapidly removed from the chest and rinsed with cold saline, and the ventricles were freeze-damped. Total elapsed time between decapitation and freeze-damping of turtle hearts or between opening the thoracic cavity and freeze-clamping of rat and rabbit hearts was always <5 min. Frozen tissue was stored at ~85°C until analyzed.

**Statistical analysis.** We analyzed the levels of HSPs by one-way ANOVA, followed by pairwise multiple comparison with the Student-Newman-Keuls method. Data not meeting the equal variance assumption were transformed before ANOVA. We accepted as statistically significant a level of P < 0.05. Data are presented as means ± SE.

**RESULTS**

**Normoxic Levels of HSPs**

During normoxia, hearts from the most anoxia-tolerant species, the painted turtle, expressed the highest levels of HSP60 (22.6 ± 2.0 mg/g total protein) followed by softshell turtles (11.5 ± 0.8 mg/g), rabbits (6.8 ± 0.9 mg/g), and rats (4.5 ± 0.5 mg/g, Fig. 1). The differences were significant (Fig. 2, P < 0.05). HSP72/73 levels, however, were not significantly different among these species (Fig. 3).

**HSP Changes After Anoxia and Recovery**

HSP60 levels in hearts from both painted and softshell turtles did not deviate significantly from control values after either 12 h of anoxia or 12 or 24 h of recovery (Fig. 4).

The pattern of changes observed in HSP72/73 was quite different in our two turtle species (Fig. 5). In
painted turtles myocardial HSP72/73 was significantly elevated after 12 h of submergence anoxia (3.9 ± 0.2 mg/g anoxic vs. 2.8 ± 0.1 mg/g normoxic, P < 0.05). After 12 h recovery, HSP72/73 returned to control level (2.7 ± 0.1 mg/g) and remained there through 24 h (2.6 ± 0.2 mg/g). In softshell turtles, HSP72/73 decreased significantly after 12 h of anoxia (1.3 ± 0.2 mg/g anoxic compared with 2.4 ± 0.4 mg/g normoxic, P < 0.05). HSP72/73 levels were still slightly below control after 12 h of recovery (2.1 ± 0.1 mg/g) and then rose to significantly above control after 24 h of recovery (4.1 ± 0.7 mg/g, P < 0.05).

We failed to detect expression of HSP25, HSP90, or HSP104 in hearts of either turtle species under any of our experimental conditions. We suspect that the commercial antibodies available for these stress proteins are unable to bind to the turtle proteins, although we cannot rule out their true absence in these species.

Plasma Lactate Changes After Anoxia and Recovery

Both painted and softshell turtle plasma lactate concentrations rose dramatically after 12 h of submergence anoxia (0.72 ± 0.09 mM normoxic vs. 24.13 ± 3.88 mM anoxic and 0.6 ± 0.20 mM normoxic vs. 18.64 ± 4.45 mM anoxic for painted and softshell turtles, respectively; P < 0.05, Fig. 6). However, painted turtle lactate concentrations were not significantly different compared with control levels after 12 and 24 h normoxic recovery (8.09 ± 0.84 mM and 4.75 ± 1.19 mM, respectively). Plasma lactate concentrations of softshell turtles also decreased during recovery but were still significantly higher than the control level after 12 (15.66 ± 2.31 mM) and 24 h (5.8 ± 1.48 mM).

DISCUSSION

Turtle hearts have a high degree of anoxia and ischemia tolerance compared with hearts from mammals (6, 13, 15, 19–21, 41–43). Although it is widely accepted that much of this tolerance is mediated by a rapid and dramatic decrease in metabolic rate in response to anoxic or ischemic stress (16, 18, 42, 43), the specific molecular mechanisms governing this pro-
cess remain obscure. Understanding the mechanisms underlying hypoxia and ischemia tolerance in turtles can help shed light on the injury seen in the highly hypoxia- and ischemia-sensitive mammalian heart. Different turtle species have also been shown to have widely varying degrees of anoxia tolerance, at least in vivo (40). For example, the most highly anoxia-tolerant turtle species known, the painted turtle, can survive an anoxic exposure of up to six times longer than the most sensitive species, the softshell turtle (these experiments were carried out at 10°C). In the present study, we tested the hypothesis that hearts from anoxia-tolerant species (turtles vs. mammals and painted vs. softshell turtle) would have differing degrees of stress protein expression, both under normoxic conditions and in response to anoxia and recovery. Although we screened turtle heart tissue for a range of stress proteins, our failure to detect any expression of many of these led us to concentrate our analysis on HSP60, a predominantly constitutive, mitochondrial stress protein, and HSP72/73, the inducible and constitutive forms of the 70-kDa stress protein family.

HSP60

HSP60 is a constitutive stress protein that resides in the mitochondrial matrix. It is known to be essential for cell growth and viability (1). The presence of HSP60 also appears to be a critical factor for normal protein import into and/or retranslocation to mitochondria, and is thus of fundamental importance for mitochondrial function and biogenesis (7, 28). HSP60 deficiency has been associated with a severe decrease in mitochondrial enzyme activities that can lead to fatal clinical syndromes (1). Ornatsky et al. (35) reported that the HSP60 level in the nonstimulated skeletal muscle was proportional to muscle oxidative capacity. HSP60 was moderately elevated by brief ischemia (4 × 5-min coronary occlusion) in rabbit heart (30).

In our study, myocardial expression of HSP60 under normoxic conditions did appear to correlate with in vivo anoxia tolerance. The most highly anoxia-tolerant animal studied, the painted turtle, had myocardial HSP60 levels that were five times higher than rat hearts, over three times higher than rabbit hearts, and about twice as high as softshell turtle hearts. Softshell turtle myocardial HSP60 levels were also much higher than that observed in both mammal species (P < 0.05). Knowlton et al. (27) reported low levels of HSP60 in normal human myocardium (2.72 ± 0.75 g/mg total protein), lower than either rabbit or rat hearts. We therefore maintain that high constitutive levels of HSP60 may be one of the evolutionary advantages turtles exploit to survive anoxia. Douglas et al. (12), working on another hypoxia-tolerant turtle species, the red-eared slider turtle (Trachemys scripta elegans), found that anoxic exposure induced changes in translatable mRNA populations that were organ specific. In our study we have not demonstrated a direct cause and effect relationship between normoxic HSP60 expression and anoxia tolerance, because this would require manipulating tissue levels of the stress protein and seeing whether anoxia tolerance had been altered; however, the observed correlation between HSP60 levels and hypoxia tolerance is very suggestive that HSP60 levels are an important factor in resistance to hypoxia. Experiments are currently underway in our laboratory to manipulate HSP60 levels and test directly this hypothesis.

The changes we observed in myocardial HSP60 expression during anoxia and recovery in both painted and softshell turtles were modest, and none were significantly different from the control values. This indicates that, as in mammals, in the turtle HSP60 is predominantly a constitutive, rather than an inducible, stress protein.

HSP70

The HSP70 family of stress proteins has been the most intensively studied in both pro- and eukaryote systems. HSP70 facilitates the proper folding and assembly of nascent proteins under unstressed conditions and functions to renature proteins damaged by stress. In the nonmammalian literature, Norris et al. (34) reported that the level of HSP72 (the inducible form), but not HSP73 (the constitutive form), correlated with increased thermotolerance in tropical and desert fishes.
Dillmann et al. (10) were the first to show that HSP70 increased in dog hearts in response to prolonged ischemia. Currie et al. (8) then reported that the increased HSP70 was associated with enhanced post-ischemic ventricular recovery. Hutter et al. (17) found that the amount of induced HSP72 correlated with the degree of myocardial protection. Furthermore Mestril et al. (31) and Heads et al. (14) demonstrated that a high degree of tolerance to ischemic injury and thermal stress exists in rat myogenic cells that highly expressed HSP70. Plummer et al. (36) reported that transient mouse constitutively expressing human HSP70 can protect heart from ischemia injury. Recently Nakano et al. (33) demonstrated that using antisense (to HSP72 mRNA) to block the hypoxia-induced endogenous increase in HSP72 increased the susceptibility of adult cardiac myocytes to hypoxia and reoxygenation.

In our studies, normoxic levels of HSP72/73 were not significantly different among either the turtle or mammal species examined, and for all four species the absolute concentrations of HSP72/73 were lower than that of HSP60, especially in the turtles. We looked at combined HSP72/73 levels because the available monoclonal antibodies to HSP72 did not bind to the turtle proteins. Our data show that painted turtles could express additional HSP72/73 in response to 12 h of anoxia, whereas the opposite seemed to be true for the more hypoxia-sensitive softshell turtles (myocardial levels of HSP72/73 decreased significantly after 12 h of anoxia). The observed rapid (by 12 h) return of HSP72/73 levels to control in painted turtles is also consistent with this species having a more finely tuned stress protein response than that of softshell turtles. In the latter species, we did see an increase in HSP72/73 expression, but only after 24 h of postanoxic recovery. We do not know whether these different patterns are related to their hypoxia tolerance capacity.

Although it is apparent that a wide variety of stresses will induce the expression of specific heat shock proteins, the specific biochemical steps required for the initiation of the stress response are not known. Intracellular acidosis has been suggested as a likely candidate for the trigger for anoxia-induced stress protein expression (26). Although we did not measure blood or tissue pH in this study, we did collect blood lactate data that can be correlated with pH measurements from literature on responses to anoxia in turtles (15, 22). Both turtle species had very high mixed venous lactate concentrations, indicating severe extracellular metabolic acidosis during submergence consistent with the earlier reports of Uitsh et al. (40) and Herbert and Jackson (15). For softshell and painted turtles, 12 h anoxic blood lactate concentrations were 31 and 34 times higher, respectively, than during normoxia. A few studies have shown that changes in intracellular pH and/or ATP levels may be the potential triggers for HSP expression (3, 5, 32, 45), but the results were controversial. Experiments are currently under way in our laboratory in which 31P nuclear magnetic resonance spectroscopy is used to monitor intracellular acid-base state and cell energetics to try and determine the relationship (if any) between heart cell pH and high-energy phosphate concentrations and the induction of the stress response.

In conclusion, we have quantified HSP60 and HSP72/73 in myocardial tissue from two turtle species with differing degrees of hypoxia tolerance, and from the hypoxia-sensitive rat and rabbit. Under normoxic conditions, hearts from the most hypoxia-tolerant species, the painted turtle, expressed the highest level of HSP60, followed by softshell turtle, rabbit, and rat. Following anoxic exposure or anoxia plus recovery, we observed no significant changes in myocardial HSP60 expression in hearts from either turtle species. HSP72/73 levels showed no significant differences among these species under normoxic conditions. The two turtles showed different patterns of HSP72/73 changes during anoxic submergence and recovery. We conclude that the high constitutive level of HSP60 in painted turtle hearts may contribute to the extraordinary degree of anoxia tolerance seen in this species. The early increase in HSP72/73 expression observed during anoxia in this species may also be part of a cellular adaptation promoting anoxia tolerance.

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