Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish

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Currie, Suzanne, Bruce L. Tufts, and Christopher D. Moyes. Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R990–R996, 1999.—The physiological and biochemical signals that induce stress protein (HSP) synthesis remain conjectural. In this study, we used the nucleated red blood cells from rainbow trout, Oncorhynchus mykiss, to address the interaction between energy status and HSP gene expression. Heat shock (25°C) did not significantly affect ATP levels but resulted in an increase in HSP70 mRNA. Hypoxia alone did not induce HSP transcription in these cells despite a significant depression in ATP. Inhibition of oxidative phosphorylation with azide, in the absence of thermal stress, decreased ATP by 56% and increased lactate production by 62% but did not induce HSP gene transcription. Inhibition of oxidative phosphorylation and glycolysis with azide and iodoacetic acid respectively, decreased ATP by 79% and prevented lactate production, but did not induce either HSP70 or HSP30 gene transcription in these cells. This study demonstrates that a reduction in the cellular energy status will not induce stress protein gene transcription in rainbow trout red blood cells and may, in fact, limit induction during extreme metabolic inhibition.

ATP; ATP/ADP; heat shock protein 70; heat shock protein 30; rainbow trout; hypoxia

STRESS OR HEAT SHOCK proteins (HSPs) are a conserved group of inducible and constitutive proteins that are thought to protect cells from stresses that typically result in protein misfolding, such as hypoxia, ischemia, reactive oxygen species, or heavy metals (28, 31). The constitutive expression of these proteins indicates that, in addition to their protective function, they also have an important role in normal cell functioning, acting as molecular chaperones (14). Considerable information exists concerning the role of HSPs in folding and protection; however, the physiological and biochemical signals that induce their synthesis remain conjectural. It is noteworthy that stressful conditions are frequently accompanied by disturbances in energy metabolism, typically assessed as changes in ATP concentrations. Therefore, alterations in energy metabolism may have the potential to affect HSP function and expression at several levels.

ATP depletion is thought to cause accumulation of denatured proteins (30) and aggregation of constitutive HSP70 that ultimately triggers the heat shock response under non-heat-shock temperatures (1, 2, 25). It has also been hypothesized that during metabolic stress (i.e., decrease in ATP), the constitutive and inducible forms of HSP70 become stably bound with proteins that are unable to fold or have become denatured. As the available HSP70 levels decline, the cells respond by increasing HSP expression through a feedback mechanism yet to be described (3, 5, 29).

Several studies document a correlation between decreases in cellular energy (i.e., ATP) and HSP activation. ATP depletion was shown to be sufficient to activate the heat shock response in HeLa cells (24) and C2C12 myogenic cells (5). Ehrlich tumor cells (11), murine cell lines (30), and rat cardiomyocytes (34) also demonstrated increased synthesis of HSP70 in response to ATP depletion arising from metabolic inhibition. In contrast, some experimental models do not exhibit a correlation between ATP depletion and HSP expression. Hypoxic rat cardiomyocytes increased HSP gene expression before any significant decrease in ATP was observed (16). Furthermore, although salicylate-induced decreases in ATP levels activated heat shock factor (HSF) binding to DNA in Drosophila salivary glands, HSP gene transcription was inhibited (35). These studies have correlated changes in ATP with changes in HSP expression. However, in tissues where ADP and AMP can be further metabolized, ATP concentration alone is not a valid indicator of bioenergetic stress. In these tissues, ATP-to-ADP ratio (ATP/ADP) is a more sensitive indicator of changes in energy metabolism (e.g., Ref. 32). Thus the effect of cellular energy status on HSP synthesis remains equivocal.

In the present study, we use nucleated red blood cells from rainbow trout to address the interaction between energy status and HSP gene expression. We previously showed that thermal stress causes an induction of HSP70 synthesis in these cells, whereas severe hypoxia does not (9). It is expected that thermal and hypoxic stress would each cause deleterious or compensatory changes in energy metabolism. We have investigated this paradox by studying, in parallel, changes in HSP expression (HSP70, HSC71, HSP30) and energy metabolism (lactate production, ATP, and ADP) in response to high temperature, severe hypoxia, and inhibitors of oxidative phosphorylation and glycolysis. Collectively, these studies suggest that HSP mRNA expression does not increase as a result of bioenergetic stress in nucleated red blood cells. We have concluded that the physiology and biochemistry of the cell/tissue type is critical when evaluating stress with HSPs.

MATERIALS AND METHODS

Animals. Male and female adult rainbow trout, Oncorhynchus mykiss, (500–1,000 g) were obtained from Pure Springs Trout Farm in Belleville, Ontario, and were transported to Queen's University where they were maintained in dechlorinated freshwater tanks. The fish were acclimated at 10°C (±1°C) for at least 1 mo before experiments.

The trout were anesthetized in a buffered 3-aminobenzoic acid ethyl ester (MS-222, Sigma) fresh water mixture (1:10,000), and the dorsal aorta was cannulated using the
method of Smith and Bell (33). After surgery, the fish recovered overnight in darkened acrylic boxes supplied with aerated flowing water at 10°C.

Inhibitors. To block aerobic ATP production, sodium azide (A2), an inhibitor of cytochrome oxidase, was added to the blood to a final concentration of 5 mM. The glycolytic inhibitor iodoacetic acid (IAA; final concentration 10 mM) was added to the blood in combination with A2. We measured lactate production and ATP and ADP concentrations using several doses of A2 and IAA and determined that these concentrations were optimal.

Experimental design. Whole blood (8–14 ml) was removed from the dorsal aortic cannula of the fish and placed at 10°C in a round-bottomed flask with ~2 ml of physiological saline (in mmol/l: 124.1 NaCl, 5.1 KCl, 1.9 MgSO4, 1.5 Na2HPO4, 11.9 NaHCO3, 1.1 CaCl2, 5.6 glucose) containing 20 mmol/l EDTA to prevent clotting. In the three experimental series that are described below, whole blood (hematocrit ~25%) was aliquoted among round-bottomed flasks, treated with the appropriate inhibitor (if applicable), and held at 10°C for 10 min. Blood was then sampled (1 or 2 ml) for either metabolites or RNA analyses, as described below. This sample represented time 0. The flasks were then placed under their respective gas and temperature conditions and rotated at ~75 rpm. A 1-ml blood sample was taken for metabolite analyses after 15 min of equilibration. Samples for both metabolites and RNA (1 ml each) were taken after 2 h of equilibration.

In the first experimental series, 2 or 3 ml of whole blood were aliquoted to each of four flasks. Two flasks were treated with A2 and two flasks remained untreated. At time 0, two flasks (A2 and untreated) were transferred to 25°C and two flasks (A2 and untreated) were kept at 10°C. Blood was sampled from each treatment for later analysis of metabolites or mRNA after 15 min (metabolites only) and 2 h of equilibration. This experimental series was repeated under hypoxic conditions (100% nitrogen), with the exception of the A2 treatment.

In the final experimental series, 2 ml of whole blood were aliquoted to each of four flasks kept at 10°C. A2 was added to two flasks and one of these flasks was also treated with IAA. Three flasks (untreated, A2, A2 + IAA) were placed at 10°C, while the remaining flask (untreated) was placed at 25°C. Blood was sampled from each treatment for further analysis of metabolites and mRNA after 2-h equilibration period.

RNA isolation and Northern blot analysis. Total RNA was extracted according to the acid phenol method of Chomczynski and Sacchi (8). Total RNA (10 µg) was fractionated by standard saline citrate (SSC).

Metabolite assays. In preparation for metabolite assays, whole blood samples (1 ml) were acidified with 0.1 ml of 70% perchloric acid and centrifuged 5 min at 15,000 g. The supernatant was neutralized by addition of saturated Tris base (~0.3 ml) and 2 M KOH (~0.2 ml). Metabolites were measured spectrophotometrically at 340 nm using enzyme-coupled assays (22) modified for use on a Molecular Devices Spectramax Microplate Spectrophotometer.

Lactate was measured in samples and standards using lactate dehydrogenase (LDH). A hydrazine trap (in mM: 200 hydrazine, 500 glycine, 1 EDTA, pH 9.5) was used to drive lactate oxidation to completion. The reaction mix contained 2 mM NAD+ and 1 unit (U) LDH.

The ATP assay used the coupling enzymes glucose-6-phosphate dehydrogenase and hexokinase (HK). Addition of 1 U HK in the presence of excess glucose (10 mM) and NAD+ (2 mM) initiated the reaction.

ADP was determined by measuring the disappearance of NADH in the presence of LDH and pyruvate kinase (PK). Preincubation in the absence of PK was used to deplete the reaction mixture of pyruvate arising from the sample and from potential phosphoenolpyruvate degradation.

Statistics. A one-way ANOVA (P < 0.05) followed by Student-Newman-Keuls all pairwise comparison test (P < 0.05) was used to assess significant differences among treatment groups for metabolites, and t-tests were used to determine significant differences (P < 0.05) between 10 and 25°C. A two-way ANOVA (P < 0.05) followed by Student-Newman-Keuls all pairwise comparison test (P < 0.05) determined the significance of temperature and treatment for relative HSP band densities. A one-way ANOVA (P < 0.05) was also used to determine differences among HSP band densities in hypoxia and IAA experiments.

RESULTS

Metabolic stress in rainbow trout red blood cells. To define a metabolic stress in the rainbow trout red blood cell, we have characterized the energetic state of the cell under non-stress conditions. Metabolic rate was...
Hypoxia alone did not induce HSP transcription (Table 2). To investigate the relationship between energy status and HSP transcription we measured HSP gene transcription after treatment of rainbow trout red blood cells with metabolic inhibitors. Inhibition of oxidative phosphorylation with Az decreased ATP by 56% (Fig. 1B) and increased lactate production by 62% relative to 10°C in air (Fig. 1C) but did not induce HSP gene transcription (Figs. 2, A and B, and 3). Az also resulted in a greater decrease in ATP than that observed under normoxic or hypoxic conditions at both temperatures after 15 (data not shown) and 120 min (Fig. 1B). Az did not significantly induce expression of HSP70 or HSP30 mRNA expression after heat shock (Figs. 2, A and B, and 3) but resulted in a significantly greater decrease in ATP (Fig.

Table 1. Estimation of the energetic state of trout red blood cells under control and heat shock conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total ATP Turnover (nmol/min·g rbc)</th>
<th>Glycolytic ATP (nmol/min·g rbc)</th>
<th>Oxidative ATP (nmol/min·g rbc)</th>
<th>%Oxidative Metabolic Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56</td>
<td>11</td>
<td>45</td>
<td>80</td>
</tr>
<tr>
<td>Heat Shock</td>
<td>153</td>
<td>43</td>
<td>110</td>
<td>72</td>
</tr>
</tbody>
</table>

We have estimated 1) total ATP turnover in trout red blood cells (10), 2) anaerobic and aerobic contribution of ATP, and 3) percentage of ATP that may be attributable to oxidative phosphorylation in rainbow trout red blood cells. Cells were equilibrated under control (10°C) and heat shock (25°C) conditions in air. Glycolytic ATP contribution was calculated from lactate production (Fig. 1C) and the ATP depletion due to azide treatment (Fig. 1B). An ATP:O2 of 6 was assumed in these calculations.

Effects of heat shock and hypoxia on bioenergetics and HSP mRNA expression. A 2-h heat shock of 25°C did not significantly affect ATP concentration but resulted in a significant decrease (35%) in ATP/ADP relative to 10°C (Fig. 1A) and a significant increase (74%) in lactate production (Fig. 1C) in rainbow trout red blood cells. These metabolic alterations were accompanied by a significant increase in expression of HSP70 but not HSP30 mRNA (Figs. 2, A and B, and 3). Rainbow trout red blood cells also transcribe the constitutive member of the HSP70 family, HSC71, and this transcription was not significantly affected by heat shock (Table 1). Equilibration for 2 h under control conditions at 10°C significantly decreased ATP/ADP by 26% (Fig. 1A); however, there was no change in ATP concentration (Fig. 1B).

Fig. 1. ATP-to-ADP (ATP/ADP; A), ATP concentrations ([ATP]; B), and lactate (Lac) production (C) in rainbow trout whole blood under control conditions (air), hypoxia, and after azide treatment. Blood was equilibrated in air, 100% nitrogen (hypoxia), and with a final concentration of 5 mM sodium azide after 120 min (n = 12) at 10 or 25°C. In A and B, control was sampled at 10°C before equilibration, and in A all values were significantly lower than the control. Lactate production is significantly greater at 25°C in all treatments. *Significant (P < 0.05) difference from air; †significant (P < 0.05) difference from the hypoxic condition; ‡significant (P < 0.05) differences between 10 and 25°C. All values are expressed as means ± SE. rbc, Red blood cells.
and a significantly greater increase in lactate production (Fig. 1C) relative to heat shock.

Inhibition of oxidative phosphorylation with Az and glycolysis with IAA resulted in a >75% decrease in ATP (Fig. 4B). Lactate production was prevented (Fig. 4C), attesting to the efficacy of the IAA treatment. Despite these bioenergetic alterations, there was no induction of either HSP70 (Fig. 5) or HSP30 (data not shown) gene transcription in these cells.

The transcription of the constitutive form of HSP70, HSC71, was not significantly affected by severe hypoxia, inhibitors, or any combination of these treatments (Table 2).

**DISCUSSION**

Metabolic stress, characterized by a depletion of cellular ATP, has been proposed to be an important component of the trigger that induces stress protein synthesis. A reduction in ATP is thought to cause the HSF to bind to the heat shock element in the promoter region of the heat shock gene, thereby inducing the heat shock response (3, 28, 29). We have shown that heat shock alone has no significant effect on ATP concentrations but induces increased gene transcription of the major stress protein, HSP70, in rainbow trout red blood cells (Figs. 2 and 3). Although HSP30 mRNA also increased after heat shock, the increase was not statistically significant, which may be attributed to the high variability associated with these data. Despite the maintenance of ATP levels after heat shock, however, there is a significant decline in ATP/ADP (Fig. 2).

**Table 2. Densitometric quantification of relative HSC71 mRNA band density in rainbow trout whole blood**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Air (10°C)</th>
<th>Hypoxia (10°C)</th>
<th>Az (25°C)</th>
<th>Az + IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>10°C</td>
<td>1.9 ± 0.7</td>
<td>1.8 ± 0.4</td>
<td>1.6 ± 0.8</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>25°C</td>
<td>3.4 ± 1.9</td>
<td>1.7 ± 0.9</td>
<td>1.0 ± 0.8</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6). Blood was equilibrated 2 h in air, 100% nitrogen (hypoxia), with sodium azide (Az), and with both Az and iodoacetic acid (IAA). Values are normalized to the control condition (time 0 10°C) condition that was assigned an arbitrary value of 1. HSC71, constitutive form of heat shock protein 70; N/A, not applicable.
A decrease in ATP/ADP (increase in ADP) signals an energetic deficiency and results in a regulatory response. Any alteration in the adenylate ratio indicates that metabolism is changing, regardless of ATP concentration ([ATP]). Thus, in our study, heat shock alone probably does not cause a metabolic stress in the cell, but accelerates the rate of cellular respiration to maintain constant ATP levels. It is therefore important to examine both [ATP] and ATP/ADP to obtain a more veritable depiction of the energetic state of the cell. In terms of metabolic stress and HSP induction, however, analysis of ATP levels alone is probably sufficient.

Hypoxia causes a greater metabolic disturbance than heat shock alone, as indicated by the decrease in ATP/ADP (Fig. 1A), the depletion of ATP (Fig. 1B), and the increase in lactate production (Fig. 1C). Hypoxia has also been shown to induce stress proteins in many cells and tissues (13, 16, 17, 27), however, the results from the present study demonstrate that severe hypoxia has no effect on transcription of either HSP70 or HSP30 (Fig. 2C) in rainbow trout red blood cells. Our previous study also demonstrated that this severe hypoxic condition did not result in HSP70 protein synthesis in these cells (9). These results may be somewhat surprising considering that, unlike their mammalian counterparts, the nucleated red blood cells of lower vertebrates rely heavily on oxidative metabolism for energy (72–80% in the present study).

Changes in ATP have the potential to affect several aspects of HSP function and expression. In fact, stress proteins have an important role in energy-dependent processes, such as protein translocation into cellular organelles (4). Moreover, the binding and release of denatured proteins and short peptides to HSP70 is dependent on the hydrolysis of ATP (26). ATP is thus considered essential for proper HSP70 functioning (29). The limited information that exists on the role of ATP in the function of the small HSPs (i.e., HSP30) suggests that these HSPs do not require ATP for their function (7). As was the case with severe hypoxia, metabolic inhibition with Az and a combination of Az and IAA resulted in a significant decline in [ATP] and ATP/ADP (Figs. 1 and 4) without any significant effect on HSP70 or HSP30 mRNA (Figs. 2 and 5). This may not be
unexpected in the case of HSP30 synthesis, because the few data that are available on the small HSPs indicate that ATP depletion may actually render the stress protein inactive (21).

In addition to the role of ATP in HSP function, HSP70 expression has been shown to correlate with ATP depletion in several mammalian cells and this energetic stress is thought to trigger HSP synthesis (3, 16, 30, 34). This trigger relies on the assumption that the release of HSP70 from proteins is effectively blocked by depletion of intracellular ATP and the subsequent reduction in available HSP70 induces HSP expression (3). In the present study, however, it is unlikely that ATP would ever limit HSP70 release from proteins because the Michaelis-Menten constant for ATP [1–2 μM (26)] is several orders of magnitude lower than the lowest ATP level observed (Fig. 1B). Moreover, other studies, including the present one, demonstrate no correlation between reduced ATP levels and increased HSP70 gene transcription (16, 17, 35). There are examples where reductions in ATP will activate HSF binding but will not induce HSP transcription (5, 27, 35), which may be related to the particular HSF involved or an energy requirement at several steps in the activation of transcription (27). Although we cannot comment on HSF activation in our study, the absence of HSP transcription during severe hypoxia or metabolic inhibition observed in this study suggests that ATP-independent activation pathways of the stress response must exist.

The energetic cost of mounting a heat shock response is largely unknown. Transcription and translation are energetically expensive processes, and in fish, protein synthesis is estimated to cost 40% of the whole animal oxygen consumption (23). We have shown that rainbow trout red blood cells will not increase HSP transcription after metabolic inhibition alone (low ATP), indicating that a decrease in ATP is not part of the HSP induction mechanism in these cells. The lack of a significant heat shock response in metabolically inhibited cells (Fig. 2, A and B) suggests that under these conditions of extreme energetic stress, low ATP levels may actually limit HSP expression. Under moderate but significant decreases in ATP (i.e., heat shock and hypoxia), however, the heat shock response does not appear to be energetically limited because rainbow trout red blood cells are still capable of significantly increasing HSP70 (Fig. 2C) and HSP30 (data not shown) gene expression.

Most mammalian tissues are susceptible to the cellular damage caused by energetic stress, such as hypoxia and/or ischemia, and will respond by synthesizing HSPs (6, 27, 28). It is clear from these studies that energy stress of the magnitude observed in this study is insufficient to elicit a heat shock response in fish red blood cells. Rainbow trout red blood cells, however, may commonly be exposed to reduced oxygen levels in the circulation, particularly postexercise, and thus may be inherently hypoxia tolerant (9) as was also suggested by Ferguson et al. (10). Unlike many mammalian cells, trout red blood cells may therefore be able to cope with reduced ATP levels without requiring the protection of HSPs because hypoxia is not regarded as a potential stress. Another tissue of the rainbow trout, the myocardium, also failed to increase HSP70 synthesis after short-term hypoxic exposure (12). In addition, anoxia-tolerant turtle (Chrysemys picta bellii) hepatocytes do not synthesize stress proteins in response to reduced oxygen levels (19, 20), further suggesting that the physiology of the organism and/or tissue may be critical when assessing stress with HSPs.

In conclusion, we have shown that a reduction in cellular energy status will not induce stress protein gene transcription in rainbow trout red blood cells. This suggests there may be multiple activation pathways for the stress response, some of which may be ATP-independent. Indeed, during extreme energetic stress, such that occurs during a combined heat shock/metabolic inhibition, HSP mRNA expression actually appears to be energetically limited. The relationship between ATP and HSP expression observed in other studies may be related to cell-type specific differences in gene regulation. Nucleated red blood cells, however, appear to possess strategies that allow them to cope with reduced levels of ATP without HSP expression, thereby demonstrating that the physiology of the tissue and organism must be considered when evaluating stress protein gene transcription and synthesis. Moreover, the absence of increased HSP gene transcription in response to ATP depletion indicates that stress proteins may not be valid indicators of metabolic stress.

Perspectives

The failure of both severe hypoxia and metabolic inhibition to induce increased HSP mRNA transcription in the red blood cells of rainbow trout suggests that this cell type may be adapted to conditions of low ATP and/or oxygen levels and do not require the protection potentially afforded by HSPs. Low ATP levels do not likely limit these cells during a moderate metabolic stress of heat shock and hypoxia because, although ATP is significantly reduced, HSP gene expression is induced. It should be noted, however, that insufficient ATP levels might be responsible for the lack of a heat shock response in metabolically inhibited heat-shocked cells. Although reduced ATP levels have been implicated in the induction mechanism of the heat shock response in many cells and organisms, future studies examining 1) HSP synthesis in hypoxia-sensitive tissues (i.e., heart and brain) of the rainbow trout and 2) HSP expression in tissues of hypoxia-tolerant and -intolerant organisms are warranted to determine potential alternate signaling pathways of the heat shock response. Such pathways most likely involve accumulation of denatured proteins; however, the factors that cause proteins to denature are variable and probably depend on the physiology and biochemistry of the cell and/or organism. A complete understanding of the regulation of the heat shock response will only be obtained through integrated studies combining molecular, biochemical, and physiological approaches.

NOTE ADDED IN PROOF

Airaksinen et al. recently demonstrated that hypoxia induced the synthesis of specific proteins in rainbow trout gill.
epithelial cells [Airaksinen, S., C. M. I. Räbergh, L. Sistonen, and M. Nikinmaa. Effects of heat shock and hypoxia on protein synthesis in rainbow trout (Oncorhynchus mykiss) cells]. J. Exp. Biol. 201: 2543–2551, 1998. However, these proteins were different than those synthesized after a heat shock. Our study does not eliminate the possibility that HSP genes other than HSP70 and HSP30 are transcribed in rainbow trout red blood cells in response to a bioenergetic stress.

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REFERENCES


