Cold induced changes of adenosine levels in common eelpout (Zoarces viviparus): a role in modulating cytochrome c oxidase expression

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Accepted 14 February 2008

SUMMARY

Exposure of ectothermic organisms to variations in temperatures causes a transient mismatch between energy supply and demand, which needs to be compensated for during acclimation. Adenosine accumulation from ATP breakdown indicates such an imbalance and its reversal reflects a restoration of energy status. We monitored adenosine levels in blood serum and liver of common eelpout (Zoarces viviparus) during cold exposure in vivo. Furthermore, we tested its effect on the pattern of thermal acclimation in hepatocytes isolated from cold- (4°C) versus warm- (11°C) exposed fish. Adenosine levels increased during cold exposure in vivo and reached a transient maximum after 24 h in serum, but remained permanently elevated in liver. Whole animal cold acclimation induced a rise of liver citrate synthase activity by 44±15%, but left cytochrome c oxidase activity (COX) and RNA expression of the respective genes unchanged. Cold incubation of hepatocytes from warm-acclimated fish failed to cause an increase of mitochondrial enzyme activities despite increased COX4 mRNA levels. Conversely, warm acclimation of hepatocytes from cold-acclimated fish reduced both enzyme activities and COX2 and COX4 mRNA levels by 26–37%. Adenosine treatment of both warm- and cold-acclimated hepatocytes suppressed COX activities but activated COX mRNA expression. These effects were not receptor mediated. The present findings indicate that adenosine has the potential to regulate mitochondrial functioning in vivo, albeit the pathways resulting in the contrasting effects on expression and activity need to be identified.

Key words: hepatocytes, primary culture, adenosine, temperature acclimation, cytochrome c oxidase, citrate synthase, RNase protection assay.
as a ‘retaliatory metabolite’ (Newby et al., 1990). These effects include a reduction of protein synthesis rate (Tinton et al., 1995), a decrease of oxygen consumption (Krumschnabel et al., 2000) or a stimulation of anaerobic glycolysis (Lutz and Nilson, 1997). Adenosine is usually examined in the context of hypoxic or anoxic exposure (Lutz and Kabler, 1997; Reipschläger et al., 1997; Renshaw et al., 2002). However, since thermal acclimation in fact involves compensation for temperature-induced hypoxia (Pörtner, 2002) adenosine may also play a role in temperature adaptation.

The purpose of this study was to examine (1) if acute temperature changes affect adenosine concentrations in ectothermic marine fish, (2) if adjustments in mitochondrial function observed in vivo can be induced in isolated fish hepatocytes with temperature as a single factor, (3) if adenosine participates in the regulation of mitochondrial adjustments to temperature, (4) how adenosine action on mitochondrial functions might be mediated. This study was performed in the common eelpout, *Zoarces viviparus*, which has become a model organism to study thermal acclimation and limitation (Pörtner and Knust, 2007). Here we describe, for the first time, that adenosine is a suitable effector to modulate the thermally induced cellular acclimation response.

**MATERIALS AND METHODS**

**Animals**

Adenosine concentrations were determined in tissues of laboratory-born offspring (first generation) of common North Sea eelpout *Zoarces viviparus* L., caught in the German Bight near Helgoland. Fish were raised in the aquarium of the Alfred Wegener Institute, Bremerhaven at 11.0±0.5°C and 30‰ salinity and fed twice per week with small shrimps (*Neomysis integer, Crangon crangon*). Animals (mass: 13.5±1.2 g, mean ± s.e.m.) were acutely transferred to 4.0±0.5°C and sampled before (t=0) and after 1 and 3 days of cold incubation. For the preparation of hepatocytes North Sea eelpout, caught in the German Bight in spring 2004 (mass: 67.3±25.9 g) were acclimated to either 4.0±0.5°C (representing mean winter habitat temperature) or 11.0±0.5°C (close to optimum temperature) and a salinity of 30% for at least 2 months. During the acclimation period specimens were fed once per week with *Crangon crangon*.

**Determination of adenosine concentrations**

Blood was collected from fish anaesthetised with 0.5 g l⁻¹ MS-222 (3-amino-benzoic-methanosulfonate) by opening the caudal vein. Livers were excised, immediately frozen in liquid nitrogen and animals were killed by a cut through the spinal cord. The liver of the first fish was carefully excised and suspended in 3.5 volumes ice-cold TCA and processed as described for serum samples, but the pH of the extracts was adjusted to 9.0–9.4 with 2 mol l⁻¹ NaOH.

Adenosine was determined by capillary electrophoresis (Beckman, Fullerton, CA, USA) using a method modified after Casey et al. (Casey et al., 1999). Extracts were supplied with 0.4 mmol l⁻¹ uric acid as an internal standard and filtered through a 0.2 μm syringe filter. Samples were separated on a 50 μm diameter uncoated fused silica capillary with a current of 30 kV at 40°C. Adenosine peaks were identified by migration time and sample spiking. Adenosine concentrations were calculated from the area ratio of adenosine:uric acid using a calibration curve created with concentrations between 0.5–50 μmol l⁻¹ adenosine.

**Isolation of hepatocytes**

Hepatocytes were isolated following a procedure modified after Mommsen et al. (Mommsen et al., 1994). For each cell culture two fish from the same acclimation temperature were prepared simultaneously to obtain a sufficient number of cells. Animals were anaesthetised with 0.5 g l⁻¹ MS-222 and killed by a cut through the spinal cord. The liver of the first fish was carefully excised and weighted, transferred immediately to ice-cold solution 1 (magnesium-free Hank’s medium, containing 240 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Hepes, 5.5 mmol l⁻¹ glucose, 5.4 mmol l⁻¹ KCl, 4.2 mmol l⁻¹ NaHCO₃, 0.4 mmol l⁻¹ KH₂PO₄, 0.3 mmol l⁻¹ Na₂HPO₄, pH 7.4) and perfused through the vena hepatica with solution 1 to remove blood cells. The second liver was prepared accordingly. Subsequently both livers were transferred to one vial and each organ was perfused two times with 2 ml g⁻¹ fresh mass ice-cold collagenase solution [solution 1 + 1% bovine serum albumin (BSA) + 750 i.u. ml⁻¹ collagenase type IV]. Between perfusions livers were gently massaged for about 10 min. After finely chopping the tissue the homogenate was gently shaken on ice for 60 min. Finally, the suspension was filtered through 250 μm mesh-size gauze, and hepatocytes were collected by centrifugation (4 min at 70 g, 0°C) and washed by repeated centrifugation (2 min at 70 g, 0°C) in solution 1 containing 1% BSA to remove collagenase, lipids and erythrocytes. Cells were resuspended in culture medium (Leibovitz L-15 medium + 103 mmol l⁻¹ NaCl + 10 mmol l⁻¹ Hepes + 1% BSA + 5 mmol l⁻¹ glucose + 1% penicillin–streptomycin; pH 7.8 at 4°C) and shaken on ice until being dispersed for primary cell culture. Cell density and viability was determined in a Fuchs-Rosenthal haemocytometer dish by Trypan Blue exclusion.

**Cell culture and incubation conditions**

Cells were incubated in polystyrene-6-well plates. To each well 2 ml of culture medium was added and plates were pre-cooled to 4.0°C. The hepatocyte suspension was equally portioned between wells to a concentration of 2×10⁶ viable cells per well. Culture dishes were briefly shaken by hand to spread cells on the bottom. Cells were incubated under air atmosphere at 4.0°C or 11.0°C (±0.1°C), and 5 μmol glucose were added per 10⁶ cells per day. In parallel to the control group, cells were exposed to adenosine at both acclimation temperatures using the same basic culture conditions. Cells were supplied with 100 nmol ml⁻¹ adenosine directly after dispersion and once every 24 h thereafter. To investigate the potential role of adenosine receptors, one group of cells was incubated for 30 min with 100 nmol ml⁻¹ 8-phenyltheophylline (8-PT), a selective adenosine A₁-receptor antagonist, always prior to addition of adenosine, and in another group adenosine was replaced with 100 nmol ml⁻¹ 5’-(N-ethylcarboxamido)adenosine (NECA), a non-selective adenosine receptor agonist. Owing to limited cell numbers, incubations with 8-PT or NECA were only performed with cells from warm-acclimated fish at 11°C.

For sampling of hepatocytes, culture dishes were transferred onto ice without shaking and 1 ml of culture medium was removed. Cells were resuspended in the remaining culture medium and precipitated.
by centrifugation (2 min at 1000 g, 0°C). After residual medium was carefully removed, cells were immediately frozen in liquid nitrogen. Samples were collected after 48 and 72 h of incubation. Since no differences were seen between the two time points (multi-factorial ANOVA) values were pooled and treated as replicate samples.

In an additional incubation series the viability of hepatocytes during cell culture was determined by Trypan Blue exclusion. Therefore, cells incubated at 4 or 11°C were harvested after 48 and 72 h by resuspension in their culture medium. Three sub-samples were taken for each well and mixed with 1:6 volumes of 0.4% Trypan Blue dye (Sigma, Steinheim, Germany). Numbers of cells that excluded or took up the dye were counted in a Fuchs-Rosenthal haemocytometer dish.

**Enzyme activity**

Cells were homogenized in 150 μl ice-cold buffer (20 mmol l⁻¹ Tris–HCl, 1 mmol l⁻¹ EDTA, 0.1% Tween 20, pH 7.4) by shaking on a Vortex Genie2 (Scientific Industries, New York, NY, USA) for 2 min at the highest level. COX and CS activities were determined at 20°C in a thermostatted spectrophotometer (Beckman, Fullerton, CA, USA) according to Lucassen et al. (Lucassen et al., 2003). Prior to measurement of CS activity, homogenates were sonicated in a bath sonicator (Branson, Danbury, CT, USA) for 5 min at 0°C.

**RNA isolation and construction of probes**

Total RNA from hepatocytes was isolated using the RNaseasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol for animal cells. RNA was quantified in a spectrophotometer (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Accordingly, the amount of each radiolabelled probe was lowered to 20 000 cts min⁻¹ per sample, and hepatocyte RNA was supplied with an equal quantity of yeast RNA, to improve the formation of pellets. Sample RNA was simultaneously hybridized to all antisense probes at 42°C. RNase treatment was performed with an RNaseA/T1 dilution of 1:100. RNA:RNA hybrids were precipitated and separated by denaturing PAGE (Lucassen et al., 2003). To obtain equal intensities for protected fragments, specific radioactivity was applied as follows: 1000 Ci mmol l⁻¹ for CS and COX4 probes, and 45 Ci mmol l⁻¹ for COX2 and β-actin probes.

The RNA was adjusted to low RNA amounts (2 μg) following the manufacturer’s instructions. Accordingly, the amount of each radiolabelled probe was lowered to 20 000 cts min⁻¹ per sample, and hepatocyte RNA was supplied with an equal quantity of yeast RNA, to improve the formation of pellets. Sample RNA was simultaneously hybridized to all antisense probes at 42°C. RNase treatment was performed with an RNaseA/T1 dilution of 1:100. RNA:RNA hybrids were precipitated and separated by denaturing PAGE (Lucassen et al., 2003). Radioactivity was detected and quantified in a phosphor storage imaging system (Fuji, Tokyo, Japan) using the AIDA software package (raytest, Straubenhardt, Germany).

**Statistical analyses**

Statistical significance was tested at the P<0.05 level. Outliers were identified on the 95% significance level with Nalimov’s test (Nalimov, 1980). Adenosine concentrations were analysed using one-factor ANOVA and the post-hoc Student–Newman–Keuls test. Differences in freshly isolated hepatocytes were analysed using a t-test. The impact of acclimation temperature in primary cell culture was observed with multi-factorial ANOVA. The effects of different treatments during cell culture in each acclimation group were analysed in a pairwise manner using repeated measures ANOVA and the post-hoc Student–Newman–Keuls test. Linear

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**Table 1. List of primers for RT-PCR and RACE**

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<tr>
<th>Fragment</th>
<th>Term</th>
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<th>Position</th>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>CTCAAGCCGAGACTAAAGG</td>
<td>399–380</td>
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</table>

All sequences are written from 5' to 3'. The positions correspond to the position of the following genes: *Tunus obsesus* cytochrome c oxidase subunit IV (COX4) ([Hüttemann, 2000], GenBank accession no. AF204870); *Z. viviparus* cytochrome c oxidase subunit II (COX2-a) ([Lucassen et al., 2003], GenBank accession no. AF227660) and subunit IV (COX4-3’; COX4-5’; COX4-a) (GenBank accession no. EF175142).
Adenosine effects on COX

Adenosine effects on COX regressions and squared correlation coefficients were calculated using SigmaStat 3.0. Data are given as means ± s.e.m. (N=4–10).

RESULTS

Adenosine levels during cold acclimation

Adenosine concentrations in blood serum and liver of unstressed, long-term warm-acclimated Zoarces viviparus were found to be below or close to the detection limit (Fig. 1; 0 days). Adenosine levels in serum were significantly increased after 24 h of acute cold exposure. After 3 days, serum adenosine concentrations were found to be significantly lower than on the first day, but they were still elevated compared to the warm-acclimated group. Adenosine levels in the liver of Z. viviparus were also significantly increased after 24 h of cold exposure. Concentrations remained high during 3 days of cold exposure (Fig. 1).

Primary culture of hepatocytes

Cellular viability directly after isolation was always >90%. Hepatocyte appearance under the microscope remained stable under culture conditions and cells showed very little uptake of Trypan Blue dye. The fraction of cells that took up the dye was similar at both incubation temperatures and never exceeded 16.8%. The total number of viable cells was maintained at initial levels at both temperatures during the whole incubation period (Fig. 2). The activities and mRNA levels of both mitochondrial enzymes, as well as total RNA contents, were widely maintained at a high level in cells incubated at their origin temperature under control conditions, indicating a good conservation of cellular functions.

Effects of temperature and adenosine on enzyme activities

The effect of temperature acclimation in vivo on mitochondrial enzyme activities was determined in freshly isolated hepatocytes of Z. viviparus acclimated at 4 and 11°C. Citrate synthase (CS) activity per 10^6 cells was significantly higher, by 44±10%, in cells from cold-acclimated compared with those from warm-acclimated animals (Fig. 3A), whereas activities of cytochrome c oxidase (COX) remained unaffected by whole animal acclimation (Fig. 3D). During cell culture, the original acclimation temperature in vivo influenced

![Fig. 1. Adenosine levels in blood serum (open circles) and liver (filled circles) of eelpout (Z. viviparus) during 1 or 3 days of cold exposure. The fish were acclimated to 11°C (t=0) and transferred to 4°C. Data points marked by < include samples with adenosine concentration below detection limit. For these samples the detection limit of the method was used for calculations. *significant difference from other time points; †significant difference from control (t=0). Values are means ± s.e.m. (N=9–10).](image1)

![Fig. 2. Cellular viability during primary culture of hepatocytes from common eelpout (Z. viviparus). The number of viable cells (circles), expressed relative to the initial cell number, and the fraction of dead cells (triangles) in total cell counts were determined for cells from warm-acclimated animals during primary culture at either 11°C (shaded symbols) or 4°C (open symbols). Values are means ± s.e.m. (N=3).](image2)

![Fig. 3. Activities of CS (A–C) and COX (D–F) in hepatocytes prepared from cold- (white bars) and warm- (grey bars) acclimated eelpout (Z. viviparus). Activities were determined in freshly isolated hepatocytes (A,D; N=4) and in cells after incubation at either 4 or 11°C (B,C,E,F; N=5–8) under control conditions (white bars) or with the addition of adenosine (hatched bars). *significant difference between acclimations; †significant difference from the control group incubated at the same temperature; ‡significant difference from the corresponding group incubated at 4°C. Values are means ± s.e.m.](image3)
the response of both enzymes to incubation temperature. Warm incubation of hepatocytes from 4°C-acclimated eelpout caused a significant decrease of CS and COX activities, by 26±4% and 30±4%, respectively, compared with their cold-incubated counterparts (Fig. 3B,E). By contrast, enzyme activities in cells isolated from warm-acclimated fish were not altered by cold or warm incubation (Fig. 3C,F). Adenosine treatment of isolated hepatocytes had no impact on CS, but significantly affected COX activities. In cells from cold-acclimated eelpout, adenosine treatment resulted in a reduction of COX activities by 16±8% at 4°C and by 18±8% at 11°C compared with their respective control cells (Fig. 3E). In cells from warm-acclimated Z. viviparus COX activities remained more or less unaffected by the 4°C incubation temperature, whereas adenosine treatment during warm incubation significantly reduced COX activities, by 25±12% compared to the untreated control at 11°C (Fig. 3F).

Specific mRNA expression
Expression of CS mRNA was neither affected by whole animal acclimation or incubation temperature, nor by adenosine. The mRNA levels for CS were very similar in all treatments (Fig. 4A–C). The expression of COX mRNA was measured by use of the mitochondrial encoded COX2 and the nuclear encoded COX4 subunits, both displaying a similar pattern. In accordance with unchanged enzyme activities, long-term temperature acclimation of Z. viviparus (in vivo) resulted in equal mRNA levels for both subunits in freshly isolated hepatocytes (Fig. 4D,G). However, temperature significantly affected the response of hepatocytes to different incubation conditions. In cells from cold-acclimated Z. viviparus, warm incubation without adenosine induced a significant reduction in COX2 and COX4 mRNA levels, by 34±6% and 37±6%, respectively (Fig. 4E,H). By contrast, adenosine treatment, which had caused a drop in COX activities, significantly increased the expression of COX2, by 27±7% at 4°C and 95±7% at 11°C, and of COX4 by 81±14% at 11°C. The increase in COX2 and COX4 expression was less pronounced in hepatocytes from warm-acclimated eelpout, but followed the same pattern. COX4 mRNA levels were maintained at significantly, by 34±10%, higher levels in cold- than in warm-incubated cells under control conditions, and were increased to levels found in 4°C controls, when treated with adenosine at 11°C (Fig. 4I). For COX2 expression, no effect occurred as a result of incubation temperature, but adenosine treatment at 11°C resulted in mRNA levels 66±20% higher than in control cells (Fig. 4F).

Effects of adenosine receptor antagonists and agonists
A potential role for adenosine–receptor interactions in eliciting adenosine effects was tested by the addition of 8-PT, an antagonist for adenosine A1 receptors, and 5′-(N-ethylcarboxamido)adenosine (NECA)
(NECA), a potent non-selective agonist for adenosine receptors (Ralevic and Burnstock, 1998). 8-PT- and NECA-treated samples were compared to the analogous control and adenosine-treated groups of the respective preparations.

Hepatocytes treated with the receptor blocker 8-PT in addition to adenosine (Fig. 5) exhibited COX activities identical to the ones measured in cells treated with adenosine alone, both significantly lower than the activities determined for the control group. This suggests that adenosine A1 receptors are not involved in the adenosine effects described above. This assumption is supported by the observation that the adenosine agonist had no effect either (Fig. 4). COX activities in cells treated with NECA instead of adenosine were almost identical to those of the control group ($P=0.984$). Thus, a receptor-mediated hormone-like action of adenosine on COX activity can be excluded as unlikely.

**DISCUSSION**

**Temperature effects on adenosine concentration**

Adenosine accumulation often occurs from the degradation of intracellular ATP, when energy demand exceeds energy supply. The metabolite can be released from the cell by specialized nucleoside transporters and can be distributed to the whole organism (Buck, 2004). Therefore, adenosine might act as a hormone-like effector in response to bioenergetic challenges such as temperature changes. However, although there is no literature available concerning the effect of temperature, elevated adenosine levels have frequently been reported following anoxic or hypoxic conditions. In the brain of epaulette shark, adenosine levels increased during acute anoxia, whereas the levels of phosphorylated adenylates (ATP+ADP+AMP) remained virtually unchanged (Renschaw et al., 2002). Since acute temperature changes result in functional hypoxia (Pörtner, 2001; Pörtner, 2002), similar effects may occur during thermal response. Sartoris et al. (Sartoris et al., 2003) found unchanged ratios of phosphorylated adenylates during thermal acclimation of common eelpout. In the present study, adenosine concentrations were found to increase significantly in blood serum and liver during 24 h after a temperature shift from optimum (11°C) to a typically experienced low temperature (4°C). Increased levels were still visible after 3 days of incubation (Fig. 1). Changes in adenosine concentrations are typically small but significant and therefore elicited by only minor reductions in ATP levels. These observations suggest that adenosine might be a suitable signal to support metabolic adaptation in response to such bioenergetic disturbances.

Adenosine concentrations observed in eelpout after cold exposure were similar to those previously reported for the heart of short-nosed sculpin during acute anoxia (Mac Cormack and Driedzic, 2004). Hypoxic conditions usually cause an increase in adenosine levels for minutes or hours (Renschaw et al., 2002; Lutz and Kabler, 1997). By contrast, cold-induced adenosine accumulation in Z. viviparus persisted for at least 3 days (Fig. 1), possibly as long as the hypoxic challenge remained uncompensated for. After 3 days of cold exposure liver adenosine concentrations remained high, whereas serum adenosine had already significantly decreased (Fig. 1). In our previous study on eelpout, cold compensation of energy metabolism in liver, monitored by mitochondrial enzymes, was shown to become visible after 4 days (Lucassen et al., 2003). In line with thermally induced hypoxia and functional insufficiency an extended exposure to temperature change may result in a prolonged elevation of adenosine levels until the seasonal acclimation process is well underway. This would allow adenosine to contribute to the cold acclimation process.

**Impact of temperature and adenosine on mitochondrial enzymes**

To test for a potential role of adenosine as a systemic effector in thermal adaptation, we investigated its influence on the acclimation response of isolated hepatocytes from cold- versus warm-acclimated fish. Therefore, we monitored the activity and expression of the mitochondrial key enzymes citrate synthase (CS) and cytochrome c oxidase (COX) in isolated hepatocytes. Elevated activities of the mitochondrial matrix enzyme CS in conjunction with conserved capacities of membrane bound COX typify the cold acclimation response of eelpout liver (Lucassen et al., 2003). By contrast, both enzymes typically increase in parallel in red and white muscle (Lannig et al., 2003; Lucassen et al., 2006). In line with earlier observations, cold acclimation of Z. viviparus in vivo had no impact on COX activities (Fig. 3D), but resulted in an increase of CS activities (Fig. 3A). The mRNA levels of CS (Fig. 4A) and of two COX subunits, the mitochondrial-encoded subunit COX2 (Fig. 4D) and the nuclear-encoded subunit COX4 (Fig. 4G), were not affected by in vivo temperature acclimation. The acclimation profile of freshly isolated hepatocytes is thus in line with the situation in liver of thermally acclimated specimens (Lucassen et al., 2003).

The consecutive response of isolated hepatocytes to temperature clearly depended on the previous acclimation temperature of the cells in vivo. Warm incubation of cells from cold-acclimated fish induced a muscle type reduction of the activities of both mitochondrial enzymes (Fig. 3B,E) and a decrease of mRNA expression of both COX subunits (Fig. 4E,H). In line with an earlier study on catfish hepatocytes (Koban, 1986), this suggests that fish liver cells without any systemic input may display basic adjustment to warming.

By contrast, cold incubation of hepatocytes from warm-acclimated eelpout left enzyme activities unchanged (Fig. 3C,F) and solely increased the mRNA levels of COX4 (Fig. 4I). These findings are in line with the concept that a cold-induced mismatch of energy demand and supply becomes effective at a high organisational level, the intact animal, as a consequence of limitations in oxygen supply (Pörtner, 2001; Pörtner, 2002). The decrease in the metabolic rate of isolated cells in the cold parallels the reduction of energy demand and occurs at ample oxygen supply. This experimental situation alleviates the pressure to acclimate, an observation, which indicates that cold acclimation in vivo occurs in response to systemic signal(s).

Adenosine probably participates as a modulator of thermal acclimation. When applied to isolated hepatocytes, the metabolite had no effect on CS, but distinctly affected COX activities. Although in vivo acclimation of Z. viviparus changed neither the initial activities nor the mRNA expression of COX, the cellular response to adenosine was found to depend on the thermal origin of the cells. Responsiveness to adenosine was enhanced in hepatocytes from cold-acclimated fish, however, the effects were more pronounced at higher incubation temperatures.

The adenosine effect comprised two major components, a decrease of COX activity (Fig. 3E,F) and a concomitant increase of COX mRNA expression (Fig. 4E,F,H,I). The activity of COX, the terminal oxygen-consuming step of the respiratory chain, is often used as an approximation for the aerobic capacity of the cells (Kadenbach et al., 2000). Thus, it can be assumed that adenosine reduces the capacity of aerobic energy production. This is in line with observations by Krummschnabel et al. (Krummschnabel et al., 2000), who found reduced oxygen consumption rates under the acute effect (10-30 min) of adenosine in trout hepatocytes in parallel to a decrease of protein synthesis rate. They assumed that the deceleration of oxygen uptake was due to diminished cellular ATP
demand caused by adenosine. Such an effect may be paralleled by the
reduction of COX capacities as observed in the present study.

The suppressing effect on aerobic capacities is contrasted by the
stimulating effect of adenosine on the expression of COX. Since this
effect was more pronounced in cells incubated at 11°C, adenosine
treatment of isolated hepatocytes abolished the warming induced
reduction of COX expression. As a result, COX mRNA remained
at similar levels at both incubation temperatures (Fig. 4E,H,I),
resembling the expression pattern obtained during in vivo temperature
acclimation (Fig. 4D,G). Adenosine treatment thus results in a
discrepancy between the levels of transcription (increased) and the
capacity of the enzyme (decreased). Similarly, loose coordination
between message levels and enzyme functional capacity was found
in the time-course of whole animal acclimation (Lucassen et al.,
2003). Adenosine may thus influence the coordination of
transcriptional and translational activities or cause posttranslational
modification of the enzyme proteins. Besides, the discrepancy might
result from incomplete response of the cell culture within the
experimental time. However, while establishing the cell culture
system we found steady state levels for COX activities established
within the first 48-h, which were conserved for the following 3 days
(data not shown). Because of restricted survival time of eelpout
hepatocytes in primary culture, a further extension of the incubation
period was not feasible. A more delayed response of the functional
levels compared to the mRNA response seems to be rather unlikely.

The question arises of how adenosine exerts these effects. A
receptor-mediated action was investigated by application of the
adenosine A1 receptor antagonist 8-PT and the non-selective receptor
agonist NECA. Both ligands have been used to block and stimulate
adenosine receptors in other fish species, respectively (Krumshnabel et al.,
2000; Rosati et al., 1995). However, 8-PT could not prevent the effects of adenosine, and NECA failed to reduce COX activities in eelpout liver cells (Fig. 4). Furthermore, hepatocytes continuously consumed adenosine, a process stimulated at elevated temperature (data not shown). These observations suggest diffusive entry, and intracellular action of adenosine. Assuming a receptor-mediated action in accordance with earlier studies on fish hepatocytes, adenosine concentrations about one
order of magnitude higher than the observed concentrations have
been used in our cell culture system. Higher adenosine levels might
be necessary to compensate possible insensitivity of the isolated
cells, but also facilitate the diffusive entry of the metabolite. Since
the actual concentration in incubated cells was not measurable
because of the small sample size, we cannot exclude higher intracellular adenosine levels in vitro compared to in vivo, which
might have influenced the response of the cells in vitro.

Although no data exist for an intracellular action in fish, adenosine
may act through different mechanisms in hepatocytes. First,
adenosine can be reconverted to AMP by adenosine kinase and give
rise to subsequent ATP synthesis (Bontemps et al., 1983). ATP is
known as an allosteric inhibitor of COX but also acts as a
noncompetitive inhibitor of CS in fish (Hochachka and Lewis, 1970),
thus adenosine treatment might result in reduced activities of both
enzymes which has, however, not been observed here. Accumulation
of ATP upon thermal acclimation of the cells appears unlikely.
Second, high intracellular adenosine concentrations prevent the
hydrolysis of S-adenosylhomocystein (SAH), a competitive inhibitor
of most S-adenosylmethionine (SAM)-dependent methyltransferases
(Kloor and Oswald, 2004), which are involved in the methylation
of many molecules, e.g. proteins, DNA and RNA (Chiang et al.,
1996). In knockout mice deficient for the synthesis of hepatic SAM,
the levels of COX1 and COX2 proteins were found to be only half
of those in wild-type mice, whereas the levels of COX2 mRNA
remained unaltered, indicating a translational downregulation of
COX (Santamaria et al., 2003). Although the underlying mechanisms
still need to be investigated, the inhibition of SAM-dependent
methyltransferases by adenosine may account for the mismatch
between the expression and activity levels of COX, observed in
adenosine-treated eelpout hepatocytes. The adenosine-related
increase in the expression of both the nuclear and the mitochondrial
encoded COX subunits is most remarkable and suggests coordinated
regulation of nuclear and mitochondrial genes, thereby substantiating
the observed effects. However, with the data at hand and the sparse
literature available, the mechanism of how adenosine affects COX
transcription remains to be elaborated.

Conclusions and perspectives

In summary, the lack of cold acclimation in isolated fish hepatocytes
in vitro and the differences between warm acclimation patterns in
whole animals versus cells isolated from cold-acclimated specimens
indicate the involvement of systemic control in thermal acclimation.
The accumulation of adenosine observed during cold exposure in vivo would allow for a role for adenosine in thermal acclimation. Our findings suggest that adenosine specifically modulates mitochondrial functioning. mRNA from nuclear and mitochondrial encoded COX subunits were found to be increased under adenosine treatment, resulting in an expression pattern in isolated hepatocytes similar to the one found during whole animal acclimation. By contrast, functional levels of COX were decreased in the presence of adenosine, possibly mediated by the inhibition of
methyltransferases. The detailed mechanisms of action including
the stimulating effect of adenosine on COX transcription await
further investigation. Since in vivo thermal acclimation of liver
mitochondria involves the modulation of CS, the lack of an effect
of adenosine on CS activity and expression levels indicate that
further signals remain to be identified.

LIST OF ABBREVIATIONS

8-PT  8-phenylthiotheolinine
COX  cytochrome c oxidase
COX2/4  cytochrome c oxidase subunit 2/4
CS  citrate synthase
MS-222  3-amino-benzoic-methanesulfonylate
NECA  S-(N-ethylcarboxamido)adenosine
RLM-RACE  RNA ligase-mediated rapid amplification of cDNA ends
RPA  ribonuclease protection assay

The author would like to thank Dr Felix Mark for his introduction to cell isolation
techniques. This work contributes to the MARCOPOLI research program of the
Alfred Wegener Institute (POL4: Response of higher marine life to change).

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