J. exp. Biol. **156**, 233–248 (1991)
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DESENSITIZATION OF ADRENALINE-INDUCED RED BLOOD CELL H^+ EXTRUSION *IN VITRO* AFTER CHRONIC EXPOSURE OF RAINBOW TROUT TO MODERATE ENVIRONMENTAL HYPOXIA

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Accepted 5 October 1990

Summary

The sensitivity of red blood cell Na^+/H^+ exchange to exogenous adrenaline was assessed *in vitro* using blood withdrawn from catheterized rainbow trout (*Oncorhynchus mykiss*) maintained under normoxic conditions [water P_{O_2} (Pw_{O_2})= 20.66 kPa] or after exposure to moderate hypoxia (Pw_{O_2}=6.67–9.33 kPa) for 48 h, which chronically elevated plasma adrenaline, but not noradrenaline, levels. Peak changes in whole-blood extracellular pH over a 30 min period after adding 50–1000 nmol l^{-1} adrenaline were employed as an index of sensitivity; the blood was pre-equilibrated to simulate arterial blood gas tensions in severely hypoxic fish (Pa_{O_2}=2.0 kPa, Pa_{CO_2}=0.31 kPa). Blood pooled from normoxic fish displayed a dose-dependent reduction in whole-blood pH after addition of adrenaline. Blood pooled from three separate groups of hypoxic fish, however, displayed diminished sensitivity to adrenaline, ranging from complete desensitization to a 60 % reduction of the response. Subsequent experiments performed on blood from individual (i.e. not pooled) normoxic or hypoxic fish demonstrated an inverse correlation between the intensity of H^+ extrusion (induced by exogenous adrenaline addition) and endogenous plasma adrenaline levels at the time of blood withdrawal. However, acute increases in plasma adrenaline levels *in vitro* did not affect the responsiveness of the red blood cell to subsequent adrenergic stimulation. The intensity of H^+ extrusion was inversely related to the Pa_{O_2} *in vivo* between 2.67 and 10.66 kPa, and directly related to the logarithm of the endogenous plasma adrenaline level. The results suggest that desensitization of Na^+/H^+ exchange in chronically hypoxic fish is related to persistent elevation of

Key words: red blood cell, catecholamines, hypoxia, Na^+/H^+ exchange, *Oncorhynchus mykiss*.

levels of this catecholamine. This desensitization can be reversed *in vitro* as a function of time, but only when blood is maintained under sufficiently aerobic conditions.

Introduction

It is well documented that red blood cells (RBCs) of rainbow trout possess a Na^+/H^+ exchanger that can be activated by application of exogenous catecholamines *in vitro* (Baroin *et al.* 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986; see also reviews by Nikinmaa, 1986; Nikinmaa and Tufts, 1989). Although direct evidence is lacking, it is apparent from measurements of intracellular pH (pHi) and/or electrolyte levels, that RBC Na^+/H^+ exchange is also stimulated by elevation of endogenous catecholamine levels during periods of stress *in vivo*. Examples of such stresses include severe hypoxia (Fievet *et al.* 1987, 1988; Boutilier *et al.* 1988; Motais *et al.* 1989; see also review by Thomas and Motais, 1990), hypercapnia (Perry *et al.* 1987; Perry and Kinkead, 1989; Vermette and Perry, 1988) and exhaustive exercise (Primmett *et al.* 1986; Milligan and Wood, 1987).

Adrenergic activation of the Na^+/H^+ exchanger and consequent extrusion of H^+ from the RBC is thought to be important in (i) minimizing fluctuations of RBC pHi during extracellular acidosis or (ii) contributing to an actual elevation of RBC pHi during severe hypoxia. This alkalization of the RBC, in conjunction with associated responses induced by activation of Na^+/H^+ exchange, such as RBC swelling (see review by Motais and Garcia-Romeu, 1987), reduction of intracellular nucleoside triphosphate (NTP) levels (Tetens and Christensen, 1987; Ferguson and Boutilier, 1989) and increased cooperativity of haemoglobin O_2-binding (Thomas and Motais, 1990), aids blood oxygen transport during periods of stress by elevating or sustaining haemoglobin O_2-affinity (Nikinmaa, 1983; Tetens and Christensen, 1987; Claireaux *et al.* 1988; see also review by Perry and Wood, 1989) while still permitting adequate delivery of O_2 to the tissues.

There are large inter- and intraspecific variations in the response of the teleost RBC Na^+/H^+ exchanger (Milligan and Wood, 1987; Jensen, 1986; Fuchs and Albers, 1988; Salama and Nikinmaa, 1988; Tufts and Randall, 1989; Hyde and Perry, 1989, 1990). Although the underlying basis of interspecific differences remains unresolved, it is clear from studies on the rainbow trout that extracellular variables such as pH and P_{O_2} can profoundly modify intraspecific responses. Trout RBCs become increasingly responsive to catecholamines during extracellular acidosis (Nikinmaa *et al.* 1987*b*; Borgese *et al.* 1987) or after reduction of haemoglobin O_2-saturation (Motais *et al.* 1987). Furthermore, RBCs of tench and carp, normally insensitive to catecholamines, display adrenergic responses during severe hypoxia (Nikinmaa *et al.* 1987*a*; Salama and Nikinmaa, 1988) or hypoxic hypercapnia (Jensen and Weber, 1985; Fuchs and Albers, 1988).

Until now, all studies on intraspecific variability in RBC adrenergic responses have focused exclusively on *acute* changes in blood pH and/or P_{O_2}. *Chronic*

perturbation of blood respiratory status, for example by environmental hypoxia, is undoubtedly a common situation in nature, but has not been examined previously as a possible influence on the RBC response. In the present study we have tested the hypothesis that long-term pre-exposure (48 h) of rainbow trout to moderate hypoxia would decrease the sensitivity of RBC Na^+/H^+ exchange to exogenous adrenaline *in vitro* and that the underlying mechanism of this desensitization would be related to a persistent elevation of plasma catecholamine levels *in vivo*.

Materials and methods

Experimental animals

Rainbow trout (*Salmo gairdneri* Richardson=*Oncorhynchus mykiss* Walbaum) weighing between 200 and 400 g were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and transported to the University of Ottawa. Several different batches of trout were obtained over the 1 month duration (May) of the experiments. Fish were held indoors in large tanks supplied with flowing aerated and dechlorinated City of Ottawa tapwater (see Perry *et al.* 1989, for water ionic composition). The temperature of the holding and experimental water was 10±1 °C, photoperiod was kept at 12 h light:12 h dark. Fish were fed daily but not for 48 h before experimentation.

Animal preparation

Fish were anaesthetized in a solution of 1:10 000 (w/v) ethyl-*m*-aminobenzoate (MS 222; adjusted to pH 7.5 with $NaHCO_3$) and placed on an operating table that permitted continuous irrigation of the gills with the same solution. Indwelling cannulae (Clay Adams PE 50 polyethylene tubing) were implanted into the dorsal aorta (Soivio *et al.* 1972). After surgery, fish were placed into individual black Perspex boxes (3 l volume) supplied with aerated flowing water, and allowed to recover for 24 h prior to experimentation.

The normoxic groups of fish were maintained under these conditions for a further 48 h during which the partial pressure of oxygen in the water (Pw_{O_2}) was 20.66±0.67 kPa (standard error of the mean; S.E.M.).

The hypoxic groups were placed in identical boxes and, after the initial 24 h recovery period, hypoxia was imposed for a further 48 h period. This was achieved by bubbling nitrogen into a water equilibration column. By manipulating the rates of gas and water flows into the equilibration column, the desired degree of hypoxia (6.67 kPa$<Pw_{O_2}<$9.33 kPa) was reached within a few minutes. After these initial adjustments, Pw_{O_2} rarely changed by more than a few pascals during the next 48 h and only occasionally was it necessary to readjust water or gas flow.

In each experiment, separate groups of six fish were exposed simultaneously to normoxia or hypoxia.

Blood sampling and storage

At the end of the 48 h period of hypoxia or normoxia, approximately 3 ml of

blood was withdrawn slowly from the dorsal aortic cannula of each fish into a pre-heparinized syringe. The blood sampling was terminated immediately if fish struggled or became agitated. Arterial blood P_{O_2} (Pa_{O_2}) was determined immediately on a subsample (0.5 ml). The blood was then retrieved from the measuring chamber of the P_{O_2} electrode to determine haemoglobin concentration ([Hb]). A separate subsample was used for measurement of endogenous adrenaline and noradrenaline concentrations; after centrifugation (12 000 *g* for 2 min) the plasma was stored at −80 °C for no longer than 1 week for subsequent assay of catecholamine levels.

Experimental protocol

The standard protocol consisted of measuring the change in whole-blood pH (i.e. extracellular pH, pHe) in flasks containing 1 ml of blood after the addition (25 μl bolus injection) of L-adrenaline bitartrate dissolved in Cortland saline (Wolf, 1963) to yield final nominal concentrations ranging from 50 to 1000 nmol l^{-1}. The blood was maintained at ambient water temperature in a shaking water bath and gassed continuously with a humidified gas mixture (0.3 % CO_2; 2 % O_2; remainder N_2) supplied by a Wösthoff gas-mixing pump (model M301-A/F). A hypoxic gas mixture was utilized to amplify the magnitude of the adrenergic Na^+/H^+ exchange (Motais *et al.* 1987). Whole-blood pH was measured on 50 μl samples immediately before addition of adrenaline and 5, 10, 15, 20 and 30 min thereafter.

These experiments were performed on samples of blood either pooled from six fish or on samples from individuals.

Experiments using pooled blood

The blood samples from each of the six fish were combined in a 50 ml tonometer flask containing ammonium heparin (10 i.u. ml^{-1}) and placed on ice. A sample of this pooled blood (0.5 ml) then was removed to determine haemoglobin concentration, [Hb], and plasma catecholamine levels.

Series 1. In this series the aim was to establish dose–response curves in blood pooled from normoxic fish under optimal conditions of sensitivity. This was accomplished by allowing the blood to remain on ice for 3 h after sampling while being gassed with humidified air. The flasks were shaken frequently during this 3 h period preceding the experiment.

Series 2. In this series the responses of blood pooled from normoxic fish were compared with the responses of blood pooled from hypoxic fish. Three separate groups (six fish each) of hypoxic fish were tested. The normoxic blood was equilibrated initially with air and then placed on ice for 2 h, whereas the hypoxic blood was equilibrated initially with a gas mixture containing 2 % air and 98 % N_2 and also placed on ice for 2 h. In one of the hypoxic groups in this series, and in the normoxic group, small blood samples were taken at six different times during the 48 h exposure period in order to follow the changes in plasma adrenaline and noradrenaline levels over time.

Series 3. The objective of this series was to observe the evolution of sensitivity

as a function of (i) time and (ii) blood oxygenation after sampling. To do so, the experiments were performed at times varying between 1 and 10 h after sampling for both the normoxic and hypoxic blood pools.

Experiments using blood from individuals

Series 4. Blood was sampled and incubated separately from each fish at the end of the 48 h hypoxic or normoxic exposure. Because of the small individual blood sample volumes, the experiments were always performed using a single adrenaline concentration of 1000 nmol l^{-1}. However, they offered the possibility of establishing relationships between individual *in vivo* Pa_{O_2} and catecholamine values and the sensitivity of the blood to adrenaline *in vitro*. In addition, some individual blood samples were acutely 'spiked' with 50–600 nmol l^{-1} of exogenous adrenaline immediately after blood sampling to evaluate the effects of acute, as opposed to chronic, elevations in catecholamine levels.

Analytical procedures

Whole-blood pH (i.e. extracellular pH) and Pa_{O_2} were determined by using Radiometer microcapillary pH (G299A) and P_{O_2} (E5046) electrodes maintained at ambient water temperature in conjunction with a Radiometer PHM71 acid–base analyzer and BMS3 MK2 blood microsystem. Pw_{O_2} was continuously monitored from the output of the water equilibration column using a Radiometer P_{O_2} electrode (housed in a thermostatted cuvette) and PHM71 meter.

Plasma adrenaline and noradrenaline levels were determined on alumina-extracted plasma samples using high performance liquid chromatography (HPLC) in conjunction with electrochemical detection, according to the basic method of Woodward (1982) as modified by Perry and Vermette (1987).

Haemoglobin measurements were performed in duplicate on 20 μl blood samples using a commercial spectrophotometric assay kit (Sigma).

Standardization of data

The blood haemoglobin concentration differed from specimen to specimen and, for a given blood, the changes in pHe after addition of adrenaline varied as a linear proportional function of the haemoglobin concentration. Thus, to ensure comparable data, all results have been expressed on the basis of a blood haemoglobin concentration of 10 g 100 ml^{-1}.

Results

Series 1

A decrease in whole-blood pH (pHe) was observed after the addition of adrenaline to blood pooled from normoxic fish (Fig. 1). The time course and intensity of acidification varied with the concentration of adrenaline in the flasks after its addition. It is noteworthy that, for the lower concentrations (75 and

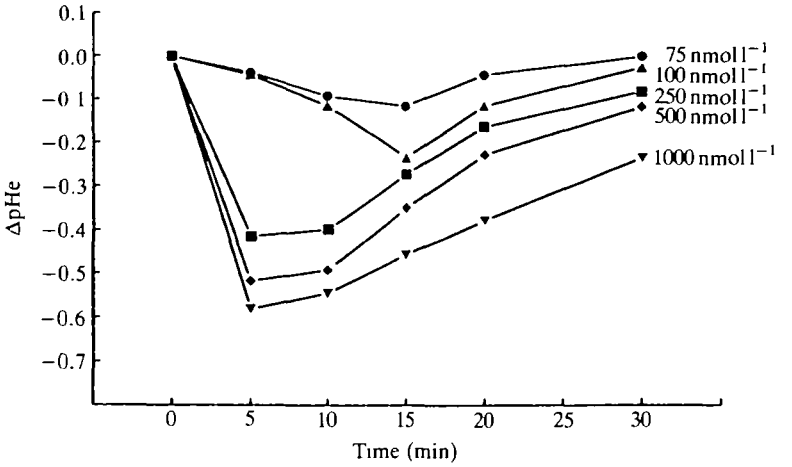

Fig. 1. Temporal and dose-dependent changes in whole-blood pH (ΔpHe) after addition of adrenaline (75–1000 nmol l^{-1} final nominal concentrations; ●, 75 nmol l^{-1}; ▲, 100 nmol l^{-1}; ■, 250 nmol l^{-1}; ◆, 500 nmol l^{-1}; ▼, 1000 nmol l^{-1}) to blood pooled from normoxic trout (N=6), kept on ice for 3 h under normoxic conditions, and then equilibrated with a hypoxic gas mixture (P_{O_2}=2.0 kPa, P_{CO_2}=0.31 kPa).

100 nmol l^{-1}), the acidification peaked after 15 min whereas, for higher concentrations (250–1000 nmol l^{-1}), the maximal reduction of pHe occurred within the initial 5 min after addition. Moreover, a marked increase in sensitivity was observed between 100 and 250 nmol l^{-1}. Regardless of the concentration of adrenaline employed, pHe gradually returned towards pre-addition values. The highest concentration of adrenaline utilized in this and subsequent experiments (1000 nmol l^{-1}) caused the largest change in pHe. Preliminary experiments, however, demonstrated that even higher concentrations of adrenaline caused still greater reductions in pHe (data not shown).

Series 2

Exposure to moderate hypoxia (Pw_{O_2}: 6.67–9.33 kPa) for 48 h caused a 60–100 % reduction in the size of the RBC adrenergic response. Fig. 2 compares the adrenaline-induced pHe variations in blood pools withdrawn from three separate groups of hypoxic fish with those obtained using blood from normoxic fish. It appears that the intensity of the pHe changes was not only related to the imposed adrenaline concentrations, but also to the Pa_{O_2} of the individual blood samples constituting the pools. Thus, the largest pHe changes caused by adrenaline were measured on the blood pooled from the normoxic group which, of course, had the highest mean Pa_{O_2} (13.12±0.99 kPa; normoxic group). In contrast, smaller changes or even a total absence of pHe variations resulted from adrenaline addition after 48 h of exposure to hypoxia. Note that, in the three groups of hypoxic fish examined, the diminished response to adrenaline appeared to vary as

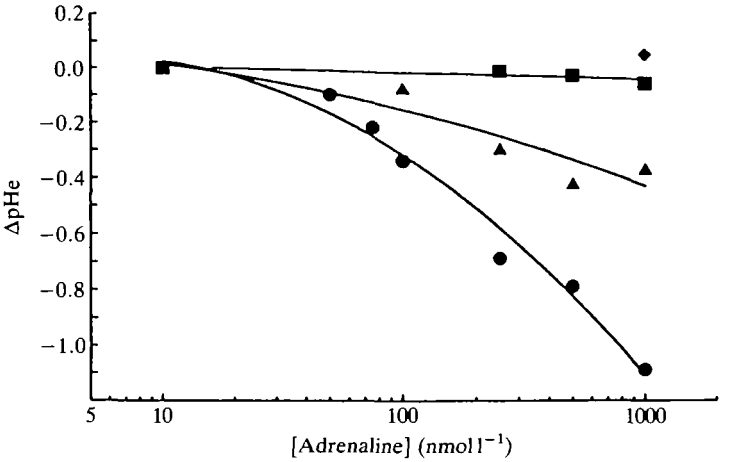

Fig. 2. Peak changes in whole-blood pH (ΔpHe) after addition of various levels of adrenaline (50–1000 nmol l^{-1} final nominal concentrations) to blood pooled from normoxic trout (●; N=6) or from three separate groups of hypoxic trout (Pw_{O_2}= 6.67–9.33 kPa for 48 h) displaying a range of mean Pa_{O_2} values [▲, Pa_{O_2}= 5.13±0.75 kPa (N=6); ■, Pa_{O_2}=3.39±0.68 kPa (N=6); ◆, Pa_{O_2}=2.0±0.23 kPa (N=6)]. Curves were drawn by eye. Blood from hypoxic trout was maintained on ice for 3 h under hypoxic conditions. All other details as in Fig. 1.

a function of the mean Pa_{O_2} of the particular group, such that the least responsive group had the lowest Pa_{O_2} and *vice versa*.

Series 3

The apparent hypoxia-induced desensitization of RBC proton release is a reversible phenomenon. Fig. 3 demonstrates that the sensitivity of the pooled blood evolves as a function of time after blood withdrawal, but only when the blood is maintained under sufficiently aerobic conditions. In this experiment, two pools of blood were assessed during a 9-h period by measuring the peak changes in pHe after addition of 1000 nmol l^{-1} adrenaline to samples at regular intervals.

The first pool was composed of blood from hypoxic fish. Two hours after blood withdrawal the RBC proton release was totally unresponsive to adrenaline, as indicated by the absence of changes in pHe (Fig. 3). This pool then was divided into two sub-pools. The first sub-pool was continuously equilibrated with hypoxic gas (P_{O_2}=2.0 kPa) and remained completely insensitive to adrenaline during the following hours. In contrast, the second sub-pool was equilibrated with normoxic gas (P_{O_2}=20.66 kPa) and regained a progressively greater sensitivity with time thereafter.

The second pool was composed of blood from normoxic fish and then divided evenly into two sub-pools (as above). The initial pool displayed a pronounced sensitivity to adrenaline which was further increased as a function of time when blood was kept under normoxic conditions. In contrast, the blood exhibited only a marginal increase in sensitivity over time when equilibrated with hypoxic gas.

Series 4

The following results were obtained using blood from individual fish (i.e. not pooled). This enabled us to correlate the adrenaline-induced pHe changes with the exact Pa_{O_2} or endogenous adrenaline value existing in the blood *in vivo* at the time of sampling. Fig. 4A shows that (i) when Pa_{O_2} was above 10.66 kPa, the change in pHe was always approximately −0.3 pH unit for an adrenaline concentration of 1000 nmol l^{-1}, and (ii) when it was below 10.66 kPa, progressive desensitization

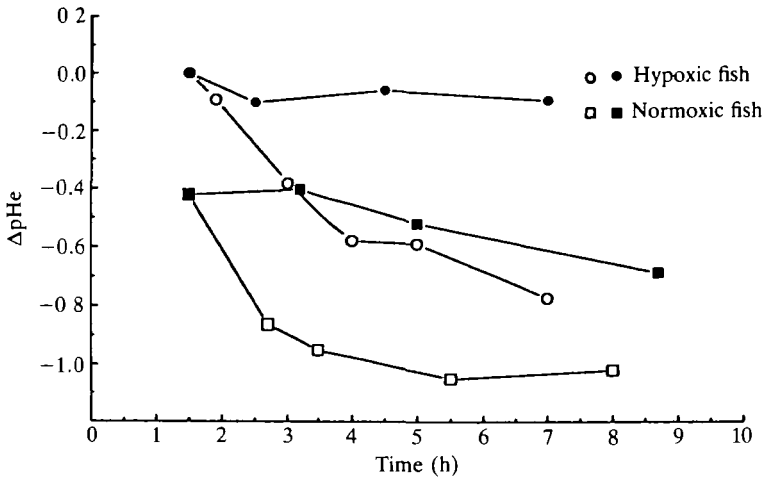

Fig. 3. Peak changes in whole-blood pH (ΔpHe) after addition of 1000 nmol l^{-1} adrenaline to blood pooled from hypoxic (circles, N=6) and normoxic (squares, N=6) trout at varying times after blood withdrawal. Filled symbols represent blood maintained or assessed under hypoxic conditions (P_{O_2}=2.0 kPa, P_{CO_2}=0.31 kPa), whereas open symbols represent blood maintained under normoxic conditions (P_{O_2}=20.66 kPa) or assessed under hypoxic conditions.

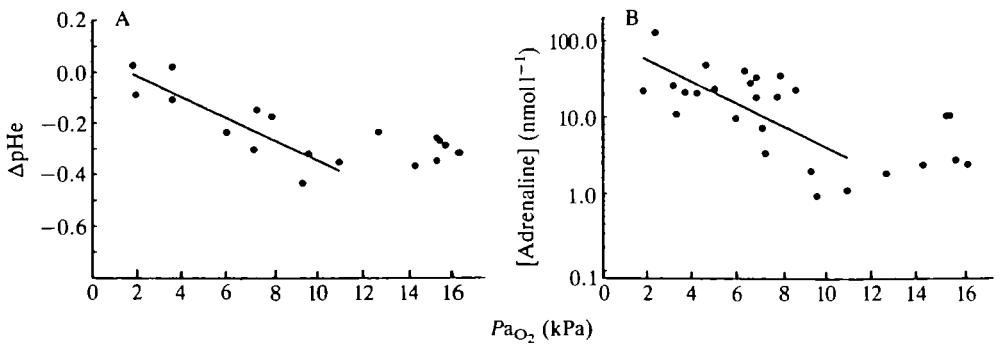

Fig. 4. Relationships between arterial blood oxygen tension (Pa_{O_2}) and (A) peak changes in whole-blood pH (ΔpHe) after addition of 1000 nmol l^{-1} adrenaline and (B) arterial plasma adrenaline concentration in blood samples from individual rainbow trout. The linear regression equations for Pa_{O_2} values below 10.9 kPa are (A) $\Delta pHe = -0.0056 Pa_{O_2} + 0.07$; r=0.87 and (B) $\log[\text{adrenaline}] = -0.019 Pa_{O_2} + 2.04$; r=0.65.

occurred as a direct function of Pa_{O_2}. Fig. 4B demonstrates that the logarithm of the adrenaline concentration in the blood was also related to Pa_{O_2} in a similar manner. This observation supports the hypothesis of a direct relationship between the levels of circulating adrenaline and the sensitivity of the RBC Na^+/H^+ exchanger to exogenous adrenaline *in vitro*.

It is likely, however, that the levels of adrenaline in the blood during sampling did not accurately reflect the levels that prevailed during the preceding 48 h of hypoxia. Indeed, in the one hypoxic group (Pa_{O_2}=5.13±0.1 kPa, N=6) in which catecholamine levels were monitored throughout the 48 h exposure (from series 2), plasma adrenaline concentration increased to very high levels (approximately 300 nmol l^{-1}) at the midpoint of the exposure and then returned towards levels observed in the normoxic fish by 48 h (Fig. 5A). Thus, the relationship shown in Fig. 4B presumably would have been even more obvious had the history of the adrenaline levels been recorded for each fish. Fig. 5 also demonstrates that, under our particular experimental conditions, no significant changes in plasma noradrenaline levels were observed during hypoxia.

Finally, Fig. 6 illustrates the excellent correlation between the logarithm of adrenaline concentration *in vivo* (at the time of blood sampling) and the intensity

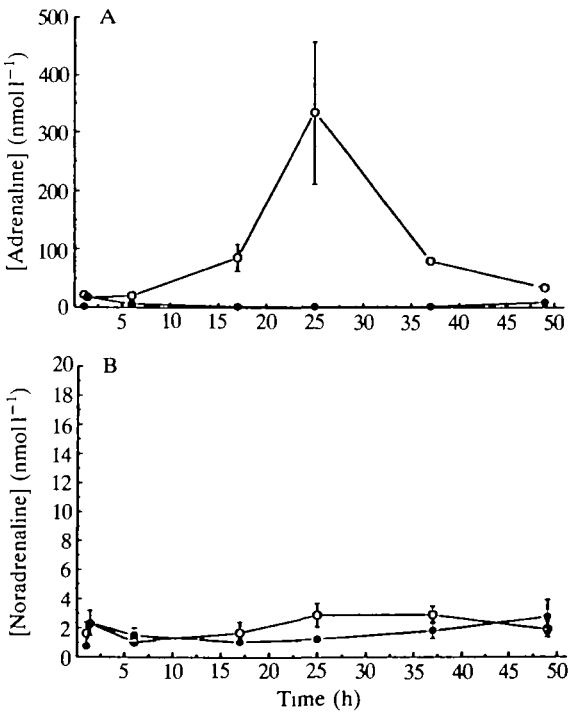

Fig. 5. The effects of chronic (48 h) exposure to moderate hypoxia (○, N=6) or normoxia (●, N=6) on (A) plasma adrenaline and (B) plasma noradrenaline levels. All values shown are means 1 S.E.M.; where not indicated, the standard error lies within the symbol.

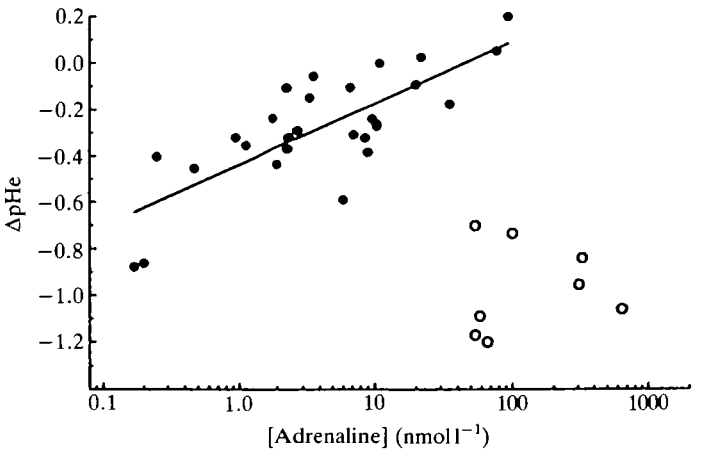

Fig. 6. The relationship between the peak changes in whole-blood pH (ΔpHe) after addition of 1000 nmol l^{-1} adrenaline and the levels of endogenous adrenaline in arterial plasma at the time of blood withdrawal (filled symbols; ΔpHe= 0.265log[adrenaline]−0.439; r=0.77) or after acutely 'spiking' several blood samples with exogenous adrenaline to achieve initial levels ranging from 50 to 600 nmol l^{-1} (open symbols).

of the proton release after the addition of 1000 nmol l^{-1} adrenaline *in vitro*. In contrast, *acute in vitro* elevations of adrenaline levels clearly did not reduce the responsiveness of RBCs to subsequent application of 1000 nmol l^{-1} adrenaline, as indicated by the pronounced reductions of pHe, regardless of the initial adrenaline concentration (Fig. 6; open circles). In these experiments, adrenaline was added to the flasks immediately after blood sampling. After a 3 h waiting period, the final concentration of adrenaline was applied.

Discussion

The addition of catecholamines to the blood of rainbow trout *in vitro* causes reproducible effects on both intracellular and extracellular acid–base and ionic status. These adrenergic effects have been discussed elsewhere in detail (Nikinmaa and Tufts, 1989; Thomas and Motais, 1990; Motais and Garcia-Romeu, 1987) but are reviewed here briefly because they form the basis of our assessment of the consequences of chronic moderate hypoxia. Immediately after addition of adrenaline or noradrenaline, a Na^+/H^+ exchanger on the RBC membrane is activated; it causes an elevation of intracellular Na^+ concentration, extrusion of H^+ and consequent cell swelling owing to water entry. The RBC intracellular pH rises proportionately and both the bicarbonate and non-bicarbonate buffer systems react accordingly. The net effect, however, is an elevation of intracellular HCO_3^- levels, thereby stimulating Cl^- influx and HCO_3^- efflux *via* the well-known HCO_3^-/Cl^- anionic exchanger and thus raising intracellular Cl^- concen-

tration, swelling the cell further and finally attenuating the internal pH elevation. However, as the amount of Cl^- entering the cell is not as great as the amount of Na^+, the resulting effect is a persistent increase of RBC pHi.

The massive entry of Na^+ and Cl^- into the RBC is matched by an equivalent output of H^+ and HCO_3^-. In the absence of extracellular carbonic anhydrase, these two ions form CO_2 at the uncatalysed rate. This is very slow at physiological pH and temperature (between 25 and 90 s; Edsall, 1968). Indeed, it has recently been demonstrated that the slow rate of this reaction is responsible for a large, but temporary, extracellular acid–base disequilibrium during which whole-blood pH (pHe) is markedly reduced (Motais *et al.* 1989). A large portion of the CO_2 produced in the plasma at the uncatalysed rate diffuses into the red cell, where it is rapidly converted to H^+ and HCO_3^- in the presence of carbonic anhydrase. This phenomenon sustains the anionic and cationic exchangers and cell swelling, as long as CO_2 is reintroduced into the RBC by way of this cycle.

This extracellular acid–base disequilibrium is precisely what we have measured *in vitro* in the present study. Because it is easy to measure and of large amplitude, the pHe variation allows accurate quantification of the intensity of the Na^+/H^+ exchange after the addition of adrenaline. Moreover, because of (i) the transient uncoupling between extra- and intracellular compartments and (ii) the absence of steady-state (i.e. equilibrium) conditions in the plasma, it is incorrect to calculate H^+ flux from pHe or RBC pHi variations using the buffer capacities of either compartment. Other techniques frequently used to assess the intensity of RBC Na^+/H^+ exchange, such as measurements of RBC pHi, water content or NaCl levels, are considerably more difficult and often yield highly variable data. For these reasons, measurements of pHe variations appeared to us to be the most appropriate and practical approach for quantifying the intensity of adrenergically activated Na^+/H^+ exchange.

The observation of a dose-dependent variation of pHe after the addition of a catecholamine to blood is not original. This phenomenon has been observed *in vitro* by many investigators, either using washed cells placed in artificial media (Baroin *et al.* 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986) or using whole blood (e.g. Perry and Vermette, 1987; Tetens *et al.* 1988; Motais *et al.* 1989; Ferguson and Boutilier, 1989).

This study and others (Tetens *et al.* 1988; Ferguson and Boutilier, 1989) have demonstrated a rapid (between 5 and 15 min depending on the dose) maximal decline of pHe after adrenaline addition and then a gradual return to control levels. This indicates that for high doses of adrenaline (greater than 250 nmol l^{-1}) the period of maximal disequilibrium (i.e. lowest pHe) is reached after approximately 5 min. For this reason, we chose to assess the sensitivity of the RBC Na^+/H^+ exchange to exogenous adrenaline after 5 min, at which time the intensity of this exchange is presumably greatest. In addition, each assessment of RBC Na^+/H^+ exchange intensity was performed using blood equilibrated with 2.0 kPa P_{O_2}. This protocol not only simulated the Pa_{O_2} values prevailing in the most severely impacted of the hypoxic exposure groups (Fig. 2) but also served to

amplify the responses of the RBC Na^+/H^+ exchange to adrenaline (Motais *et al.* 1987).

The results of this study support our hypothesis that pre-exposure of rainbow trout to moderate hypoxia *in vivo* subsequently reduces the sensitivity of the RBC Na^+/H^+ exchange to exogenous adrenaline *in vitro*. With the available data, a plausible explanation for this phenomenon is desensitization of RBC beta receptors caused by chronic elevation of plasma adrenaline level which, in turn, reflects the severity of the depression of Pa_{O_2}. This argument is supported by (i) the pronounced and prolonged elevation of plasma adrenaline levels during hypoxia (Fig. 5), (ii) the inverse correlation between the intensity of exogenous adrenaline-induced RBC Na^+/H^+ exchange and endogenous plasma adrenaline concentrations (Fig. 6), and (iii) the inverse relationship between Pa_{O_2} (below 10.66 kPa) and arterial plasma adrenaline levels (Fig. 4). It is also noteworthy that Van Dijk and Wood (1988) found that RBC pHi regulation after exhaustive exercise was markedly attenuated in 'winter' trout with chronically elevated plasma catecholamine levels, despite very large post-exercise mobilization of adrenaline and noradrenaline.

In higher vertebrates, the phenomenon of beta receptor desensitization owing to chronic elevation of catecholamines is well documented (see review by Lefkowitz *et al.* 1985), but it has not previously been described in fish. There are at least two mechanisms underlying the process of beta receptor desensitization in mammals which may or may not be mutually exclusive. For example, cell surface beta receptors may be functionally internalized and thus become inaccessible to adrenergic agonists or, alternatively, they may remain in position but become functionally uncoupled from adenylate cyclase. In the absence of direct measurements of cell surface beta receptors in the present study, we cannot distinguish between these two possibilities. It is noteworthy, however, that apparent RBC surface beta receptors have been shown to be highly mobile in both carp (Marttila and Nikinmaa, 1988) and trout (S. Reid; personal communication). Although a reduction of functional RBC beta receptors is the most obvious explanation of the desensitization of the Na^+/H^+ exchange, we cannot exclude other possibilities, including desensitization of the Na^+/H^+ exchanger itself (Garcia-Romeu *et al.* 1988) or an uncoupling between cyclic AMP and activation of the exchanger. Clearly, further experiments are warranted to differentiate between these possibilities.

The reduction of Na^+/H^+ exchange activity in blood obtained from hypoxic fish could be reversed gradually but only if the blood was maintained under sufficiently aerobic conditions. This differential ability to recover sensitivity depending upon available oxygen cannot be attributed to differential rates of endogenous catecholamine oxidation because we have demonstrated that acute *in vitro* adjustments of adrenaline do not affect the sensitivity of the RBC Na^+/H^+ exchange to subsequent application of adrenaline (Fig. 6). We suggest, however, that the recovery of sensitivity reflects reincorporation of nascent cytosolic beta receptors into the RBC membrane. In support of this idea, it was recently

demonstrated that, during hypoxia, cultured myocytes of chick embryo display a reduction of cell surface beta receptors which subsequently reappear during reoxygenation (Marsh and Sweeney, 1989).

We can exclude elevated noradrenaline levels as a contributing factor in the desensitization of RBC Na^+/H^+ exchange because plasma levels remained constant throughout the 48 h period of hypoxia, despite the large increases in adrenaline levels. This observation confirms the notion that adrenaline is generally the predominant catecholamine released into the circulation of trout during environmental disturbances, with notable exceptions (see Perry *et al.* 1989, for a literature review of plasma catecholamine levels during a variety of stresses). The very high levels of adrenaline measured in this study differ greatly from those reported in a similar study (Boutilier *et al.* 1988) in which exposure of rainbow trout for 24 h to a similar Pw_{O_2} provoked only a small rise in this catecholamine. This discrepancy may be related to seasonal differences, since the fish used in the study of Boutilier *et al.* (1988) were maintained at considerably lower water temperatures.

Previous studies that have examined the relationship between blood P_{O_2} and the sensitivity of RBC Na^+/H^+ exchange to catecholamines (Motais *et al.* 1987; Marttila and Nikinmaa, 1988) have exposed RBCs or fish to acute hypoxia. The results demonstrated an increased sensitivity of RBC Na^+/H^+ exchange to catecholamines. The results of the present study, utilising chronic hypoxia, are exactly opposite. This important difference between acute and chronic hypoxia demonstrates that the history of the fish in its environment prior to sampling must be considered as an important variable when working *in vitro*.

A further complicating factor is a progressive time-dependent increase in the sensitivity to adrenaline *in vitro*, even when blood from control normoxic fish is used. This enhancement of sensitivity occurs only when the blood is oxygenated sufficiently (Fig. 3). It is unclear whether this increase in sensitivity reflects a re-establishment of the conditions that prevailed *in vivo* or whether it reflects a deviation from the normal situation *in vivo*. We have no reason to suspect that blood sampling, itself, can evoke a loss of sensitivity. Indeed, levels of adrenaline immediately after sampling in normoxic fish were very low (Fig. 5) and acute elevation of adrenaline levels *in vitro* (this study) or *in vivo* (S. Thomas and S. F. Perry, unpublished observations) does not reduce RBC Na^+/H^+ exchange sensitivity. These results are in apparent disagreement with those of Garcia-Romeu *et al.* (1988), demonstrating rapid desensitization of the Na^+/H^+ exchanger *in vitro* after acute addition of isoproterenol. There are a variety of hormones, such as neurohypophyseal hormones (Guibbolini and Lahlou, 1987) and insulin (Houslay *et al.* 1984), that can impair cyclic-AMP-mediated responses *in vivo*. Thus, we consider it more likely that the increasing sensitivity *in vitro* is an abnormal condition, perhaps caused by oxidative metabolism of hormones or other factors that otherwise depress the agonist-induced activity of RBC Na^+/H^+ exchange, oxidation of surface ligands which may normally limit receptor sensitivity, or mobilization of cytosolic beta receptors to the cell surface.

Regardless of which hypothesis is correct, it is clear that whole blood *in vitro* is a dynamically changing tissue; care must be taken in the interpretation of results from such experiments.

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