

ADENOSINE RECEPTOR BLOCKADE AND HYPOXIA-TOLERANCE IN RAINBOW TROUT AND PACIFIC HAGFISH

I. EFFECTS ON ANAEROBIC METABOLISM

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Summary

The physiological properties of adenosine may be essential in the control of energy metabolism for the survival of animals exposed to oxygen shortages. Accordingly, we tested the hypothesis that adenosine modulates metabolic regulation in rainbow trout and Pacific hagfish exposed to acute hypoxia. Treatment of hypoxic rainbow trout (P_{wO_2} =3.33 or 4.00 kPa) with the adenosine receptor (AR) blocker theophylline was associated with greater increases in plasma [lactate], more rapid and pronounced metabolic acidosis, higher tissue [lactate], and lower heart creatine charge and glycogen content than in the hypoxic controls. The recruitment of anaerobic metabolism in hypoxic trout treated with enprofylline, an AR blocker with very weak affinity, was intermediate to that of the hypoxic theophylline-injected

and control groups. In hagfish, plasma [lactate] increased following exposure to a P_{wO_2} of 1.33 kPa but did not increase following exposure to 3.33 kPa and, like plasma acidosis, it was greatest in the animals treated with theophylline. These findings indicate that AR blockade results in a more rapid and pronounced recruitment of anaerobic metabolism following acute hypoxic exposure, and while rainbow trout and Pacific hagfish show marked differences in their responses to hypoxia, adenosine appears to play an important protective role in both species.

Key words: adenosine, hypoxia, methylxanthines, anaerobic metabolism, *Oncorhynchus mykiss*, rainbow trout, *Eptatretus stouti*, Pacific hagfish.

Introduction

Rapid detection of declining oxygen tensions (Burlison *et al.* 1992) and quick adjustments in cardiovascular and ventilatory activity (Smith and Jones, 1978; Randall, 1982, 1990; Fritsche and Nilsson, 1993) enable most fish species to tolerate moderate hypoxia (van den Thillart and van Waarde, 1985). These regulatory mechanisms improve oxygen extraction (Jensen *et al.* 1993) and reduce the need to recruit anaerobic metabolism as a compensatory measure to meet energy requirements (van den Thillart, 1982). Even with severe hypoxia, extensive use of anaerobic metabolism is a last resort, given the inefficiency, reduced capacity and metabolic waste problems associated with these pathways (Hochachka, 1991). Hence, without the ability to depress metabolism, most fish species exposed to severe hypoxia must maximize oxygen delivery while minimizing the use of anaerobic metabolism to compensate for any energy deficit. This tight energy budgeting can be achieved by selectively favouring vital over non-vital

metabolic functions and by closely adjusting the rate of energy consumption to the metabolic supply of each tissue.

In the hypoxia-sensitive terrestrial mammals, such a regulatory role has been attributed to adenosine (Mullane and Williams, 1990; Ribeiro, 1991). The many actions of adenosine redress any imbalance between energy demand and availability (Berne *et al.* 1982; Newby, 1984; Newby *et al.* 1990).

There is evidence that adenosine may be important in the control of anaerobic metabolism and energy supply in the anoxic crucian carp (Nilsson, 1991; Nilsson *et al.* 1994). However, fish from the genus *Carassius*, such as crucian carp, are an exception amongst fish, in having the ability to produce and excrete ethanol as the main metabolic end-product during anoxia (Johnston and Bernard, 1983) and to reduce energy consumption by up to 70% under anoxic conditions (van Waversveld *et al.* 1989). While adenosine may also be

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involved in the modulation of the cardiovascular system of several fish species (Nilsson *et al.* 1994; Nilsson and Holmgren, 1993), its possible metabolic role has not been investigated in fish that do not produce ethanol under severe hypoxia.

Whereas cytoplasmic production of adenosine has been demonstrated in a number of mammalian aerobic tissues (Stone *et al.* 1990), and in the brain of some lower vertebrates (Nilsson and Lutz, 1992), adenosine production has not been measured in fish. However, the main adenosine receptor subtypes found in most mammalian tissues, A₁ and A₂ (Ramkumar *et al.* 1988; Reddington and Lee, 1991; Collis and Hourani, 1993), have been characterized in a number of different fish species including the hagfish *Eptatretus deani* (Lucchi *et al.* 1992; Siebenaller and Murray, 1994). So far, in fish, A₁ adenosine receptors have been detected in brain and heart membranes, while A₂ adenosine receptors have only been characterized in brain membranes (Siebenaller and Murray, 1994). The binding affinities and specificities of these receptors are similar to those of mammals (Siebenaller and Murray, 1986, 1994; Murray and Siebenaller, 1987). Altogether, this evidence argues for the presence of specific adenosine receptors and a physiological relevance of adenosine as a neuromodulator in fish.

The objective of this study was to assess the possible role of adenosine in metabolic regulation during severe hypoxia in fish. To this end, rainbow trout and Pacific hagfish were given the adenosine receptor blocker theophylline under hypoxic and normoxic conditions, and their responses were monitored and compared with those of control sham-injected fish. In one experimental series, an additional adenosine receptor blocker, enprofylline, was used to separate the endogenous actions of adenosine from possible adenosine-independent actions of these blockers. Comparisons were made between Pacific hagfish and rainbow trout because of the known differences in their ability to tolerate hypoxia. Hagfish, with one of the lowest standard metabolic rates of vertebrates (Munz and Morris, 1965), tissues with a large anaerobic potential (Sidell *et al.* 1984) and resting cardiac energy demands which can be met almost entirely by anaerobic metabolism (Forster, 1991), can tolerate hypoxic and even anoxic habitats. Rainbow trout, which must adopt a number of cardiovascular and ventilatory strategies to try to maintain oxygen uptake (Randall, 1982) and meet the oxygen requirements of their tissues (Dunn and Hochachka, 1986), are relatively hypoxia-intolerant. In view of these differences, and given the possible protective role of adenosine in hypoxia outlined above, differences may also be expected in the possible physiological actions of adenosine in the hypoxia-tolerance of rainbow trout and Pacific hagfish. Although hagfish and rainbow trout differ in many respects, they both produce lactate as an end-product of anaerobic metabolism and seem to lack the ability to enter a state of metabolic arrest under severe hypoxic conditions (Boutilier *et al.* 1988; Davison *et al.* 1990; Forster, 1990). Hence, to extend survival during acute hypoxia, they must contend both with the problems of metabolic acidosis and with the maintenance of vital functions on a fixed energy budget.

Materials and methods

Three different experimental series involving the exposure of fish to hypoxic conditions were conducted. Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were exposed either to a P_{wO_2} of 4.00 kPa for 6 h (series I) or to a P_{wO_2} of 3.33 kPa for 1 h (series II). Pacific hagfish [*Eptatretus stouti* (Lockington)] were exposed to a P_{wO_2} of either 1.33 or 3.33 kPa for 1 h (series III). Experimental protocols, sampling regimes and analytical procedures described below were similar for all three experiments. Specific information regarding the conditions of a given experimental series is given only when they differ from the common approach.

Experimental animals

Rainbow trout of either sex were obtained from West Creek Trout Farm (Aldergrove, BC, Canada) and kept in 2000 l outdoor fibreglass tanks supplied with flow-through dechlorinated tap water. The fish were acclimated to these conditions for a minimum of 3 weeks prior to the experiments. They were fed with a commercial trout food and kept on a maintenance ration. Series I experiments were carried out in June at a mean water temperature of 10 °C, while series II experiments were carried out in February at a mean water temperature of 7 °C. The trout for these experiments had mean body masses of 461±24 g (series I; mean ± S.E.M., $N=24$) and 1144±32 g (series II; $N=40$).

Pacific hagfish (mean body mass 183±7 g; $N=36$) were collected from Trevor Channel on the west coast of Vancouver Island with baited traps, at a depth of approximately 30 m. They were kept at Bamfield Marine Station in an 800 l fibreglass tank with flow-through salt water (30‰). The fish were acclimated to this water for at least 2 weeks prior to the experiments. Both acclimation and experiments were carried out at a mean water temperature of 10 °C. The fish were starved over the duration of the study.

Surgical procedure

Trout were anaesthetized in a buffered (NaHCO₃) MS-222 solution at a dilution of 1:10 000 and transferred to an operating table where they were forced-ventilated with a buffered, cooled and aerated MS-222 solution of dilution 1:16 000. The dorsal aorta was chronically cannulated with polyethylene (PE) 50 tubing (Clay Adams), using the technique of Soivio *et al.* (1975), and fish were allowed to recover in a flow-through black Perspex box. The cannulae were filled and flushed with heparinized (50 i.u. ml⁻¹ sodium heparin) Cortland saline (Wolf, 1963). The trout were left undisturbed for 48 (series I) and 72 h (series II) prior to the hypoxic exposure experiments.

The hagfish were anaesthetized in a seawater solution of MS-222 (1:400) for approximately 10 min and transferred to an operating table for cannulation. The operating table used was filled with water and chilled prior to surgery. The surgery involved making a 2.5 cm incision along the midline of the ventral surface between gill apertures 7 and 10, dissecting out the ventral aorta from the surrounding tissue, stopping the circulation momentarily, and securing a T-shaped PE cannula

into the ventral aorta *via* a small incision made into the wall of this vessel. The cannula consisted of a 5 mm piece of PE 240 tubing, which allowed blood to flow through the ventral aorta, and a long piece of PE 50 tubing from which blood samples could be withdrawn. Once the cannula was in place, a small hole was made through the skin on one side of the ventral incision in order to guide the cannula to the outside. The cannula was then secured in place and the incision closed with skin sutures. The surgery took between 10 and 15 min to complete, at which time the hagfish were placed in a flow-through black Perspex box to recover for 48 h before the start of the experiments.

Experimental protocol, blood sampling and drug injections

All experiments were carried out on fish kept in black Perspex boxes supplied with a aerated normoxic water or hypoxic water of known oxygen partial pressure (P_{wO_2}). Oxygen-stripping was performed by bubbling nitrogen gas through ceramic air stones (Point Four Systems) in a 100 l reservoir. Water flow from the reservoir to the fish box was kept constant for the various treatments within each of the three series of experiments, and gas flow through the reservoir was adjusted to achieve the desired P_{wO_2} . During transfer from normoxic to hypoxic conditions, P_{wO_2} decreased exponentially, and 95% of the transformation from one state to the other was achieved within 10 min.

The sampling regime used throughout each hypoxic trial was as follows: (1) removal of a blood sample to assess resting control values; (2) injection of either an adenosine receptor (AR) blocker or saline; (3) transfer from normoxic water to hypoxic water taken from the reservoir; and (4) removal of blood samples at set times following the transfer of water sources. The sampling regime used throughout each normoxic trial did not involve step 3.

Two different AR blockers were used in this study: theophylline (1,3-dimethylxanthine) and enprofylline (3-propylxanthine; Sigma, St Louis, MI, USA). At low doses (e.g. 1–5 mg kg⁻¹), the actions of methylated xanthines have been attributed to their ability to block ARs (Bruns, 1988). However, methylxanthines can also inhibit cyclic nucleotide phosphodiesterase, the enzyme that breaks down cyclic AMP to 5'-AMP (Rall, 1990). Taking advantage of their different properties, theophylline and enprofylline have been used previously in concert to delineate the endogenous actions of adenosine (Persson, 1988). *In vitro* enprofylline is approximately six times more potent than theophylline as a competitive inhibitor of cyclic nucleotide phosphodiesterase (Ukena *et al.* 1985, 1993). However, although these two drugs do not discriminate between the two main AR subtypes (A₁ and A₂), enprofylline has a much lower affinity than theophylline for both receptor subtypes (Ukena *et al.* 1985).

In series I, four different experimental groups of six fish each were used to investigate the effects of AR blockade on hypoxia tolerance: (1) normoxic sham; (2) normoxic theophylline; (3) hypoxic sham; and (4) hypoxic theophylline. Throughout the course of a trial, blood samples of 700 µl were taken at 0, 10,

30, 120 and 360 min following the transfer of water sources. Each blood sample was replaced by an equivalent volume of Cortland saline. Theophylline was dissolved in saline to a concentration of 8 mg ml⁻¹ and given as a bolus injection at a dose of 4 mg kg⁻¹, followed by 0.2 ml of saline.

Series II involved five different experimental groups of eight fish: (1) normoxic sham; (2) normoxic enprofylline; (3) hypoxic sham; (4) hypoxic enprofylline; and (5) hypoxic theophylline. Throughout the course of a trial, blood samples of 900 µl were taken at 0, 10, 30 and 60 min. Each blood sample was replaced by an equivalent volume of saline. Enprofylline and theophylline were dissolved in saline to a concentration of 1.5 mg ml⁻¹ and infused at a rate of 0.5 ml min⁻¹. Both AR blockers were injected at a dose of 4 mg kg⁻¹, followed by 0.2 ml of saline. Immediately following the 60 min sampling time, fish were killed by a 2 ml injection of Somnitol (65 mg ml⁻¹ sodium pentobarbital). As soon as ventilation ceased, a cross-sectional slice of tissue was taken immediately posterior to the dorsal fin and freeze-clamped with aluminium tongs pre-cooled in liquid nitrogen. The fish was then beheaded, and the heart was removed from the pericardial chamber and freeze-clamped. Tissue samples were stored at -80 °C until homogenized. The time between removal of the terminally anaesthetized fish from the holding box and freeze-clamping of the tissues was kept to less than 30 s.

Series III involved six different experimental groups of six fish: (1) normoxic sham; (2) normoxic theophylline; (3) 3.33 kPa P_{wO_2} hypoxic sham; (4) 3.33 kPa P_{wO_2} hypoxic theophylline; (5) 1.33 kPa P_{wO_2} hypoxic sham; and (6) 1.33 kPa P_{wO_2} hypoxic theophylline. Throughout the course of a trial, blood samples of 500 µl were taken at 0, 10, 30 and 60 min. Each blood sample was replaced by an equivalent volume of hagfish saline (3.0% NaCl and heparin at 50 i.u. ml⁻¹). Theophylline was dissolved in hagfish saline to a concentration of 6 mg ml⁻¹ and given as a bolus injection at a dose of 4 mg kg⁻¹, followed by 0.2 ml of hagfish saline.

In all three experimental series, blood samples were collected in 1.5 ml microcentrifuge tubes. From this initial sample, aliquots of whole blood were taken immediately for measurement of arterial blood P_{O_2} and arterial blood O₂ content in series II. The blood was then centrifuged at 11 000 revs min⁻¹ for 2 min, and plasma was removed for measurement of blood pH (pHe) and later measurement of plasma [lactate]. The plasma sample for determination of lactate was deproteinized with ice-cold 0.6 mol l⁻¹ perchloric acid (PCA), centrifuged at 11 000 revs min⁻¹ for 1 min, and the supernatant frozen in liquid nitrogen and stored at -80 °C for later analysis.

In a separate experiment, the *in vivo* concentration of plasma theophylline following bolus injection of the drug was assessed in rainbow trout (mean mass 441 ± 12 g) either kept in normoxic water ($N=6$) or exposed to a P_{wO_2} of 4.00 kPa ($N=6$) for a period of 12 h at 12.5 °C. Throughout the course of a trial, blood samples of 150 µl were taken at 0, 5, 10, 20, 30, 60, 120, 240, 360, 540 and 720 min. Each blood sample was replaced by an equivalent volume of saline. Theophylline was dissolved

in Cortland saline to a concentration of 8 mg ml^{-1} and given at a dose of 4 mg kg^{-1} , followed by 0.2 ml of saline.

Finally, while plasma [theophylline] was not monitored in series I–III, samples of plasma were taken in series II to assess plasma $[\text{K}^+]$, an indicator of possible theophylline overdose.

Analytical techniques

Measurement of water oxygen tension, arterial blood P_{O_2} (P_{aO_2}) and arterial blood O_2 content were made using thermostatted Radiometer P_{O_2} electrodes (E5046; Radiometer, Copenhagen, Denmark) and Radiometer PHM71 acid–base analyzers. Arterial blood O_2 content was measured using the method described by Tucker (1967), using $50 \mu\text{l}$ blood samples collected in a gas-tight Hamilton syringe, and with the chamber thermostatted at 40°C . The P_{O_2} electrodes were calibrated using nitrogen gas and air-saturated water. Whole-blood pH was measured using a thermostatted Radiometer G297/G2 glass capillary electrode with a PHM71 acid–base analyzer. Calibration of the pH electrode was performed using Radiometer precision buffer solution standards S1519 and S1500. Whole-blood [lactate] was determined using the NAD^+ -linked assay described by Bergmeyer (1985) and modified for use with microtitration plates and a Titertek Multiskan spectrophotometer (Flow Laboratories, Mississauga, Ontario, Canada). Plasma [theophylline] was determined by ^{125}I -labelled theophylline radioimmunoassay (clinical assay no. 1592; Baxter Healthcare Corp., Cambridge, MA, USA). Plasma $[\text{K}^+]$ was measured with a Perkin-Elmer atomic absorption spectrophotometer, model 2380.

Tissue metabolite preparation and assay

The frozen cross sections of skeletal muscle were immersed in a shallow bath of liquid nitrogen, and samples (approximately 0.8 g) of red and white muscle were dissected free from skin and bone. These frozen muscle samples, and heart samples of similar size, were ground to a fine powder in a mortar cooled in liquid nitrogen. The powder was then transferred to pre-weighed chilled test tubes containing 4 ml of ice-cold 0.6 mol l^{-1} PCA and re-weighed. The mixtures were homogenized with three 20 s passes of an Ultra-Turrax tissue homogenizer. Homogenization of the tissues was performed with the tubes in a slurry of salt water and ice. While stirring at low speed, one sample of $250 \mu\text{l}$ was removed and frozen at -80°C for later glycogen analysis. The remaining homogenate was centrifuged at $12\,000 \text{ g}$ for 5 min at 2°C . A 1 ml sample of the supernatant was removed and stored at -80°C for later determination of tissue adenosine triphosphate (ATP), creatine phosphate (PCr) and creatine (Cr) levels. Lastly, a further $500 \mu\text{l}$ sample of the supernatant was removed and neutralized with $3 \text{ mol l}^{-1} \text{ KHCO}_3/0.5 \text{ mol l}^{-1}$ triethanolamine, recentrifuged at 7500 g for 5 min, and the resulting supernatant frozen at -80°C for later determination of lactate.

Glycogen was measured in digested samples of tissue homogenate, and glycogen level is presented as the difference between the concentration of glucosyl units per gram tissue post-digestion and prior to digestion. Glycogen homogenates

were digested by incubating for 4 h at 40°C with amyloglucosidase (Boehringer Mannheim, 10 mg ml^{-1}) in acetate buffer (Bergmeyer, 1985). The enzymatic reaction was halted with 70% PCA and glucose was determined in the extract after neutralization with $3 \text{ mol l}^{-1} \text{ KHCO}_3$. Tissue glucosyl units, lactate and Cr were determined using the standard NADH - or NAD^+ -coupled enzymatic procedures described in Bergmeyer (1985) and modified for use with microtitration plates and a Titertek Multiskan spectrophotometer.

Concentrations of PCr and ATP were determined using high-performance liquid chromatography (HPLC) based on Harmsen *et al.* (1982) with modifications. The HPLC incorporates a Waters 625 LC system controller coupled to a Waters 996 photodiode array detector set at 210 nm (Millipore Corp., USA). $50 \mu\text{l}$ samples of filtered tissue homogenates were passed through a $250 \text{ mm} \times 4.6 \text{ mm}$ Partisil 10 SAX strong anion exchanger column (Whatman, Maidstone, Great Britain) at a flow rate of 1.5 ml min^{-1} . Elution was isocratic for the first 5 min, using $0.01 \text{ mol l}^{-1} \text{ H}_3\text{PO}_4$ (pH 2.85; solvent A), followed by a linear gradient from solvent A to $0.75 \text{ mol l}^{-1} \text{ KH}_2\text{PO}_4$ (pH 4.40; solvent B) over 15 min. These conditions were then maintained for 5 min, followed by a linear gradient from solvent B to solvent A over 1 min. The column was re-equilibrated for 8 min with solvent A before the next run. Integration of the separated metabolites was performed using the Millennium 2010 Chromatography Manager software program (version 1.1, Millipore Corp., USA). Concentrations were calculated on the basis of linear standard curves constructed for both metabolites.

As one possible measure of the energy state of the tissues, the creatine charge was calculated as $[\text{PCr}]/([\text{Cr}]+[\text{PCr}])$ according to Connett (1988).

Statistical analysis

All data are presented as mean ± 1 S.E.M. The statistical significance of observed effects of treatment exposure within a group were tested using one-way repeated-measures analysis of variance (ANOVA). To compare pre-treatment means with means at subsequent sampling times, Dunnett's test was used. Where appropriate, the statistical significance of observed differences between the means from all treatments at a particular sampling time were tested using one-way ANOVA. Since the tissue and plasma [lactate] means were positively correlated with the variance, the nonparametric Kruskal–Wallis one-way ANOVA on ranks test was used to determine differences between the means from all treatments at a particular sampling time. To determine which group(s) differed from the others, the Student–Newman–Keuls test was used. The significance level for all statistical test was $P < 0.05$.

Results

The mean control P_{aO_2} and arterial blood O_2 content values for all five experimental groups of rainbow trout in series II were $16.45 \pm 0.23 \text{ kPa}$ ($N=8$) and $14.6 \pm 0.4 \text{ vols\%}$ ($N=8$)

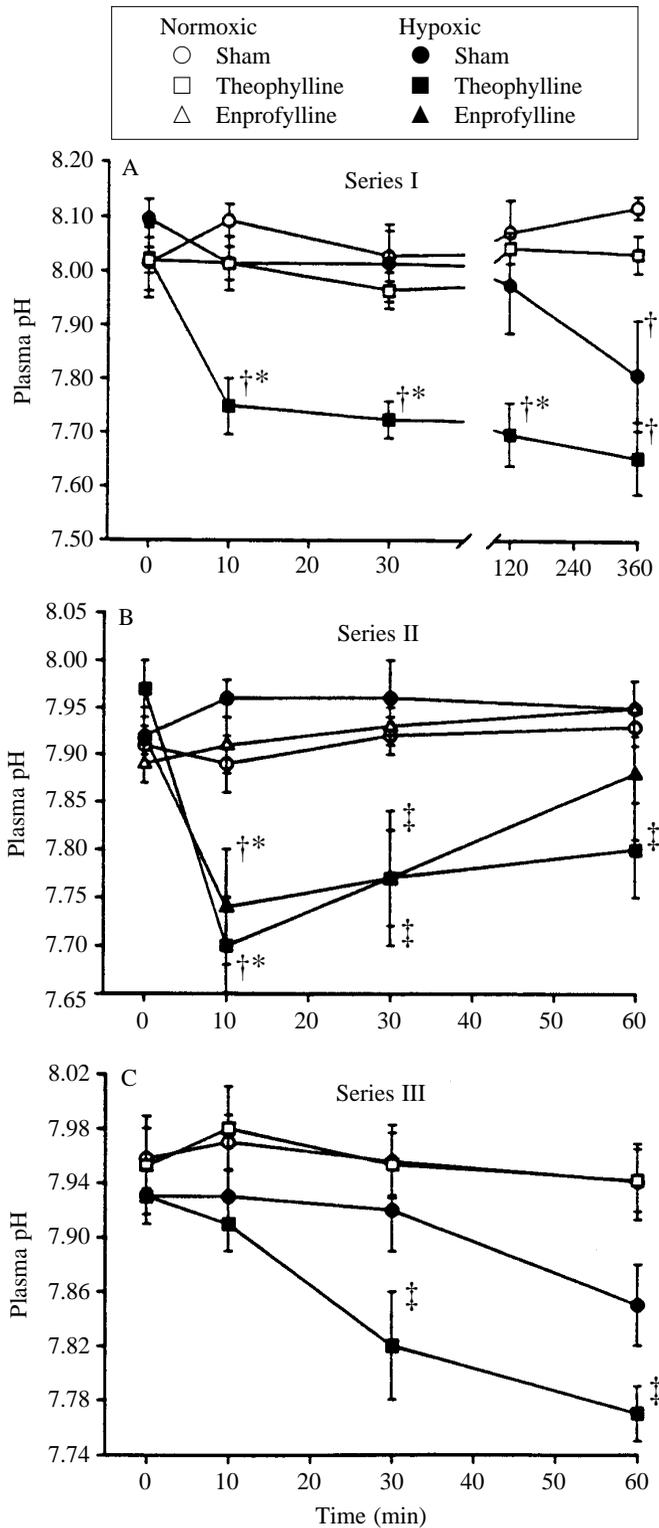


Fig. 1. Plasma pH of rainbow trout (A, series I) and (B, series II) and Pacific hagfish (C, series III) in relation to exposure duration to normoxia (open symbols) and hypoxia (filled symbols; $P_{wO_2} = 4.00$ kPa in series I, 3.33 kPa in series II and 1.33 kPa in series III). Animals were injected with saline (circles), theophylline (squares) or enprofylline (triangles). Time zero values are controls. ‡ indicates a significant difference from the control value in a given treatment. † indicates a significant difference from the control value in a given treatment and from values for both normoxic treatments at a given sampling time. * indicates a significant difference from the hypoxic sham value at a given sampling time ($P < 0.05$). Values are means ± 1 S.E.M. Values of N for all figures are given in Materials and methods.

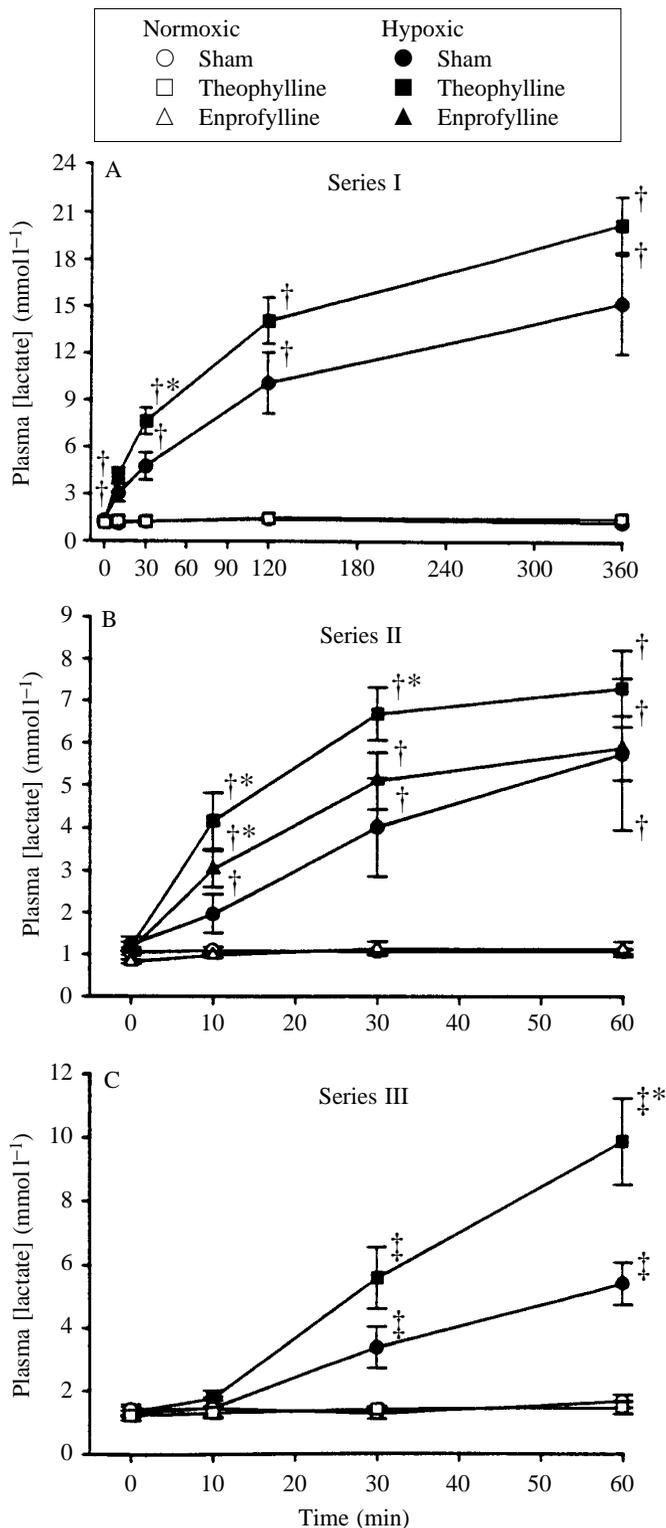
values in all three hypoxic groups for the remainder of the hypoxic challenge.

Compared with normoxic and hypoxic sham animals, hypoxic rainbow trout injected with the AR blockers theophylline or enprofylline showed a rapid and pronounced blood acidosis (Fig. 1). In series I, pHe decreased significantly in the hypoxic theophylline group after 10 min of hypoxia and remained low for the duration of the trial, whereas pHe did not decrease significantly until 360 min in the hypoxic sham group (Fig. 1A). In series II, pHe also decreased significantly after 10 min of hypoxia in the theophylline and enprofylline groups (Fig. 1B). Recovery towards the normoxic values was observed only in the enprofylline group, and pHe was unchanged throughout the hypoxic exposure in the sham groups. In series III, a significant blood acidosis was observed only in the hypoxic theophylline group exposed to a P_{wO_2} of 1.33 kPa (Fig. 1C). The mean pHe values of the sham and theophylline-injected hagfish exposed to a P_{wO_2} of 4.00 kPa were 7.95 ± 0.01 and 7.97 ± 0.01 respectively. These values remained the same throughout the trials, and were similar to the mean pHe of the normoxic sham (7.96 ± 0.01) and normoxic theophylline (7.96 ± 0.01) groups. Normoxic groups of both species injected with theophylline (Fig. 1A,C) or enprofylline (Fig. 1B) showed no changes in pHe compared with normoxic sham animals.

Hypoxia resulted in an increase in plasma [lactate] in both trout and hagfish, the increase being much greater in the small summer trout (series I) than in the larger winter trout (series II), even though the latter were exposed to a more severe hypoxia. Relative to hypoxic sham fish, increases in plasma [lactate] with hypoxic exposure were greater in the animals injected with the AR blockers (Fig. 2). In series I, plasma [lactate] increased in both hypoxic groups, and the hypoxic theophylline group had a significantly higher concentration than the hypoxic sham group at the 30 min sampling time (Fig. 2A). In series II, the increase in plasma [lactate] was greatest in the hypoxic theophylline group, intermediate in the hypoxic enprofylline group and lowest in the hypoxic sham group (Fig. 2B). In series III, no changes were observed in the mean lactate concentrations of the 4.00 kPa P_{wO_2} hypoxic sham (1.61 ± 0.12 mmol l^{-1}) and 4.00 kPa P_{wO_2} hypoxic theophylline (1.57 ± 0.10 mmol l^{-1}) groups. In the sham and theophylline-injected hagfish groups exposed to a P_{wO_2} of 1.33 kPa (Fig. 2C), however, there was a gradual increase in plasma

respectively. No changes were observed from these mean values in the normoxic sham and normoxic enprofylline groups throughout the 60 min trials.

In the hypoxic sham, enprofylline and theophylline groups, P_{aO_2} and arterial blood O_2 content fell to 2.16 ± 0.15 kPa and 5.7 ± 0.3 vol% from the control values after only 10 min of hypoxic exposure. P_{aO_2} and O_2 content remained at these low



[lactate], resulting in a five- and eightfold increase, respectively. As with pH_e, the injections of theophylline (Fig. 2A,C) or enprofylline (Fig. 2B) in normoxic groups had no effect on resting control concentrations of plasma lactate. In series II, only the tissues from the hypoxic theophylline group showed significant increases in tissue [lactate] when compared with the normoxic groups (Fig. 3).

Fig. 2. Plasma [lactate] of rainbow trout (A, series I; B, series II) and Pacific hagfish (C, series III) in relation to exposure duration to normoxia (open symbols) and hypoxia (filled symbols; $P_{wO_2}=4.00$ kPa in series I, 3.33 kPa in series II and 1.33 kPa in series III). Animals were injected with saline (circles), theophylline (squares) or enprofylline (triangles). Time zero values are controls. † indicates a significant difference from the control value in a given treatment. ‡ indicates a significant difference from the control value in a given treatment and from values for both normoxic treatments at a given sampling time. * indicates a significant difference from the hypoxic sham value at a given sampling time ($P<0.05$). Values are means ± 1 S.E.M.

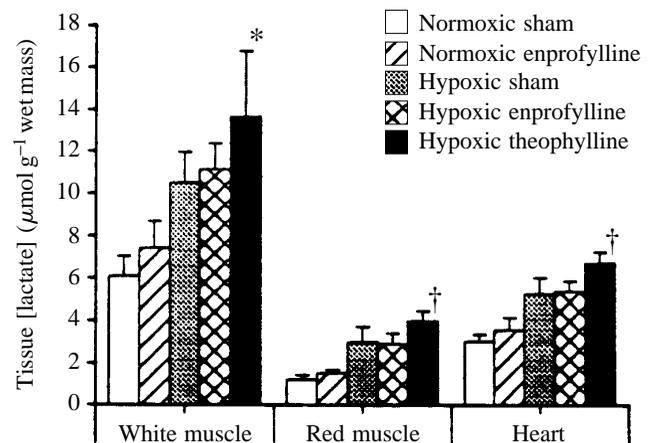


Fig. 3. Tissue [lactate] in white muscle, red muscle and heart of rainbow trout exposed to 60 min of normoxia or hypoxia ($P_{wO_2}=3.33$ kPa). Animals were injected with saline, enprofylline or theophylline. * indicates a significant difference from the normoxic sham value. † indicates a significant difference from values for both normoxic treatments ($P<0.05$). Values are means ± 1 S.E.M.

Significant changes in levels of tissue metabolites other than lactate were also only associated with the hypoxic group infused with theophylline (Table 1). Compared with normoxic groups, significant decreases in [glycogen], creatine charge and [creatine phosphate] were observed in heart tissue of theophylline-injected rainbow trout. A significant decrease in creatine charge was also observed in the red muscle of the theophylline-injected group.

In vivo plasma [theophylline] decreased exponentially in the first 120 min following bolus injection of the drug (Fig. 4). The concentration decreased more gradually and followed a linear pattern between 120 and 720 min. Relative to normoxic fish, the concentrations at 10, 20, 30 and 60 min were significantly higher in the hypoxic trout. No significant differences in plasma $[K^+]$ were observed within or between groups throughout the 60 min trials. Plasma $[K^+]$ ranged from a low of 2.33 ± 0.30 mmol l⁻¹ in the normoxic sham group to a high of 3.19 ± 0.46 mmol l⁻¹ in the hypoxic enprofylline group.

Discussion

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Table 1. Concentration of metabolites in heart, red muscle and white muscle after 1 h of either normoxia or hypoxia ($P_{wO_2}=3.33$ kPa) in rainbow trout at 7 °C

Tissue	Experimental condition	[Creatine] (mg g ⁻¹ wet mass)	[Creatine phosphate] (mg g ⁻¹ wet mass)	Creatine charge‡	[ATP] (mg g ⁻¹ wet mass)	[Glycogen] (mg g ⁻¹ wet mass)
Heart	Normoxic sham	5.06±0.35	4.12±0.37	0.45±0.03	2.15±0.10	60.25±2.86
	Normoxic enpro.	4.76±0.60	4.47±0.23	0.52±0.03	2.10±0.10	62.46±3.53
	Hypoxic sham	4.17±0.34	2.95±0.45	0.40±0.04	1.87±0.08	53.06±5.96
	Hypoxic enpro.	4.81±0.30	3.41±0.46	0.40±0.05	2.11±0.15	53.96±3.53
	Hypoxic theo.	5.90±0.32	2.88±0.24†	0.33±0.03†	2.02±0.07	41.90±5.30*‡
Red muscle	Normoxic sham	5.12±0.50	5.85±0.50	0.53±0.01	2.16±0.09	24.25±1.77
	Normoxic enpro.	5.80±0.49	6.85±0.64	0.54±0.02	2.29±0.10	21.93±1.60
	Hypoxic sham	7.18±1.24	5.70±0.46	0.47±0.03	2.24±0.15	23.81±1.85
	Hypoxic enpro.	5.94±0.63	4.48±0.28	0.44±0.04	2.00±0.07	16.23±1.74
	Hypoxic theo.	6.91±0.54	5.10±0.86	0.41±0.04*‡	2.09±0.17	18.69±3.24
White muscle	Normoxic sham	20.32±1.73	13.53±0.77	0.41±0.03	4.66±0.20	33.08±2.44
	Normoxic enpro.	23.74±0.85	13.55±1.08	0.36±0.02	4.89±0.20	33.07±2.03
	Hypoxic sham	24.84±1.21	11.14±1.73	0.30±0.04	4.33±0.34	32.51±1.87
	Hypoxic enpro.	20.44±0.85	13.55±1.08	0.36±0.02	4.89±0.20	30.06±3.30
	Hypoxic theo.	23.38±0.92	12.18±1.25	0.34±0.03	4.76±0.25	28.16±1.19

Animals were infused either with saline or with one of two adenosine receptor blockers: enprofylline (enpro.) or theophylline (theo.).

*Significantly different from normoxic value ($P<0.05$).

†Significantly different from normoxic enprofylline value ($P<0.05$).

‡Creatine charge = $[PCr]/[PCr]+[Cr]$.

Values are means ± S.E.M.; values of N are given in Materials and methods.

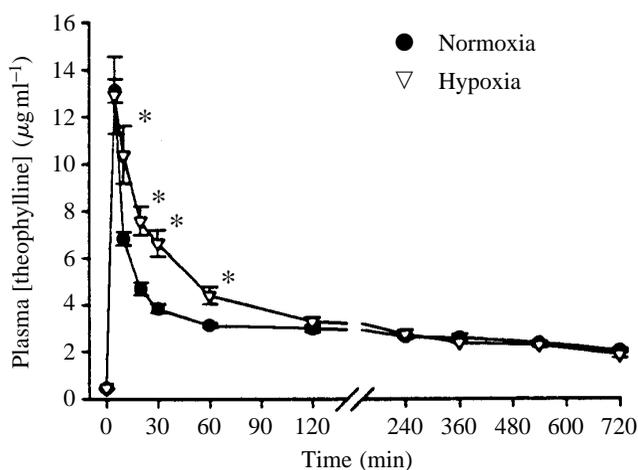


Fig. 4. Plasma [theophylline] of rainbow trout injected with 4 mg kg⁻¹ theophylline in relation to exposure duration to normoxia (circles) and hypoxia (triangles; $P_{wO_2}=4.00$ kPa). Time zero values are controls. * indicates a significant difference between the hypoxic and normoxic values ($P<0.05$). Values are means ± 1 S.E.M.

study, theophylline and enprofylline, are varied, as in other systems (see Bruns, 1988), our data clearly indicate that the differences seen between the hypoxic treatments result from adenosine receptor blockade. Injected at a dose of 4 mg kg⁻¹, theophylline had a maximum *in vivo* concentration of 73 µmol l⁻¹ (13.2 µg ml⁻¹) after 5 min and decreased exponentially thereafter. Theophylline was cleared more rapidly from the blood of normoxic than hypoxic fish, an

observation for which we have no clear explanation. Given that the IC₅₀ (the concentration required for 50% inhibition) values of theophylline for the various phosphodiesterase isoenzymes are in the range 155–630 µmol l⁻¹, but its affinity for A₁ and A₂ adenosine receptors is in the 10 µmol l⁻¹ range (Ukena *et al.* 1993), the *in vivo* concentrations used in all our experimental series are not likely to cause phosphodiesterase inhibition. Hypokalaemia is the most consistent metabolic abnormality associated with theophylline toxicity (Kearney *et al.* 1985; Memon, 1993), but the theophylline and enprofylline dosage used in our experiments had no effect on plasma [K⁺]. Irrespective of the parameter measured, the normoxic fish given the AR blockers were similar in every respect to the normoxic sham fish, indicating no phosphodiesterase inhibition. Similarly, aminophylline (which forms theophylline) injected intraperitoneally at a dose of 75 mg kg⁻¹ had no effect on the routine oxygen consumption of crucian carp (Nilsson, 1991). The effects of the methylxanthines were only observed under severe hypoxic conditions, when anaerobic metabolism was recruited to compensate for an imbalance between energy supply and demand. These conditions favour the production of adenosine (Meghji, 1991), indicating that the methylxanthines were acting on adenosine receptors. Significant differences were observed between the high-affinity AR blocker theophylline and the low-affinity receptor blocker enprofylline. In every instance where AR blockade treatment caused a significant departure from the response in the hypoxic sham group, the response due to theophylline treatment was greater than that due to enprofylline

treatment, further supporting the conclusion that the effects observed were due to AR blockade.

In all three experimental series presented in this study, the accumulation of plasma lactate following acute hypoxic exposure was significantly greater in the animals receiving AR blockers than in the sham hypoxic treatments. Measurement of heart, white and red muscle tissue [lactate] in series II also showed an increase in glycolytic activity following theophylline treatment under hypoxic conditions. Therefore, although lactate turnover was not measured, these results suggest an increase in lactate production with theophylline treatment.

The activation of anaerobic metabolism is associated with the development of a metabolic acidosis. The acidosis results from an imbalance between H^+ production from ATP hydrolysis and H^+ consumption by the fermentation of glycogen (Hochachka and Mommsen, 1983). Although there was a significant increase in plasma [lactate] in the hypoxic sham group of series I and II, and in the 1.33 kPa P_{wO_2} hypoxic sham group of series III, a significant plasma acidosis only developed in series I after 360 min of hypoxia. In the hypoxic sham treatments, the metabolic acidosis is compensated by a respiratory alkalosis resulting from an increase in gill ventilation (Thomas and Hughes, 1982; Tetens and Lykkeboe, 1985). In sharp contrast to this general pattern, the animals injected with the AR blockers were characterized by a rapid and pronounced plasma acidosis. Since there was no difference in P_{aO_2} and O_2 content between the three hypoxic treatments in series II, the magnitude of the hyperventilatory response was also probably the same between these three treatments. Furthermore, in all three experimental series, no differences were observed between the various hypoxic groups in the magnitude of red blood cell alkalization following β -adrenergic stimulation of the Na^+/H^+ exchanger (see Bernier *et al.* 1996). While this rapid movement of protons from the red blood cells to the plasma will contribute to the metabolic acidosis (Fievet *et al.* 1987, 1988), it cannot explain the abrupt and marked metabolic acidification observed in the AR-blocked animals. This metabolic acidosis must result from the more rapid increase in anaerobic metabolism observed in the fish that received the AR blockers, which exceeds the buffering capacity of the blood and the compensatory alkalosis.

Significant depletion of glycogen and PCr stores in fish is usually only observed after prolonged exposure to severe hypoxic or anoxic conditions (Jorgensen and Mustafa, 1980a,b; van Waarde *et al.* 1983, 1990; Dunn and Hochachka, 1986; Boutilier *et al.* 1988; Dalla Via *et al.* 1994). Although the hypoxic treatment in series II resulted in a significant increase in plasma [lactate] in all three hypoxic groups, the exposure to a P_{wO_2} of 3.33 kPa for 60 min was not severe enough to result in significant changes in levels of any tissue metabolites in the hypoxic sham and hypoxic enprofylline groups. Significant decreases in energy reserves were only observed in the oxidative tissues, heart and red muscle of animals treated with theophylline (Table 1). No appreciable changes were observed in the glycogen and PCr stores of white

muscle following hypoxic theophylline treatment. This apparent difference between the aerobic and anaerobic tissues in maintaining their energy reserves may be related to differences in the possible interactions between adenosine and these tissues. In fish glycolytic muscles, a tight inverse stoichiometric relationship exists between ATP and IMP concentrations (Mommsen and Hochachka, 1988; Schulte *et al.* 1992), making the formation of adenosine in these tissues very unlikely. In mammals, the capacity for adenosine formation is much smaller in glycolytic tissues in comparison with oxidative tissues (Newby *et al.* 1990; Stone *et al.* 1990). Hence, since adenosine has very localized actions (Stiles, 1991), it is possible that adenosine effects on fish white muscle are minimal but that there are marked effects in heart and red muscle.

Altogether, our results indicate that adenosine receptor blockade may reduce the hypoxia-tolerance of rainbow trout and Pacific hagfish by increasing the rate of anaerobic metabolism during acute hypoxic exposure. Similar results were obtained with crucian carp, where AR blockade caused the rate of ethanol excretion during anoxia to increase up to threefold (Nilsson, 1991). As an explanation for his results, Nilsson (1991) suggested that AR blockade prevented the decrease in neuronal activity mediated by adenosine and its associated decrease in energy consumption. We have no reason to believe that a similar scenario is not also at play in our experiments; however, unlike the experiments carried out on anoxic crucian carp, a limited amount of oxygen was available to the fish in this study. Hypoxia causes struggling in many fish, but we did not detect any differences in struggling between the groups that might account for the differences in anaerobic metabolism observed.

In addition to its various inhibitory effects in the central nervous system, adenosine is also an important neuromodulator of the respiratory and cardiovascular systems in mammals (Ribeiro, 1991). Although the cardiovascular effects of adenosine in fish are complex and unresolved, the physiology of the heart, the coronary arteries and the branchial and brain vasculature are modulated by adenosine under hypoxic conditions (Nilsson *et al.* 1994; Nilsson and Holmgren, 1993). Adenosine receptor blockade may prevent these presumably adaptive changes in respiratory and cardiovascular control and, thereby, alter the distribution of the very limited oxygen and fuel supply under conditions of severe hypoxia. Given the inefficiency and limited capacity of anaerobic metabolism to compensate for reductions in aerobic metabolism, small impairments of the cardiovascular system may result in a large amplification of the anaerobic metabolic pathways.

Severe hypoxia in fish is also characterized by marked increases in the circulating concentrations of catecholamines (Boutilier *et al.* 1988; Perry *et al.* 1991, 1993) and cortisol (White and Fletcher, 1989). These hormones cause a rapid mobilization of energy reserves in order to maintain or increase energy turnover. Overall, the catabolic properties of catecholamines and cortisol help fish to meet the energy

demand required to fuel the acute physiological responses necessary to maintain oxidative processes during hypoxia. The magnitude of their actions may limit the ability of fish to conserve energy resources over prolonged periods of acute hypoxia. In a separate paper (Bernier *et al.* 1996), we have shown that adenosine receptor blockade increases the circulating concentrations of catecholamines in hypoxic rainbow trout and Pacific hagfish. Furthermore, in mammals, adenosine can inhibit the secretion of catecholamines from the adrenal medulla (Chern *et al.* 1987, 1992; Tseng *et al.* 1994) and function as a negative-feedback modulator of β -adrenoceptor-mediated contractile and glycogenolytic responses in the myocardium (Dobson *et al.* 1987).

Results obtained in this study also reflect the marked differences in hypoxia-tolerance between Pacific hagfish and rainbow trout. Even though trout and hagfish have very different abilities to tolerate hypoxia, our results show that adenosine plays a similar modulatory role in both cases. In fact, it appears probable that adenosine plays a general role in ensuring a match between energy supply and utilization in all vertebrates.

In summary, comparison of the results obtained from fish treated with either of the adenosine receptor blockers or saline indicate that adenosine may increase hypoxia-tolerance by reducing the recruitment of anaerobic metabolism. However, adenosine may stimulate glycogenolysis in the ischaemic mammalian heart and brain (Magistretti *et al.* 1986; Janier *et al.* 1993), tissues that have been extensively investigated for the protective effects of adenosine (Berne, 1980; Belardinelli *et al.* 1987; Dobson *et al.* 1987; West *et al.* 1987; Law and Raymond, 1988; Stefanovich, 1988; Belardinelli and Shryock, 1992; Rudolphi *et al.* 1992). The common link between these various studies is the ability of adenosine to reduce metabolic demand and to preserve energy resources while maintaining energy supply. Thus, while adenosine receptor blockade may have prevented a direct or indirect inhibitory effect of adenosine on the recruitment of anaerobic metabolism, evidence from other studies portrays a more complex scenario as to the role of adenosine in hypoxia-tolerance.

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