Strategies of Hypoxia and Anoxia Tolerance in Cardiomyocytes from the Overwintering Common Frog, *Rana temporaria*

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**ABSTRACT**

Using ventricular cardiomyocytes of the common frog, *Rana temporaria*, we investigated the metabolic strategies employed by the heart to tolerate 4 mo of hypoxic submergence (overwintering) as well as acute bouts of anoxia. In contrast to what is observed for the whole animal, there was no change in oxygen consumption in cardiomyocytes isolated from normoxic frogs compared with those isolated from 4-mo hypoxic animals. Furthermore, cells from both normoxic and hypoxic frogs were able to completely recover oxygen consumption following 30 min of acute anoxia. From estimates of ATP turnover, it appears that frog cardiomyocytes are capable of a profound, completely reversible metabolic depression, such that ATP turnover is reduced by >90% of control levels during anoxia but completely recovers with reoxygenation. Moreover, this phenomenon is also observed in frogs that have been subjected to 4 mo of extended hypoxia. We found a significant increase in the stress protein, hsp70, after 1 mo of hypoxic submergence, which may contribute to the heart’s remarkable hypoxia and anoxia tolerance and may act to defend metabolism during the overwintering period.

**Introduction**

The common frog, *Rana temporaria*, like most northern-based amphibians, must cope with several months of cold winter conditions by submergence in aquatic habitats under the cover of ice and snow. In this environment, oxygen consumption by resident organisms can outpace renewal and the PO₂ of the medium may reach very low levels (Bradford 1983). To adapt to these hypoxic conditions, the frog progressively suppresses its metabolic rate by up to 70% of that seen in the resting air-breathing animal (Donohoe and Boutilier 1998). This is thought to occur through hypoperfusion and, therefore, through decreased oxygen flow to an oxyconforming skeletal muscle mass (Donohoe and Boutilier 1998; West and Boutilier 1998), which comprises 35%–40% of the animal’s mass (Putsnam 1979). Entry into a hypometabolic state in winter months serves to lower fuel demands, thereby conserving metabolic substrates for posthibernatory activities (i.e., reproduction), as well as automatically reducing the buildup of deleterious end products such as lactate and H⁺ that occurs when hypoxic animals are forced to recruit anaerobic metabolism for the production of ATP.

Coincident with the hypoperfusion and metabolic suppression of skeletal muscle is a 33% reduction in its intracellular [K⁺] over the first month of hibernation (Donohoe et al. 2000). Unlike skeletal muscle, the frog heart shows no significant decrease in ion concentrations during hypoxic submergence (Donohoe and Boutilier 1998; Donohoe et al. 2000) and has been suggested to remain aerobic throughout the overwintering period. This may not be surprising considering that circulation must be maintained in the face of hypoxic challenge because substrates must be delivered to, and waste products removed from, all organs, particularly the brain. Furthermore, the glycogen content of the frog ventricle is significantly higher than that of the skeletal muscle, consistent with the need for continued activity in the heart during hypoxia (Donohoe and Boutilier 1998). Thus, the capacity of the frog heart to remain functional in hypoxia is vital to the animal’s overwintering survival. Frog heart exhibits a remarkable tolerance to oxygen deprivation. For example, when *R. temporaria* was exposed to anoxia at 20°C, cardiac activity was sustained at 10 beats min⁻¹ (bpm) during 3 h of exposure. Moreover, the normoxic rate of 50 bpm is rapidly recovered when the tissue is reoxygenated (Wegener and Krause 1993). In spite of the information available on whole tissue, we know very little about the cellular and molecular mechanisms of hypoxia tolerance in the frog heart.

An increasing number of genes and proteins are being identified where transcription and/or translation is stimulated when cells experience environmental stress such as hypoxia. For example, hypoxia, anoxia, and heat shock are among the stressors known to induce the synthesis of the highly conserved heat shock or stress proteins. These proteins help to maintain the metabolic and structural integrity of the cell and act as a protective response to external stimuli (Feder and Hofmann 1999).
In addition to being induced by stress, some of these proteins have essential functions under normal growth conditions and may be classified as a larger group of molecular chaperones (Hartl 1996). It is now well documented that stress proteins function in the protection of mammalian cardiac tissue from hypoxia, ischaemia, and ischaemia/reperfusion injury (Plumier et al. 1995; Cumming et al. 1996; Radford et al. 1996; Nayeem et al. 1997; Brar et al. 1999). It is, therefore, conceivable that stress proteins may have an important role in the protection of the hearts of overwintering frogs against the stress of long-term hypoxia. Indeed, Chang et al. (2000) have recently shown higher constitutive levels of the mitochondrial stress protein, hsp60, in anoxia-tolerant turtle heart compared with mammals. They also demonstrated a correlation between heat-shock protein (hsp) induction and hypoxia tolerance in the hearts of two turtle species following anoxic episodes. Thus, it seems likely that stress proteins may also play a role in the hypoxia and anoxia tolerance of the frog heart.

We have developed a cardiomyocyte preparation of the frog heart to examine cellular metabolic homeostasis and hsp induction over 4 mo of cold, hypoxic hibernation. Isolated non-contracting cardiomyocytes are ideal models to study cellular cardiac metabolism because they are uncomplicated by the energy demands of contracting myofilaments. We also investigated whether the ability of cardiomyocytes to tolerate acute episodes of anoxia differs between normoxic and hibernating frogs.

Material and Methods

Animals

The frogs used in these experiments were adult male Rana temporaria (approximately 25–30 g) collected by a local supplier (Blades Biological) during the winter of 1999. At the beginning of the winter, frogs were acclimated to 3°C water for 4 wk, during which time they had direct access to air. After this acclimation period, 15 frogs were taken for control (normoxic) experiments and 30 were submerged in hypoxic water (Po2 = 60 mmHg, 8.0 kPa) in a temperature-controlled recirculated water system (Living Stream, Frigid Units, Cleveland) maintained at 3°C as described in Donohoe and Boutilier (1998). These frogs were then sampled at 1 and 4 mo of hypoxic submergence.

Isolation of Ventricular Cardiomyocytes

Frogs were killed according to the schedule 1 home office protocol (United Kingdom). Quiescent, ventricular cardiomyocytes were isolated according to modifications of several protocols (Arrio-Dupont and De Nay 1985; Fischmeister and Hartzell 1986). Briefly, the heart was cannulated in situ via the aorta, with polyethylene tubing (PE-20) filled with Ringer’s solution at room temperature (solution A in mM: 88.4 NaCl, 23.8 NaHCO3, 0.6 NaH2PO4, 1.8 MgCl2, 5.0 d-glucose, 5.0 Na-pyruvate, 1.8 mM CaCl2) gassed with 95% O2 : 5% CO2 (pH 7.6). The heart was gently cut from the body and through-perfused with approximately 1 mL of solution A. The heart was then placed in a thermostatted reservoir at 28°C and the tubing attached to a peristaltic pump (set at a rate of 2 mL min⁻¹) and through-perfused with gassed (95%O2 : 5% CO2) solution A (−CaCl2) for approximately 20 min or until the blood was completely flushed from the tissue. The reservoir was then closed off and filled with approximately 5 mL of enzyme solution B (solution A without CaCl2 and with 0.5% bovine serum albumin, 5 mM creatine, 0.4 mg mL⁻¹ trypsin, and 2 mg mL⁻¹ collagenase Type II [Worthington]). The hearts were perfused for 40–60 min with several changes of recirculated solution B until they became soft. Following perfusion, the atria and bulbous arteriosus were removed and the ventricle was gently minced with microscissors. Approximately 2 mL of solution C (solution B without collagenase and trypsin) was added to the minced tissue, and this suspension was shaken at approximately 200 rpm for 20–30 min with continuous gassing (95% O2 : 5% CO2). The suspension was allowed to settle out for 1–2 min, and the supernatant (containing the cells) was filtered through medical gauze. To the remaining suspension, 2 mL of solution C was added, and the entire volume was shaken at 200 rpm for 5–10 min and then filtered as above. This process of shaking and filtering was repeated until a homogeneous cell suspension was obtained. The cells were then filtered and centrifuged at 500 rpm for 3 min and resuspended in 2–3 mL of solution C (+0.9 mM CaCl2). This lower concentration of calcium (half Ringer’s concentration) ensures resistance to contraction in the cells for the duration of our experiments. Three ventricles were typically used to yield an adequate number of cells for experimentation (~5 × 10⁴ cells mL⁻¹).

In addition to cardiomyocyte isolation, hearts from each group of frogs were freeze clamped for later determination of ATP and protein concentration.

Cell Counts and Viability

Initial cell viability and counts were assessed using Trypan Blue Exclusion. Cells were counted using a haemocytometer; those classified as viable were elongated and spindle shaped and exhibited no uptake of the Trypan Blue dye. The percentage of viable cells was approximately 80% as assessed by this method. However, Trypan Blue as a sole criterion tends to overestimate viability because contracted and/or damaged cells are also capable of excluding the dye (Watson et al. 1994). ATP content has been reported to be a more reliable index of metabolic viability than Trypan Blue in isolated cells (Page et al. 1992), and thus, ATP concentration was measured on freshly isolated cells and throughout experimentation. However, it is noteworthy that ATP concentration is also one of the experimental variables in this study and may be expected to change with
treatment. We have, therefore, established a lower ATP concentration limit of 22 nmol 10⁶ cells⁻¹, below which cells were considered nonviable, either at the beginning or the end of the experiment. Aliquots of cells below this ATP level did not have a high proportion of spindle-shaped healthy cells and were unable to maintain oxygen consumption levels throughout the course of the experiment.

High-Resolution Respirometry

To measure oxygen consumption rates and the O₂ affinity of isolated ventricular cardiomyocytes, a high-resolution respirometer (Oroboros Oxygraph, Paar KG, Graz) was used that enables sensitive measurements of oxygen kinetics at low oxygen partial pressures (Haller et al. 1994; Gnaiger et al. 1995). The experiment was carried out at 20°C with the temperature of the Oxygraph regulated to ±0.05°C by a Peltier heat pump. The oxygen solubility of the assay medium (solution C) with the temperature of 20°C and was equilibrated with 95% O₂ : 5% CO₂ (pH 7.6). To calculate the rate of oxygen consumption (Mo₂) and Pₜₐₓ (oxygen partial pressure at half-maximal O₂ flux), the partial pressure of O₂ (Pₒ₂) was corrected to account for the time response of the oxygen sensor (time constant), O₂ leak, background correction, and internal zero calibration. Details of the calibration and data acquisition have been well described elsewhere (Haller et al. 1994; Gnaiger et al. 1995). The signals from the oxygen electrode were recorded at 1–3-s intervals on a computer-driven data acquisition system (DatLab software, Oroboros, Innsbruck), and the stir bars in each chamber were set between 300 and 310 rpm. This level of stirring was found to be the minimal rate that preserved cell viability and provided a steady O₂ flux signal. Mechanical agitation of mammalian cardiomyocytes stimulates their oxygen consumption above basal levels (Spieckermann and Piper 1985), but this essential stirring was assumed to approximate oxygen consumption rates of cells in the normal physiological range of the beating heart (Noll et al. 1992).

Before experimentation, aliquots of cells were counted, subjected to Trypan Blue exclusion, and processed for later determination of ATP, lactate, and protein concentrations. To the oxygraph chamber, 0.5–1.0 mL of cell suspension was added. If <1.0 mL of cells were added, the volume of the chamber was brought to 1.0 mL with assay medium (solution C). The oxygen concentration in the chamber was reduced to ∼25% of air saturation by blowing 95% N₂ : 5% CO₂ on the surface of the cell suspension to decrease the time required to reach anoxia. Respiration rates observed at this saturation were not different than those at 100% oxygen saturation. ATP content was also measured at this point to ensure that cells retained metabolic viability. We observed no significant differences in ATP content compared with the control sample at the start of experimentation. Steady state levels of oxygen consumption were recorded, and then cells were allowed to consume oxygen until anoxic levels were reached. In one chamber, the experiment was terminated following 30 min of anoxia, and samples were taken for metabolites and cell counting. In the second chamber, the cells were permitted to recover by lifting the lid of the chamber for several seconds until the air saturation reached approximately 80% air saturation. The cells recovered for 30 min under these conditions, and then samples were taken as above.

To determine whether the cells remained viable in the oxygraph throughout the experiment, separate experiments were conducted where ATP content was measured over time in normoxic cells under the same conditions (i.e., stirring) as described above. No significant changes in ATP content were noted over the time period of the experiments.

Metabolites and Protein

ATP and lactate concentrations were determined on neutralised cellular perchloric acid (PCA) extracts according to the methods of Passonneau and Lowry (1993). Whole-heart ATP and protein concentration was determined on frozen (−80°C) tissue that had been pulv erised in 10 vol Tris homogenisation buffer (50 mM Tris, 2% sodium dodecyl sulphate, 0.1 mM PMSF [phenylmethylsulphonylfluoride] at pH 7.6). To this slurry, 0.1 vol of 70% PCA was added and the sample was vortexed and spun at 5,000 g for 5 min. The pellet was then washed two times in 1.0 mL 0.5 M PCA. ATP content was determined on neutralised PCA extracts (supernatant), and the protein fraction (pellet) was subsequently dissolved in 1.0 mL 0.3 M NaOH at 37°C for 3 h, and protein concentration was determined according to the bicinchoninic acid method (Smith et al. 1985).

Western Blots for hsp70/hsc70 and hsp60

Frozen samples of frog heart (50–100 mg) were homogenised in 15 vol of Tris buffer (as above) using a Polytron. An aliquot of the homogenate was measured for protein content using the bicinchoninic acid method (Smith et al. 1985), and another aliquot was mixed with an equal volume of 2× Laemmli buffer, boiled for 10 min, and cooled and spun at 10,000 g for 10 min. Myocardial proteins (40 μg protein/lane) were separated on 10% SDS polyacrylamide gel electrophoresis (Laemmli). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, blocked with skim milk, and probed with either a primary antibody specific for hsp60 (SPA-807, StressGen, 2 μg/mL) or a primary antibody that recognises both the constitutive and inducible forms of hsp70 (SPA-807, StressGen, 2 μg/mL). The PVDF membrane was then reacted with horseradish peroxidase conjugated goat anti-mouse IgG (SAB-100, StressGen). The membrane was visualised using enhanced chemiluminescence (ECL-Plus reagents; Amersham).
that contracted mammalian cardiomyocytes are metabolically in active (Ponce-Hornos et al. 1990), and thus, these rounded, contracted cells were not considered viable in our study. The measurement of ATP is thought to be a much more reliable and precise means of monitoring viability in isolated cells (Page et al. 1992). Therefore, for each cell preparation >65% viable (as assessed by Trypan Blue), we have measured ATP concentration before and after the experiment. The ability of cells to maintain oxygen consumption throughout the experiment (see below) was also indicative of high cell viability and survival.

**Oxygen Consumption and \( P_{50} \)**

The metabolic rate of cardiomyocytes isolated after 4 mo of hypoxic submergence is not significantly different than that from normoxic frogs (Fig. 1). Moreover, the cells from both groups of frogs are able to completely recover their oxygen consumption following a 30-min period of acute anoxia. To determine whether cellular oxygen affinity is affected by the overwintering period, the partial pressure of \( O_2 \) at half-maximal saturation, or \( P_{50} \), was measured. Similar to oxygen consumption, \( P_{50} \) is not different between the cardiomyocytes of normoxic and 4-mo hypoxic submerged frogs (Table 1).

Because respirometry was performed at 20°C and cells were isolated in normoxia at this temperature (for technical reasons), we cannot rule out the possibility that physiological adjustments made during the 4-mo hypoxic period are not masked in the isolation and analyses procedures. This is, unfortunately, an intractable problem in dealing with cells and/or organelles from hypoxic environments. However, this study is concerned with the more long-term changes associated with prolonged hypoxia, such as protein synthesis, and it is therefore unlikely that the methodology used here would significantly impact our findings.

**Metabolites**

The ATP content in isolated cardiomyocytes or in whole hearts was not significantly different between normoxic and hypoxic submerged frogs (Fig. 2A; Table 1). However, cellular lactate concentration from the hypoxic frogs was almost twice that of the normoxic frogs (Fig. 2B). Cardiomyocyte ATP concentration was not significantly affected by the anoxic exposure, and

![Figure 1: Oxygen consumption (\( \text{MO}_2 \)) of cardiomyocytes isolated from normoxic frogs (\( n = 7 \)) and from frogs subjected to 4 mo of hypoxic submergence (\( n = 4 \)) before 30 min of acute anoxia and following 30 min of oxygenated recovery. Values are mean ± SE.](image)

**Table 1: \( P_{50} \) and whole-heart ATP concentration**

<table>
<thead>
<tr>
<th>( P_{50} ) (kPa)</th>
<th>Whole-Heart ([\text{ATP}]) (( \mu \text{mol g tissue}^{-1} ))</th>
</tr>
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<tbody>
<tr>
<td>Normoxic</td>
<td>8.42 ± 0.12 (( n = 3 ))</td>
</tr>
<tr>
<td>Hypoxic:</td>
<td></td>
</tr>
<tr>
<td>1 mo</td>
<td>NA</td>
</tr>
<tr>
<td>4 mo</td>
<td>8.01 ± 0.32 (( n = 4 ))</td>
</tr>
</tbody>
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Note. Values from normoxic frogs and frogs submerged in hypoxic water for 1 and 4 mo. All values are given as mean ± SE. NA = not available.
ATP levels were maintained into recovery in both groups of frogs. Indeed, ATP content appeared to increase after anoxia in cells from the hypoxic animals; however, this increase was not significantly higher than the control (preanoxic) value (Fig. 2A). There was an increase in glycolysis in the cardiomyocytes from both the normoxic and hypoxic animals following anoxia, as indicated by the increase in lactate concentration (Fig. 2B). Lactate levels remained high after 30 min of recovery, but lactate production actually decreased during the recovery period (data not shown).

**ATP Turnover**

There was a profound metabolic depression observed in the cardiomyocytes after anoxia from both normoxic and hypoxic frogs, as indicated by the fact that ATP turnover, as measured from oxygen consumption (aerobic) and lactate production (anaerobic), crashed after 30 min of anoxia (Fig. 3). However, this depression is reversible, as is shown by the complete recovery of ATP turnover, which attests to the anoxia tolerance of these cells and shows that the reduced ATP turnover observed under anoxic conditions was not the result of loss of viability.

**hsp Synthesis**

Given the remarkable hypoxia tolerance of frog cardiomyocytes, we used our stored heart samples to investigate whether certain stress proteins are induced during overwintering hypoxia. Levels of the highly stress inducible hsp, hsp70/hsc70, and mitochondrial stress protein, hsp60, were measured in the frog heart during the course of hypoxic submergence for up to 4 mo. Following 1 mo of hypoxic submergence, there was a significant increase in the levels of hsp70/hsc70 in the frog heart, but these levels recovered to control values by 4 mo of hypoxia (Figs. 4, 5A). Levels of mitochondrial hsp60, however, remain unchanged during 4 mo of hypoxic submergence (Fig. 5B).

**Discussion**

We have used quiescent cardiomyocytes as a model for investigating the metabolic and molecular strategies employed by the heart of the common frog to tolerate extended periods of hypoxia as well as acute bouts of anoxia. In direct contrast to what is observed for frog skeletal muscle and, indeed, for the whole animal, we can find no evidence for metabolic rate depression in cardiomyocytes isolated after 4 mo of hypoxic submergence (Fig. 1). In skeletal muscle, in contrast, 4 mo of continued hibernation results in a significant reduction in pro-
Hypoxia and Anoxia Tolerance in Frog Cardiomyocytes

425

Figure 4. Representative western blot for hsp70 in cardiomyocytes isolated from normoxic frogs and those subjected to 1 and 4 mo of hypoxic submersion. Other details in "Material and Methods."
	on leak and a hypoperfusion of the muscle mass that is thought to contribute to a deep metabolic depression (Donohoe and Boutilier 1998; St.-Pierre et al. 2000a). At the organismal level, this translates into a metabolic rate depression of up to 75%, during which time ATP demands match ATP supply (Boutilier et al. 1997; Donohoe and Boutilier 1998). Metabolic depression is also evident at the mitochondrial level, as demonstrated by a decrease in the resting oxygen consumption rate and the "state 4" P50 value in skeletal muscle mitochondria isolated from 4 mo hypoxic submerged frogs (St.-Pierre et al. 2000b). In contrast, frog cardiomyocytes do not change their affinity for oxygen after extended hibernation (Table 1).

The metabolic homeostasis achieved in the frog cardiomyocyte is further exemplified by the cell’s ability to maintain ATP concentration at a constant level over the overwintering period (Fig. 2A). Frog skeletal muscle also preserves ATP during overwintering hypoxia, but this is achieved through a profound metabolic depression (Donohoe and Boutilier 1998; West and Boutilier 1998). It is thought that the demand for ATP is reduced in skeletal muscle by lowering the gradients for Na⁺ and K⁺ across the sarcolemma (Donohoe et al. 2000), especially as there is a lower requirement for locomotion in the hibernating frog. Indeed, the skeletal muscle exhibits a sharp decrease in intracellular potassium, [K⁺], that coincides with a reduction in Na⁺/K⁺ pump activity (Donohoe et al. 2000). In the case of the heart, the cutaneous vasculature must continue to be perfused, and oxygenated blood must be distributed to the hypoxia-sensitive cerebral circulation regardless of the surrounding oxygen conditions. Presumably for these reasons, [K⁺], (Donohoe et al. 2000), cellular [ATP] (Fig. 1), and ATP turnover (Fig. 3) may be preserved in the overwintering frog, thus maintaining ionic gradients essential for the contractility of the heart. Moreover, given that the heart represents such a small fraction of body mass (<0.5%) compared with the skeletal muscle (35%–40%), a reduction in the ATP turnover of the heart would offer little advantage in terms of whole-animal energy savings.

It is interesting to note that lactate buildup is significantly higher in the cardiomyocytes of the hypoxic-submerged frogs (Fig. 2B). It would appear that part of the cardiac cellular strategy to maintain ATP levels and metabolic rate during hibernation is to increase the glycolytic capacity of the cell (i.e., the Pasteur effect). We know that frogs recruit anaerobic metabolism during the first month of hypoxic submersion, as shown by the marked increase in lactate levels (from 1.27 in control frogs to 9.22 μmol/g tissue) and the sevenfold increase in glucose. However, both plasma lactate and glucose concentrations return to presubmergence levels by 4 mo when the metabolic rate of the frog is dramatically reduced (Donohoe and Boutilier 1998). The increased anaerobiosis shown in cardiomyocytes may be necessary to maintain metabolic rate, but given that these cells can then recover from acute anoxic exposure (see below), this increased dependence on anaerobic metabolism is obviously not severe enough to cause deleterious end-product accumulation that would limit contractile performance.

Figure 5. Quantification of levels of hsp70 (top) and hsp60 (bottom) in cardiomyocytes isolated from normoxic frogs (n = 4) and from frogs submerged in hypoxic conditions (n = 4) for 1 and 4 mo. The asterisk indicates a significant difference from both control and recovery. Values are mean ± SE.
Not only can frog cardiomyocytes tolerate periods of extended hypoxia without any significant alteration in their metabolic machinery but they are also capable of a completely reversible metabolic suppression in response to an acute anoxic episode. During complete oxygen lack, these cells demonstrate a profound metabolic depression of >95% of control rates of ATP turnover (Fig. 3). The cells have the capability to completely recover their original rates of oxygen consumption (Fig. 1) and maintain ATP concentration (i.e., viability; Fig. 2) completely recover their original rates of oxygen consumption (Fig. 1) and maintain ATP concentration (i.e., viability; Fig. 2A) throughout the experiment. The anoxia-induced metabolic depression seen here leads to a significant increase in lactate (Fig. 2B), as has also been observed in frog skeletal muscle (West and Boutilier 1998) and turtle hepatocytes (Buck et al. 1993). These frog cardiomyocytes retain their capacity to depress metabolism and recover even when they are already metabolically stretched by 4 mo of cold, hypoxic submergence. One of the hallmarks of hypoxia-tolerant cells is the ability to maintain energy balance with stable ATP concentration, which is achieved by downregulating to extremely low levels of ATP turnover (Hochachka et al. 1996). It is likely that the incredibly low ATP turnover rates observed in frog cardiomyocytes after anoxia are caused by decreases in Na⁺/K⁺ ATPase and protein synthesis as, is the case for other hypoxia-tolerant cells (Land et al. 1993). These processes normally represent a large fraction of the total cellular energy demand and must be downregulated in hypoxia; otherwise, they would dominate the remaining metabolism. The minute energy that remains during anoxia in the cell must be primarily directed toward the maintenance of ion gradients (Na⁺/K⁺ ATPase), as these are critical for survival during anoxia (Büttgereit and Brand 1995). In calculating ATP turnover, we assumed a P/O ratio of 2.5, which is maximal for NAD-linked substrates (Brand 1994). However, this ratio takes no account of mitochondrial proton leak, the futile cycling of protons across the inner membrane of the mitochondria, which can be responsible for up to 50% of the oxygen consumption in rat skeletal muscle (Rolfe and Brand 1996) and approximately 70% of the respiration in resting trout cardiomyocytes (Mortensen and Gesser 1999). If we assume that proton leak constitutes 50% of the oxygen consumption in mitochondrial-rich frog cardiomyocytes and that nonmitochondrial respiration is responsible for 10% (Rolfe and Brown 1997), only 40% of oxygen consumption is then coupled to ATP synthesis. This reduces the effective P/O ratio from 2.5 to 1 (2.5 × 0.4; Brand 1994), which would correspond to a 60% decrease in our estimates of aerobic ATP turnover. Remarkably, even when a possible 50% proton leak is considered, the metabolic depression seen after anoxia is still reduced by about 90% from control. Thus, in addition to the huge energetic investment needed to maintain aerobic capacity in the heart during hibernation, the cells are still capable of a marked metabolic suppression during acute anoxia.

The molecular mechanisms underpinning such hypoxia and anoxia tolerance remain undefined. In mammals, it has been well documented that the upregulation of specific stress proteins or hsp’s protect cardiac tissue from the stresses of hypoxia and ischaemia (Plumier et al. 1995; Cumming et al. 1996; Radford et al. 1996; Nayem et al. 1997; Brar et al. 1999). However, the frog heart is much more tolerant of hypoxia than the mammalian myocardium is, and it is conceivable that hsp’s play a major protective role in this tolerance. Indeed, in turtle heart, constitutive levels of the mitochondrial stress protein, hsp60, are higher in the more anoxia-tolerant species, Chrysemys picta bellii, than in the less tolerant soft-shelled Trionyx spinifer or in mammals (Chang et al. 2000). This observation may reflect mitochondrial-specific protection from oxygen-related stresses, as has been previously suggested (Polla et al. 1996; Bornman et al. 1998; Gaudio et al. 1998). Hsp60 is also thought to be vital for normal mitochondrial function, as it assists protein folding by binding unfolded proteins within the mitochondrial matrix, thus preventing their aggregation (Gething 1997). We found distinct constitutive levels of hsp60 in frog heart, and these levels did not fluctuate during 4 mo of hypoxia (Fig. 5), suggesting that this stress protein is mainly constitutive and is not induced in response to low oxygen stress, results similar to those recently reported for turtle hearts (Chang et al. 2000).

We also investigated the highly inducible and well-studied hsp70 stress protein. We measured levels of hsp70/hsc70 using the antibody that recognises both the constitutive and inducible forms of the protein. Hsp70 has been found to have a direct role in the protection and recovery of mammalian cardiac tissue from ischaemia and anoxia. We found a considerable, significant increase in hsp70 levels after 1 mo of hypoxic submergence that had returned to normoxic level by 4 mo (Figs. 4, 5A). Thus, within the first month of overwintering, the frog heart appears to require the protective effects of at least hsp70, which is known to help preserve protein function during stress. The pattern of change in this stress protein suggests that early stages of hibernation are far more stressful than those after 4 mo of hypoxic submergence. This may at first seem counterintuitive, as one imagines that the longer the hypoxic stress the more stressful it should become for the animal and/or cell. However, earlier results from our laboratory reveal that in the first week of hypoxic exposure, a 20-fold decrease in liver glycogen and sevenfold increase in both plasma lactate and glucose occurs (Donohoe and Boutilier 1998), indicative of a classical Pasteur effect. This anaerobic support for metabolism continues for an additional 3 wk until skeletal muscle glycogen stores are almost depleted. After the first month of hypoxic hibernation, the animal effectively rescues itself by lowering its metabolic rate. While the heart does not appear to engage in this hypometabolism, the increased expression of hsp’s in the first month of hypoxic hibernation is strongly correlated with the anaerobic stress. The upregulation of hsp70 is also likely correlated with the transition to a new steady state in metabolism that is evident by 4 mo of hypoxic submergence (Donohoe and Boutilier 1998).
It is tempting to speculate that the increased synthesis of hsp’s in the first month of overwintering contributes to the heart’s overall hypoxia tolerance and may somehow serve to defend metabolism during hibernation. Chang et al. (2000) also found significant increases in hsp70 in anoxia-tolerant C. picta after a 12-h anoxic episode. Thus, upregulation of specific stress proteins, such as hsp70, may serve to orchestrate metabolic changes that equip anaerobic hearts with a remarkable ability to sustain their metabolism during extended hypoxia. Further studies would be necessary to determine whether hsp’s have any function in coordinating the extreme metabolic depression observed after acute anoxia in this tissue.

Acknowledgments

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