EFFECTS OF HYPOXIA ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): INTRAERYTHROCYTIC PHOSPHATES

ADALBERTO L. VAL*, JOANNE LESSARD AND DAVID J. RANDALL

Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 2A9

Summary

Intraerythrocytic levels of guanosine and adenosine phosphates were estimated in normoxic and hypoxic rainbow trout after intra-arterial injection with either saline or carbonic anhydrase. A significant reduction of the total pool of adenosine and guanosine was observed in hypoxic animals. Similarly, a decrease in both ATP and GTP levels occurred in hypoxic animals injected either with saline or with carbonic anhydrase. Interestingly, there was a highly significant relationship between ATP and GTP levels, indicating that they are under similar control. In addition, a significant positive relationship between nucleoside triphosphate (NTP) and Mg^{2+} levels was observed. It is possible that the availability of ATP and GTP to hemoglobin (Hb) may be proportionally smaller than their intraerythrocytic absolute levels. No clear relationship between intraerythrocytic NTP levels and plasma carbonic anhydrase infusion was observed, despite the significant effect of carbonic anhydrase on acid–base balance. There was a significant relationship between red blood cell pH and [NTP]:[Hb] in fish exposed to hypoxia for 48 h. No such relationship existed during the first 6 h of hypoxic exposure. Possible mechanisms accounting for the reduction in NTP levels during the initial phases of hypoxia are discussed.

Key words: carbonic anhydrase, ATP, GTP, trinucleotides, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Fish have many different adaptive mechanisms for coping with the oscillations in oxygen availability that often occur in the aquatic environment (reviewed by Val, 1993). The adaptive regulation of intraerythrocytic levels of nucleoside triphosphates (NTPs) according to environmental or internal oxygen tensions has been extensively reported in many fish species (Wood and Johansen, 1972; Bartlett, 1978; Weber and Jensen, 1988; Val *et al.* 1992). Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) have been described as the main NTPs in fish erythrocytes. They are negatively charged phosphates that bind to deoxyhemoglobin but not to oxyhemoglobin, and both act as negative modulators of hemoglobin oxygen-affinity (Isaacks and Harkness, 1980; Nikinmaa, 1990). Nonetheless, the mechanism(s) by which oxygen tension affects NTP levels is unknown.

In fish, catecholamines are suddenly released from chromaffin tissues during exposure to deep hypoxia (reviewed by Randall and Perry, 1992). Increased levels of circulating catecholamines cause numerous adaptive changes, many of which are directed towards enhancing oxygen transfer (Randall, 1990). Increased levels of catecholamines have been correlated with the release of red blood cells from the spleen (Nilsson and Grove, 1974; Perry and Kinkead, 1989), red cell

swelling (Nikinmaa *et al.* 1990), elevation of red cell pH (Primmett *et al.* 1986; Motais *et al.* 1989; Nikinmaa *et al.* 1990), an increase in gill epithelial permeability (Isaia *et al.* 1978) and a decrease in red cell ATP concentration (Milligan and Wood, 1987).

It has been shown that pH is an important regulatory signal for changes in the levels of 2,3-diphosphoglycerate (2,3-DPG) in mammalian red blood cells (Gerlach and Duhm, 1972). Jensen *et al.* (1990) reported that acid–base status may play a similar important role in fish red cells. These authors showed that P_{O_2} and pH significantly affected the red cell NTP content in carp, *Cyprinus carpio*. It is possible that intraerythrocytic NTP levels are affected as a consequence of activation of the Na^+/H^+ exchanger by circulating catecholamine as well as by changes in pH. It has been shown that carbonic anhydrase (CA) added to the plasma short-circuits the action of catecholamines on red cell pH (Motais *et al.* 1989; Nikinmaa *et al.* 1990). Adrenergic stimulation activates the Na^+/H^+ exchanger, which pumps the protons out of the cell, thus raising intracellular pH (pHi). An increase in pH is possible if the efflux exceeds the influx of H^+, which is the case when CA is absent from the plasma because the CO_2 hydration/dehydration is uncatalyzed. Thus, by adding CA to the plasma, we could alter the

relationship between pH and catecholamine levels and investigate the effect on red cell NTP levels.

This paper analyses the intraerythrocyte levels of adenylates and guanylates in normoxic and hypoxic rainbow trout after intra-arterial injection with either saline or carbonic anhydrase. The interrelationships between NTP levels and blood oxygen content, intracellular pH and red blood cell Mg^{2+} levels are also reported and discussed.

Materials and methods

Freshwater rainbow trout [*Oncorhynchus mykiss* (Walbaum)], weighing 300–600 g, were obtained from a local hatchery and held outdoors at the University of British Columbia in dechlorinated Vancouver tap water (8–12 °C) for at least 2 weeks before experimentation. The animals were fed once a week with commercial trout pellets, but feeding was suspended 3 days prior to surgery. Under MS-222 anesthesia (1:10 000 in NaHCO₃–buffered fresh water), fish were fitted with dorsal aortic catheters according to the procedure of Soivio *et al.* (1975). Following surgery, fish were allowed to recover for at least 48 h in a darkened acrylic box with recirculating water (8–10 °C).

Experimental protocol

Series 1: carbonic anhydrase injection after 48 h of hypoxia or normoxia

This series was designed to investigate the effect of carbonic anhydrase (CA) injection in animals under normoxic and hypoxic conditions. Eighteen animals were cannulated and allowed to recover in individual darkened Perspex boxes, as described above. After the recovery period, there was a 48 h period during which aerated water (P_{O_2}=155 mmHg; 20.6 kPa) flowed through the boxes for normoxic animals and deoxygenated water (P_{O_2}=35 mmHg; 4.7 kPa) for hypoxic ones. After the acclimation period, nine animals received a bolus injection of 1 ml kg⁻¹ body mass of bovine CA (solution of 10 000 i.u. ml⁻¹) and nine received a bolus injection of 1 ml kg⁻¹ body mass of saline (Wolf, 1963). Two blood samples of 2 ml each were removed from the dorsal aorta (one at 10 min and the other at 30 min after the injection). One part of each blood sample was centrifuged and 500 µl of plasma was removed, immediately frozen in liquid nitrogen, and then stored in a freezer at −80 °C for further analysis of catecholamines; another 500 µl of plasma was deproteinized with 70 % trichloroacetic acid and stored at −20 °C until later analysis for lactate and electrolytes. The remainder of the blood was used for hematocrit, hemoglobin concentration and ion determinations.

Series 2: continuous carbonic anhydrase infusion at the onset of hypoxia

For measurements at rest, one blood sample (1.2 ml) was taken from the dorsal aorta and immediately analyzed for plasma pH (pHe), blood and plasma total CO₂, hematocrit, hemoglobin and oxygen content. The remainder of the blood was centrifuged, plasma was separated from the red blood cells

(RBCs) and both were frozen in liquid nitrogen for further analysis of lactate, catecholamines, adenylates and guanylates and red blood cell pH (pHi). These fish were not used for the hypoxia experiments. Since we were dealing with the stress hormones, catecholamines, we tried to avoid any stress prior to the infusion and the hypoxia exposure. At the same time that hypoxia was induced (P_{O_2}=30–35 mmHg; 4.0–4.7 kPa), one group of fish was infused with saline and another with approximately 3 ml of carbonic anhydrase solution of 10 000 i.u. (to achieve 0.3 g l⁻¹ in the animal) for the duration of hypoxia exposure (6 h). Blood samples were taken as described above at 10, 30, 120 and 360 min.

Analytical procedures

Since the experiments reported in the present paper are part of a larger series, the analytical procedures of some data presented here are to be found in Lessard *et al.* (1995). Blood and plasma levels of Mg^{2+} were determined by flame photometry (Perkin–Elmer, model 2380). Blood levels of cations were corrected according to the hematocrit values in order to estimate the levels inside the red blood cell. High pressure liquid chromatography (HPLC) was used to measure ATP, ADP and AMP simultaneously with GTP, GDP and GMP. The procedure was carried out using an LKB 2152 HPLC controller and 2150 titanium pump coupled to a 2220 recording integrator. The separation was performed on an Aquapore AX-300 7 µm weak anion exchanger (Brownlee laboratories) eluting at 2 ml min⁻¹ at 55 °C (Schulte *et al.* 1992). Analyses of plasma catecholamine levels were performed by HPLC with electrochemical detection, using a Brownlee Spheri-5 reverse-phase column (Technical Marketing, Richmond, BC), a Bioanalytical Systems LC-4A amperometric detector (Mandel Scientific, Rockwood, Ontario) and a Spectra-Physics SP8700 solvent delivery system (Terochem Laboratories Ltd, Edmonton, Alberta), as described by Primmett *et al.* (1986).

Statistical methods

Statistical significance of data for the 48 h experiment series was determined by Kruskall–Wallis one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate, with a fiducial limit of significance of 5 %. When no significant difference was detected between values for the 10 and 30 min time intervals after drug administration, the data were pooled and a mean and respective standard error were calculated and submitted to further statistical analysis.

Statistical significance of data for 6 h of hypoxia was determined using a two-way ANOVA followed by a Dunnett test when comparing the hypoxia values with the resting values and an unpaired *t*-test; both with a statistical significance level of 5 %. Data are presented as mean ± standard error of the mean (S.E.M.).

Results

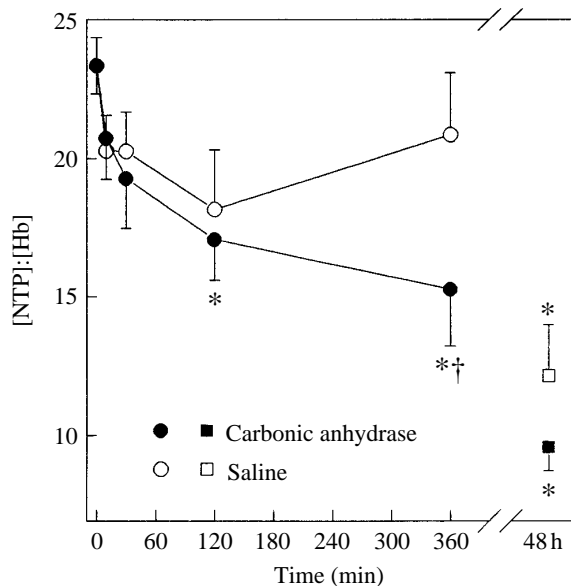The mean values of pH and hemoglobin concentrations

Fig. 1. Levels of NTP (ATP+GTP) in red blood cells of rainbow trout infused continuously with saline or carbonic anhydrase for 6 h of deep hypoxia (○, ●) or injected once with the same drug after 48 h of deep hypoxic exposure (□, ■). * indicates a significant difference between normoxic and hypoxic animals. † indicates a significant difference between saline- and carbonic-anhydrase-infused hypoxic animals. Values are mean ± S.E.M., *N*=7.
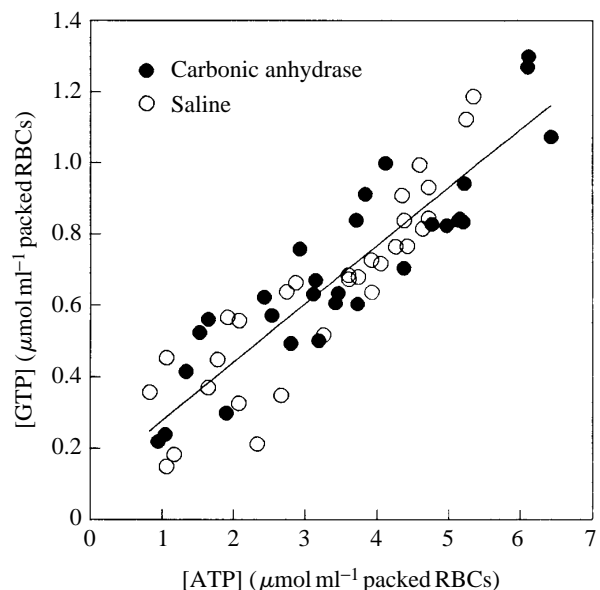

Fig. 2. Correlation between ATP and GTP concentrations in saline- and carbonic-anhydrase-injected rainbow trout previously exposed to normoxia and hypoxia for 48 h. Data were pooled to give the regression line [GTP]=0.115+0.163[ATP], *r*=0.90. RBCs, red blood cells.

depicted in the figures have been presented and discussed elsewhere (Lessard *et al.* 1995), and they were obtained from the same animals used in this study. Plasma catecholamine levels increased during hypoxia but did not differ in the CA- and saline-infused groups. These data have been reported by Lessard *et al.* (1995).

Adenylate and guanylate concentrations are expressed as ratios with hemoglobin concentration to avoid the effect of changes in RBC volume. [NTP] ([ATP]+[GTP]) decreased during the first 10 min of hypoxia and remained more or less stable for the saline-infused group (Fig. 1). The NTP levels in the CA-infused group continued to decrease after the first 10 min and became significantly different from control levels at 2 h and from that of saline-infused fish at 6 h (Fig. 1). The total pool of organic phosphates followed the same pattern as NTP, reflecting the large contribution of ATP to the total pool of organic phosphates (data not shown).

No significant differences were observed in the levels of any of the analyzed nucleoside phosphates (ATP, ADP, AMP, GTP, GDP, GMP) between 10 and 30 min post-injection after 48 h of acclimation to normoxia or hypoxia (data not shown). The expected significant decreases in ATP and GTP levels were observed in hypoxic animals, compared with normoxic ones (Fig. 1), but there were no significant differences between saline- and CA-injected animals. In addition, animals exposed to hypoxia exhibited a decrease in the levels of all intraerythrocyte adenylates and guanylates (data not shown).

A highly significant positive correlation between intraerythrocyte levels of ATP and GTP was observed when all data were combined (Fig. 2). No significant difference was detected as a result of CA injection.

There was a significant negative correlation between intracellular pH and NTP levels in animals exposed to hypoxia for 48 h (Fig. 3A). It is important to note that, in our experiments, decreased levels of ATP and GTP were accompanied by decreased levels of the total pool of adenylates and guanylates. Thus, the intracellular pH changes observed in this study could be, in part, a consequence of changed levels of organic phosphates themselves. This relationship was not observed in the first 6 h of hypoxic exposure (Fig. 3B), but only after 48 h of hypoxic exposure.

Red blood cell Mg^{2+} levels decreased significantly in CA-injected animals subjected to both normoxic and hypoxic conditions (Fig. 4A). A highly significant correlation was detected between Mg^{2+} levels and NTP levels (Fig. 4B) and the total pool of organic phosphates (data not shown), although it is not clear what fraction of the total intracellular Mg^{2+} is available for binding to organic phosphates.

Discussion

Reduced intraerythrocytic levels of ATP and GTP have been extensively reported in fish exposed to environmental or internal hypoxia (Wood and Johansen, 1972; Tetens and Lykkeboe, 1985; Monteiro *et al.* 1987; Boutilier *et al.* 1988; Weber and Jensen, 1988; Val *et al.* 1990). The reduced intraerythrocytic levels of GTP observed for rainbow trout exposed to prolonged hypoxia in the present experiment are similar to those previously reported for this species, but the
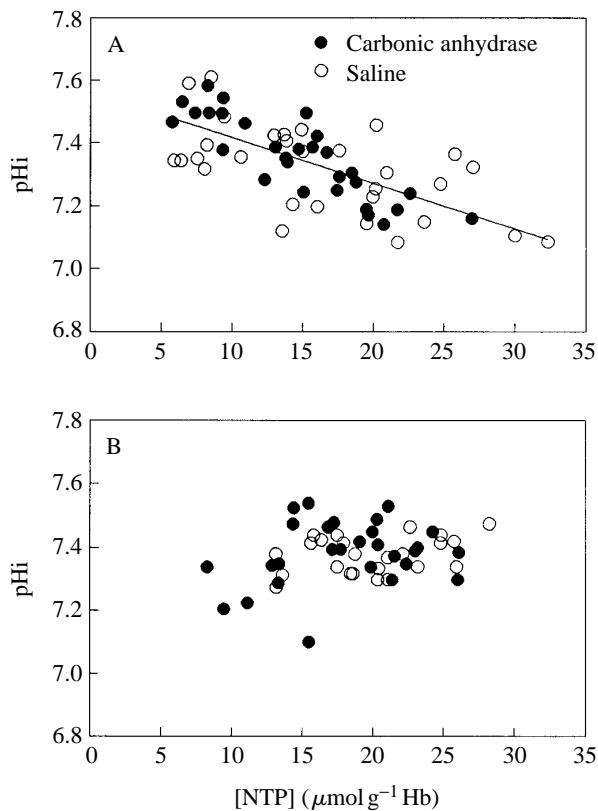
Fig. 3. Correlation between pHi and intraerythrocytic NTP levels in rainbow trout (A) following exposure to hypoxia for 48 h and (B) during the first 6 h of hypoxia. Data were pooled to give the regression line in A: pHi=7.561−0.014[NTP], r=0.69.

[NTP] (μmol ml^{-1} packed RBCs)

Fig. 4. (A) Red blood cell levels of Mg^{2+} in rainbow trout injected with saline or carbonic anhydrase after 48 h of normoxia or hypoxia and (B) the correlation between intraerythrocytic Mg^{2+} and NTP levels. Data were pooled to give the regression line [Mg^{2+}]=4.434+1.538[NTP], r=0.90. * indicates a significant difference between normoxic and hypoxic animals with respect to injection type. † indicates a significant difference between saline- and carbonic-anhydrase-infused hypoxic animals. Values in A are mean + S.E.M., N=7.

ATP levels are lower (Boutilier *et al.* 1988). The [ATP]:[Hb] and [GTP]:[Hb] ratios during normoxia were similar, but the decrease observed during the first few hours of hypoxia was somewhat smaller in saline-infused fish than that reported by Tetens and Lykkeboe (1985), although similar to that reported by Tetens and Christensen (1987).

The levels of NTPs in the fish erythrocyte reflect the balance between production and utilization of these compounds, as well as changes in volume of the RBCs. In Fig. 1, we have expressed our data as a ratio of the hemoglobin concentration to remove the effects of changes in cell volume, and there is still a large drop in NTP levels during hypoxia. Thus, changes in NTP levels during hypoxia must be due to changes in either the production or utilization of NTPs. The release of catecholamines during hypoxia increases RBC oxygen consumption (Ferguson *et al.* 1989), resulting in increased ATP utilization. *In vivo* and *in vitro* studies have related increased catecholamine levels to significant reductions in erythrocyte NTP levels (Nikinmaa, 1986; Milligan and Wood, 1987), which can be prevented by pre-treating the animals with propranolol, a β-adrenergic antagonist (Nikinmaa *et al.* 1984). The effect of catecholamines on erythrocytes diminishes during prolonged hypoxia (Fievet *et al.* 1988; Thomas *et al.* 1991) and this could account for the stabilization in NTP levels seen in saline-infused
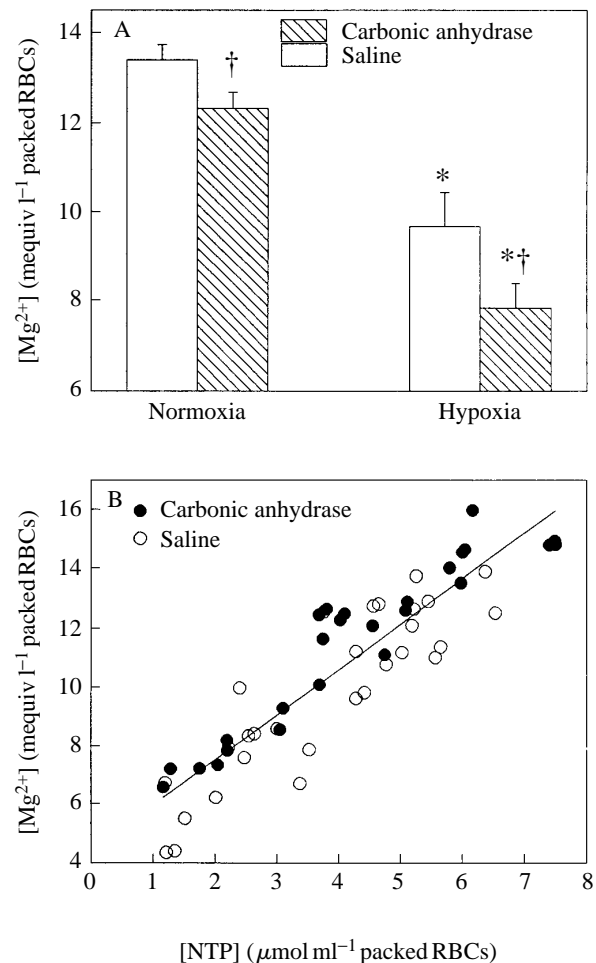
fish. Infusion of CA at the onset of hypoxia resulted in a larger drop in NTP levels during hypoxia. In these animals, plasma pH was lower than in the saline-infused fish. This reduced pH is known to enhance the effect of catecholamines on erythrocytes (Nikinmaa, 1990) and could account for the larger reduction of NTP levels in CA-infused fish.

Increased levels of circulating catecholamines induce the release from the spleen of immature red blood cells (Nilsson and Grove, 1974; Perry and Kinkead, 1989), which contain less NTP than do mature erythrocytes (Lane *et al.* 1981). Wells and Weber (1990), however, observed elevated NTP levels in cells released from the spleen, indicating that this may not be part of the cause of the reduction in NTP levels in hypoxic fish.

NTP levels may be reduced during hypoxia by a decrease in the rate of glycolysis due to an inhibition of

phosphofructokinase (PFK). PFK is inhibited by a decrease in pH and by MgATP. This seems unlikely, however, because Mg^{2+} and ATP levels both decreased and because there is no correlation between pH and NTP levels during the period over which NTP levels change during the initial phase of hypoxia. The decrease in red cell ATP levels may be a direct consequence of reduced oxidative phosphorylation caused by a reduced oxygen supply, as suggested by Greaney and Powers (1978). Blood oxygen levels, however, were not measured, but even in hypoxic trout they were probably higher than the limiting condition (5 mmHg; 0.7 kPa) reported for oxidative phosphorylation in other cell types (see Nikinmaa, 1990).

There is no relationship between pH and NTP levels at the onset of changes in the levels of organic phosphate following hypoxic exposure. After 48 h of hypoxia, however, there is a clear correlation between pH and NTP levels (Fig. 3). The initial effects of pH may be masked by the action of catecholamines, but these decrease with time (Fievet *et al.* 1988; Thomas *et al.* 1991), unmasking the action of pH, perhaps on PFK activity and, therefore, on NTP production.

There is a strong correlation between ATP and GTP levels (Fig. 2). The reason for this is not clear. However, the interconversion reactions of ATP and GTP and the possibility that both are under similar control (Val, 1993) could account for at least part of the observed relationship.

The availability of GTP and ATP to hemoglobin could be altered by the concentration of magnesium in the RBCs. GTP and particularly ATP are readily complexed with magnesium, which greatly reduces their modulatory effect on hemoglobin oxygen-affinity (see Houston, 1985). Magnesium metabolism in nucleated erythrocytes is poorly understood (Houston and Gray, 1988), as are the dynamics of its movements across the red cell membrane. A significant reduction of red blood cell Mg^{2+} levels was observed in rainbow trout exposed to hypoxia and in CA-injected animals exposed to both hypoxic and normoxic conditions (Fig. 4A). Assuming that a constant proportion of total magnesium is bound to NTP, significant changes in the total erythrocyte levels of magnesium may induce significant changes in hemoglobin oxygen-affinity by changing the levels of ATP and GTP available to hemoglobin. Such changes in total magnesium could account for an indirect regulation of hemoglobin oxygen-affinity.

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