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THE EFFECTS AND PHYSIOLOGICAL CONSEQUENCES OF RAISED LEVELS OF CORTISOL ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ERYTHROCYTE β-ADRENORECEPTORS

BY SCOTT D. REID AND STEVE F. PERRY*
Department of Biology, University of Ottawa, Ottawa, Ontario Canada K1N 6N5

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Summary

We have investigated the influence of cortisol on the β-adrenoreceptor population of rainbow trout [*Oncorhynchus mykiss* (Walbaum)] erythrocytes and determined what impact it has on the adrenergic responsiveness of erythrocytes *in vitro* to exogenous catecholamines. To do so, the erythrocyte β-adrenoreceptors were characterized in fish with chronically elevated plasma cortisol levels ($118\pm5.9\,ng\,ml^{-1}$, >10 days) and compared with shams, using radioreceptor assay techniques. The number of 'internalized', low-affinity receptors was increased when cortisol levels were raised, but the number of high-affinity, 'surface' receptors was not altered. The physiological significance of this response was ascertained by assessing the *in vitro* sensitivity (or responsiveness) of erythrocytes to adrenaline and noradrenaline ($10–1000\,nmol\,l^{-1}$) under normoxic ($P_{O_2}=16.13\pm0.55\,kPa$, $P_{CO_2}=0.41\pm0.01\,kPa$) or hypoxic ($P_{O_2}=4.13\pm0.15\,kPa$, $P_{CO_2}=0.43\pm0.01\,kPa$) conditions. Erythrocyte sensitivity to catecholamines, as determined by changes in both whole-blood pH (ΔpHe) and intracellular cyclic AMP content, was greater in hypoxic than in normoxic blood. Although cortisol further enhanced the responsiveness of erythrocytes to catecholamines, this amplification in sensitivity was observed only during hypoxia.

When the radioreceptor assay was conducted using erythrocytes from the catecholamine sensitivity experiments, results were consistent with initial receptor density data. An increase in surface receptor density was associated with hypoxia *in vitro*. This hypoxia-specific increase in surface β-adrenoreceptors was significantly enhanced in the cortisol-treated erythrocytes, showing that cortisol had a significant impact on erythrocyte β-adrenoreceptor dynamics in addition to the direct influence of hypoxia.

This study has shown (1) that, by itself, *in vitro* hypoxia simultaneously initiates the movement of internal receptors to the cell surface, where they become physiologically active, and the replenishment of the internal receptor pool,

* To whom reprint requests should be addressed.

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(2) that cortisol increases receptor availability by increasing the internal pool of low-affinity receptors in the absence of any stimulus for receptor mobilization, and (3) that the sensitivity of erythrocytes to catecholamines is directly proportional to the number of high-affinity receptors present at the erythrocyte surface. Thus, we suggest that, under conditions of chronic stress, cortisol may pre-adapt the erythrocytes to receive additional physiological inputs that can ultimately enhance respiratory performance beyond that which would be possible in the absence of chronically elevated levels of cortisol.

Introduction

Elevations in circulating cortisol levels have long been associated with stress in mammalian vertebrates (Selye, 1950). The stress response in teleostean fish is considered to be similar in nature (Schreck, 1981), including an increase in the cortisol titre (Mazeaud *et al.* 1977). For example, high cortisol levels have been observed in dying fish (Fagerlund, 1967; Strange *et al.* 1977) and in response to environmental acidification (Brown *et al.* 1984, 1986*a*), confinement (Strange *et al.* 1977; Pickering and Pottinger, 1989), excessive handling (Barton *et al.* 1986) and forced swimming regimes (Woodward and Smith, 1985; Barton and Schreck, 1987). Cortisol is thought to mediate changes in hydromineral balance and carbohydrate metabolism (Mazeaud *et al.* 1977), although the latter has recently come under question (D. E. Andersen, S. D. Reid, T. W. Moon and S. F. Perry, unpublished results). The consensus is, however, that cortisol stimulates an increase in metabolism in teleosts (Umminger, 1977; Chan and Woo, 1978). Barton and Schreck (1987) subjected juvenile steelhead trout (*Oncorhynchus mykiss* Walbaum) to bouts of forced exercise and observed a positive correlation between elevated plasma cortisol levels and oxygen consumption rates. Thus, it appears that cortisol has some influence, either directly or indirectly, on the oxygen requirements of teleosts.

Similarly, circulating levels of catecholamines (adrenaline and/or noradrenaline) are elevated during many forms of stress, including hypoxia (Butler *et al.* 1979; Tetens and Christensen, 1987; Boutilier *et al.* 1988), hypercapnia (Perry *et al.* 1987, 1989), air exposure (Fuchs and Albers, 1988) and exercise (Ristori and Laurent, 1985; Butler *et al.* 1986; Primmett *et al.* 1986; Milligan and Wood, 1987; Tang and Boutilier, 1988). The stress-related increase in circulating catecholamine levels is thought to aid blood oxygen transport directly by elevating or maintaining haemoglobin oxygen-affinity (Nikinmaa, 1983; Tetens and Christensen, 1987; Claireaux *et al.* 1988). This is accomplished, in part, through alkalization of the internal erythrocyte environment *via* stimulation of a Na^+/H^+ antiporter (Baroin *et al.* 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986; Nikinmaa, 1986; Nikinmaa and Tufts, 1989).

The initial step in the activation of the erythrocyte Na^+/H^+ antiporter is an interaction between the catecholamine and a high-affinity erythrocyte surface β-adrenoreceptor. The β-adrenoreceptors of trout erythrocytes have been characterized both pharmacologically (Baroin *et al.* 1984; Cossins and Richardson, 1985;

Tetens *et al.* 1988) and directly using radioreceptor assay techniques (Marttila and Nikinmaa, 1988; Reid *et al.* 1991). In mammals, it has been shown that administration of glucocorticoids, *in vivo* or to cultured cells, stimulates a two- to threefold increase in the steady-state levels of cell surface β-adrenoreceptors (Lee and Reed, 1977; Mano *et al.* 1979; Foster and Harden, 1980; Fraser and Venter, 1980; Lai *et al.* 1981; Scarpace *et al.* 1985). Thus, the primary objective of this study was to determine possible alterations in trout erythrocyte β-adrenoreceptor characteristics and distribution following exposure of erythrocytes to elevated cortisol titres. A secondary objective was to assess the impact that modifications, if any, in the β-adrenoreceptor population might have on erythrocyte physiology.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of either sex weighing between 172 and 273 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the University of Ottawa. Fish were held in large fibreglass tanks (Living Stream; Toledo, Ohio) supplied with flowing, aerated and dechlorinated City of Ottawa tapwater (see Perry *et al.* 1989, for water ionic composition). Fish were acclimated to those conditions for at least 4 weeks prior to experimentation. Water temperature in holding facilities varied between 12 and 15 °C. Photoperiod was kept constant at 12 h light:12 h dark. Fish were fed daily with commercial trout pellets (Purina Trout Chow) and tanks were syphoned after feeding to eliminate the build-up of organic material.

Experimental protocol

Series I. Characterization of β-adrenoreceptors following chronic or acute elevation in plasma cortisol levels

Initial experiments were conducted to determine the influence of raised cortisol levels on the β-adrenoreceptor population of trout erythrocytes through both *in vivo* and *in vitro* exposure of erythrocytes to elevated levels of cortisol.

Chronic elevations in plasma cortisol levels. Elevations in plasma cortisol concentrations were achieved using Alzet mini osmotic pumps (Alzet, California) surgically implanted into the peritoneal cavity of fully anaesthetized (ethyl *m*-aminobenzoate; MS 222, 0.1 g l^{-1} H_2O) sexually immature rainbow trout. Cortisol, as hydrocortisone 21-hemisuccinate (Sigma), was dissolved in a steroid-miscible carrier (33 % w/v Molecusol powder; Pharmatec Inc.), then placed within the osmotic pumps. The concentration of the cortisol solutions to be placed into the osmotic pumps was determined according to the average mass of the animals to be used, the pump flow rates (corrected for the water temperature) and the desired cortisol delivery rate. A nominal plasma cortisol concentration of 100 ng ml^{-1} was chosen in accordance with the levels of cortisol and the cortisol clearance rate determined in acid-stressed fish (Brown *et al.* 1986*b*). The controls for this experimental treatment group consisted of animals into which were placed

mini-osmotic pumps containing carrier without the cortisol. The suitability of this technique for elevating plasma cortisol levels chronically was determined previously in this laboratory (D. E. Andersen, S. D. Reid, T. W. Moon and S. F. Perry, unpublished results).

Following surgery, cortisol-treated and sham-operated trout were placed in 400 l rectangular fibreglass tanks supplied with well-aerated, flowing water. Fish resumed feeding within 48 h and remained unrestrained in the holding tanks for 10–13 days following implantation of the osmotic pumps. At this time, fish were selected randomly and blood samples were withdrawn from the caudal peduncle with a heparinized syringe (10 i.u. ammonia heparin ml^{-1} saline) to determine plasma cortisol levels, haematocrit, haemoglobin concentration and for the erythrocyte β-adrenoreceptor binding assay.

Acute elevations in plasma cortisol levels. To verify the results of the chronic, *in vivo* cortisol exposure experiments, they were repeated on erythrocytes exposed *in vitro* to raised cortisol levels for a shorter time (24 h). In addition to the nominal exposure concentration of 100 ng ml^{-1} cortisol, erythrocytes were exposed to 50 ng ml^{-1} cortisol for 24 h so that possible dose-dependent modifications in erythrocyte β-adrenoreceptor characteristics might be demonstrated.

Several anaesthetized fish (MS 222, 0.1 g l^{-1} H_2O) were fitted with dorsal aortic cannulae using the method of Soivio *et al.* (1975). Cannulae were checked following completion of the surgery, then flushed with heparinized (10 i.u. ml^{-1} saline) trout saline (Wolf, 1963) prior to recovery of the animals. Fish were then placed in individual black Perspex boxes (volume 3 l) supplied with well-aerated flowing water. After at least 48 h of post-surgery recovery, approximately 3 ml of blood was removed from each of several fish (N=4–5), pooled in a heparinized tonometer flask (125 i.u. ml^{-1}), which was placed in a shaking water bath, and gently mixed for 24 h at 14°C. During this time, the pool of blood (sham erythrocytes) was gassed with humidified air.

An additional pool of blood was obtained from the same group of fish and stored under identical conditions as the blood of shams. Cortisol was added to this pool to yield final concentrations of 50 and 100 ng ml^{-1}. Both pools of cortisol-treated erythrocytes were placed in the shaking water bath in which the sham blood was maintained and were gassed identically. After a 24 h incubation period, samples from all pools were taken for the determination of haematocrit and haemoglobin concentration and for the characterization of erythrocyte β-adrenoreceptors.

Series II. Catecholamine sensitivity of erythrocytes

Since the focus of this study was the possible cortisol-mediated enhancement of the erythrocyte β-adrenoreceptor population, we attempted to correlate any changes in receptor density or affinity with alterations in the responsiveness of erythrocytes to catecholamines. Therefore, as a physiological assay, experiments were conducted to determine the sensitivity or responsiveness of Na^+/H^+ exchange in erythrocytes to *in vitro* additions of catecholamines after a variety of treatments.

Normoxia. The standard protocol consisted of measuring the change in whole-blood pH (extracellular pH or pHe) in round-bottomed tonometer flasks containing 1 ml of blood after the addition (20 μl) of either adrenaline or noradrenaline (L-adrenaline or L-noradrenaline, bitartrate salt dissolved in saline) to yield final nominal concentrations ranging from 10 to 1000 nmol l^{-1}. The blood was maintained at 15°C in a shaking water bath and gassed continuously with a humidified gas mixture (0.5 % CO_2 in air) supplied by a gas-mixing pump (Wösthoff, model M301-A/F). Whole-blood pH was measured on 50 μl samples immediately before additions of adrenaline and 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20 and 30 min thereafter. In addition, two pre-catecholamine exposure blood samples of 200 μl were taken to determine total plasma CO_2 and erythrocyte cyclic AMP content, and blood oxygen partial pressure (P_{O_2}). After 5 min of catecholamine exposure, in addition to the 5 min whole-blood pH measurement, a 200 μl blood sample was removed from the sample to determine catecholamine-stimulated erythrocyte cyclic AMP concentration.

These experiments were conducted on pools of blood obtained from all series I treatment groups (acutely and chronically elevated cortisol titres and their accompanying shams) consisting usually of 4–5 fish per pool. Additional pools of blood were collected from fish used for the normoxia series II experiments. These pools were handled, maintained and gassed identically to the pools used to determine erythrocyte catecholamine responsiveness. However, these erythrocytes were used to characterize the erythrocyte β-adrenoreceptors.

Hypoxia. It has been demonstrated that acute hypoxia significantly increases the responsiveness of erythrocytes to catecholamines (Motais *et al.* 1987; Nikinmaa *et al.* 1987; Salama and Nikinmaa, 1988). Marttila and Nikinmaa (1988) have also shown that acute hypoxia results in an increase in the number of high-affinity surface β-adrenoreceptors expressed in carp (*Cyprinus carpio* Linnaeus) erythrocytes. Therefore, to assess further the manner in which cortisol-related alterations in erythrocyte β-adrenoreceptors might affect erythrocyte physiology, the erythrocyte catecholamine sensitivity assay was repeated under conditions of hypoxia.

Erythrocyte catecholamine sensitivity was assessed using pools of blood equilibrated with a humidified gas mixture of 0.5 % CO_2 in air (20 %) and nitrogen (80 %). Furthermore, additional pools of blood were collected from fish used for the hypoxia series II experiments. These pools were handled, maintained and gassed identically to the pools used for the determination of erythrocyte catecholamine responsiveness. However, these erythrocytes, as in the normoxic component of the series II experiments, were used to characterize the erythrocyte β-adrenoreceptors.

Analytical procedures

Blood analysis

Erythrocyte cyclic AMP content was determined on 40 μl of packed erythrocytes, obtained by centrifugation (12 000 ***g***, 2 min), according to the protocol of a

commercially available radioimmunoassay (Amersham Canada Inc.). Cortisol measurements were performed on 25 μl plasma samples using a commercial radioimmunoassay (Immunocorp, Mount Royal, Quebec). Haemoglobin levels were measured in duplicate on 20 μl blood samples using a commercial spectrophotometric assay kit (Sigma). Whole-blood pH (pHe) and oxygen partial pressure (P_{O_2}) were measured using Radiometer microcapillary pH (G299A) and P_{O_2} (E5046) electrodes adjusted to 15°C, in conjunction with a Radiometer PHM 71 acid–base analyzer and BMS3 MK2 blood microsystem. The change in pHe (ΔpHe) elicited by additions of catecholamines was determined as the difference between pHe prior to catecholamine addition and the maximal reduction in pHe following catecholamine stimulation. The measured values of ΔpHe were corrected for differences in blood haemoglobin content according to Thomas *et al.* (1991). Plasma P_{CO_2} was calculated from total plasma CO_2 content (Corning CO_2 analyzer) according to the Henderson–Hasselbalch equation, using the estimates for αCO_2 and pK of Boutilier *et al.* (1984). Haematocrit (Hct) was determined using heparinized microcapillary tubes centrifuged for 10 min at 12 000 ***g***.

Binding assay

The characterization of erythrocyte β-adrenoreceptors was accomplished on whole blood using both a hydrophobic ligand, 1-[4,6-propyl-^{3}H]dihydroalprenolol ([^{3}H]DHA; DHA, specific activity 2.59–4.07 TBq mmol^{-1}; Amersham) and a hydrophilic ligand, (±)-4-(3-*t*-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP 12177; CGP, specific activity 1.26–1.70 TBq mmol^{-1}; Amersham) as described by Reid *et al.* (1991). Briefly, blood samples were contained within heparinized tonometer flasks (100 i.u. ml^{-1} blood) placed on ice. The blood samples were frequently swirled prior to and during radioreceptor assays to prevent red cells from settling. Radioligand binding was initiated by the addition of 40 μl of blood to 360 μl of Cortland saline into which the radioligand (DHA, 5–100 nmol l^{-1}; CGP, 5–40 nmol l^{-1}) had been added with 10 μmol l^{-1} (±)-propranolol (Sigma) or 200 μmol l^{-1} (−)-isoproterenol (Sigma), or alone. Both propranolol, a β-adrenoreceptor antagonist, and isoproterenol, a β-adrenoreceptor agonist, compete with the radioligands for receptor binding. However, whereas propranolol competes with DHA and CGP for both apparent high- and low-affinity receptor sites, isoproterenol competes only for high-affinity receptor sites (André *et al.* 1981). The number of erythrocytes added to the incubation solution was determined by diluting 10 μl of blood in 10 ml of saline then counting the erythrocytes present using a haemocytometer (American Optical) and taking into account the dilution and volume of blood used.

Incubations were terminated, following a 45 min radioligand incubation, by transferring the erythrocytes to borosilicate filters (no. 32, Mandel Scientific), by way of a cell membrane harvester (Brandel 24R) with subsequent repeated washings (four times) with 5 ml of ice-cold Cortland saline. The filters were placed in glass liquid scintillation vials containing 8 ml of fluor (ACS II, Amersham) and

allowed to settle for at least 24 h. Sample radioactivity was then determined using an LKB Rackbeta (model 1214) liquid scintillation counter, with all counts corrected for quenching using an external standard technique. The maximal number of propranolol- or isoproterenol-displaceable binding sites (B_{max}, in disints min^{-1} $cell^{-1}$) and the apparent dissociation constants (K_D) were determined using Scatchard plot analysis (Scatchard, 1949). Receptor density (B_{max}) was then converted to, and expressed on, a receptor per erythrocyte basis by multiplying the maximal number of specific receptor sites (disints min^{-1} $cell^{-1}$) by the radioligand specific activity and Avogadro's number. The number of low-affinity, internal erythrocyte β-adrenoreceptors was calculated as the difference between the propranolol-displaceable [^{3}H]DHA binding sites (total erythrocyte β-adrenoreceptor density) and the number of isoproterenol-displaceable [^{3}H]DHA binding sites (erythrocyte DHA surface receptors; intermediate affinity) according to Reid *et al.* (1991). Similarly, specific erythrocyte β-adrenoreceptors detected using [^{3}H]CGP in combination with isoproterenol are referred to as high-affinity erythrocyte surface β-adrenoreceptors, in accordance with our previously reported findings (Reid *et al.* 1991).

Statistical analysis

All experiments were performed at least twice with differences between mean values assessed by analysis of variance (ANOVA) followed by Fisher's LSD for multiple comparison, at a 95 % level of confidence, using a commercial statistical software package (Statview 512$^+$). All values are presented as means ±S.E.

Results

Series I

Chronic elevations in plasma cortisol level

The plasma cortisol titre of fish fitted with 'cortisol-loaded' mini-osmotic pumps was 118±5.9 ng ml^{-1} (N=6) compared to 23±1.4 ng ml^{-1} (N=6) in the shams. This *in vivo* elevation in plasma cortisol levels resulted in no significant alteration in the calculated erythrocyte β-adrenoreceptor dissociation constants (K_D) of all tritiated ligand–blocker combinations (Fig. 1A). The K_D values for the isoproterenol-displaceable and propranolol-displaceable CGP binding sites are typically not different (Marttila and Nikinmaa, 1988; Reid *et al.* 1991; Fig. 2 this study). Thus, for the sake of clarity and brevity, the propranolol-displaceable CGP binding site data are not shown in this and other figures. There were, however, significant modifications in receptor density (B_{max}) in response to chronic elevations in plasma cortisol levels. As illustrated in Fig. 1B, the total (propranolol-displaceable [^{3}H]DHA binding sites) number of erythrocyte β-adrenoreceptors was increased by 21.4 % compared with the shams. Since there was no change in the number of erythrocyte surface (isoproterenol-displaceable) β-adrenoreceptors detected with either CGP or DHA, this increase in the total receptor pool was caused solely by a significant increase in the number of internal (propranolol-

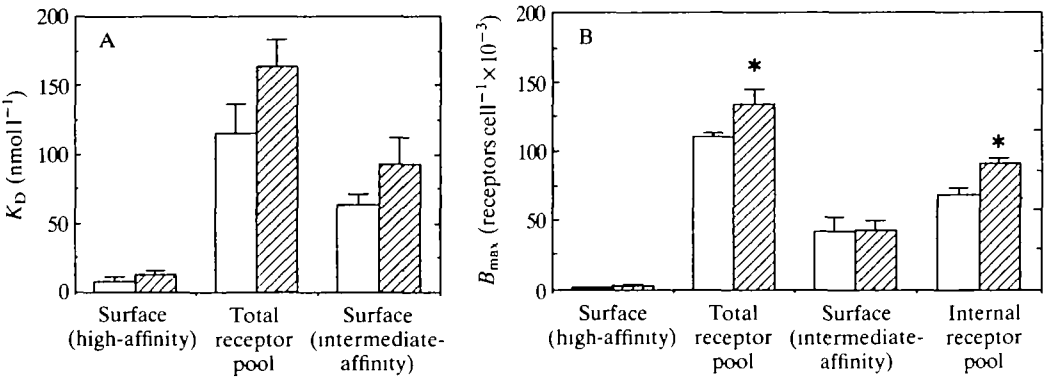

Fig. 1. The effects of chronic exposure (*in vivo*, 10–13 days) to elevated plasma cortisol levels (118±5.9 ng ml^{-1}) on erythrocyte β-adrenoreceptor dissociation constant (K_D) (A) and density (B_{max}) (B). All values are means ±1 standard error of the mean. N=6 for both shams (open histograms) and cortisol-treated (cross-hatched histograms) erythrocytes. * indicates that the cortisol-treated mean differs significantly from the corresponding sham value.

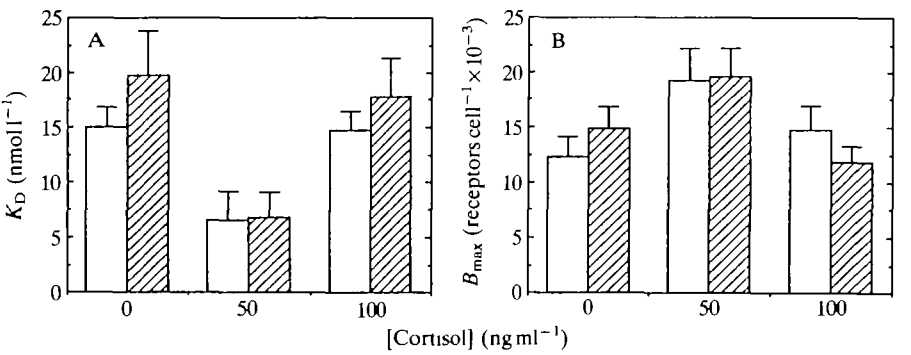

Fig. 2. Changes in (A) the dissociation constant (K_D) and (B) the density (B_{max}) of the high-affinity, surface β-adrenoreceptors after acute exposure (*in vitro*, 24 h) to 50 or 100 ng ml^{-1} cortisol. All values are means ±1 standard error of the mean. N=6 for both erythrocyte isoproterenol- (open histograms) and propranolol-displaceable (cross-hatched histograms) [^{3}H]CGP binding sites.

displaceable, isoproterenol-displaceable [^{3}H]DHA binding difference) erythrocyte β-adrenoreceptors. The calculated values for erythrocyte β-adrenoreceptor K_D and density, obtained using these specific tritiated ligands and blockers, agree with previously published values for rainbow trout erythrocytes (Marttila and Nikinmaa, 1988; Reid *et al.* 1991).

Acute elevations in plasma cortisol level

Similar findings were obtained when the receptor binding assays were conducted

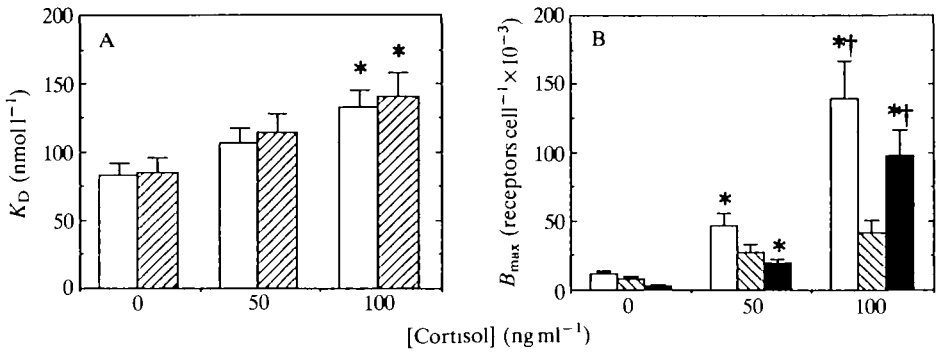

Fig. 3. Changes in (A) the dissociation constant (K_D) and (B) the density (B_{max}) of the propranolol- and isoproterenol-displaceable binding sites after acute exposure (*in vitro*, 24 h) to 50 or 100 ng ml^{-1} cortisol. All values are means ±1 standard error of the mean. N=6 for both erythrocyte isoproterenol-displaceable (open histograms, surface) and propranolol-displaceable (cross-hatched histograms, total) [^{3}H]DHA binding sites. Internal erythrocyte β-adrenoreceptor numbers (filled histograms) were calculated from the difference between the number of total and surface [^{3}H]DHA binding sites. Significant differences between sham (0 ng ml^{-1} cortisol) and cortisol-treated erythrocytes are indicated by *; significant differences between values at 50 and 100 ng ml^{-1} cortisol are indicated by †.

using pools of erythrocytes acutely (24 h) exposed to cortisol (Figs 2 and 3). No dose-dependent response in either CGP-assessed K_D or receptor density was detected when erythrocytes were exposed to 50 and 100 ng ml^{-1} cortisol for 24 h (Fig. 2A). Although, at 50 ng ml^{-1} cortisol, the K_D values for the isoproterenol- and propranolol-displaceable binding sites were somewhat reduced, the values were not statistically different from the values for shams. In addition, the number of high-affinity erythrocyte surface β-adrenoreceptors was not affected by acute exposure to the two concentrations of plasma cortisol (Fig. 2B).

Consistent with the chronic cortisol exposure findings, and in contrast to the acute, erythrocyte CGP-binding data, significant modifications in both DHA-calculated K_D and receptor density were apparent (Fig. 3). The erythrocyte DHA K_D values increased in an apparently dose-dependent manner, although only at a concentration of 100 ng ml^{-1} were the K_D values for the propranolol- and isoproterenol-displaceable binding sites significantly greater than the sham value (0 ng ml^{-1} cortisol; Fig. 3A). The total number of erythrocyte β-adrenoreceptors was significantly greater than sham values at both 50 and 100 ng ml^{-1} cortisol. Similar modifications were evident in the number of internal erythrocyte β-adrenoreceptors, as there were no changes in the DHA-binding surface receptor pool.

Series II

Since significant modifications in the characteristics of the trout erythrocyte β-adrenoreceptor population were observed both *in vivo* and *in vitro*, it was

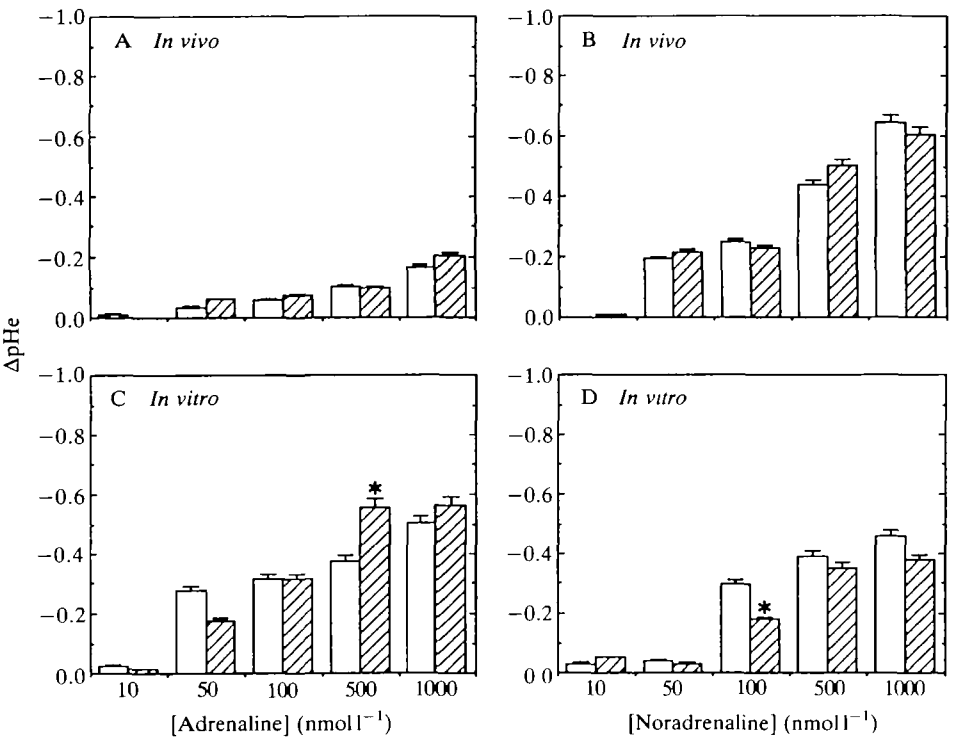

Fig. 4. Maximal reductions in whole-blood pH (ΔpHe) measured under normoxic conditions (P_{O_2}=16.13±0.55 kPa, P_{CO_2}=0.41±0.01 kPa) after additions of adrenaline (A,C) or noradrenaline (B,D) to blood in which erythrocytes were chronically (*in vivo*, 10–13 days; A,B) or acutely (*in vitro*, 24 h; C,D) exposed to elevated cortisol levels (118±5.9 or 100 ng ml^{-1}, respectively). Final nominal concentration of catecholamines was 10–1000 nmol l^{-1}. All values are means ±1 standard error of the mean. Open histograms represent ΔpHe for shams, while cross-hatched histograms represent ΔpHe for cortisol-treated erythrocytes (N=3 for both treatments). All data were corrected for differences in blood haemoglobin concentration. Experiments were conducted on blood pooled from several fish (i.e. 4–5 fish yielded N=1). * indicates that the cortisol-treated mean is significantly different from the corresponding sham value. See Materials and methods for further details.

important to determine whether these modifications were translated into significant alterations in the responsiveness of erythrocytes to catecholamines.

The maximal reductions in ΔpHe following additions of noradrenaline or adrenaline to blood exposed either chronically (*in vivo*) or acutely (*in vitro*) to elevated cortisol levels, and the respective shams, are summarized in Fig. 4. Under normoxic conditions (P_{O_2}=16.13±0.55 kPa, P_{CO_2}=0.41±0.01 kPa), the addition of 10–1000 nmol l^{-1} adrenaline or noradrenaline to blood caused a dose-dependent reduction in pHe in all treatment groups and shams (Fig. 4). Except for only two statistical differences (Fig. 4C,D), the catecholamine-stimulated maxi-

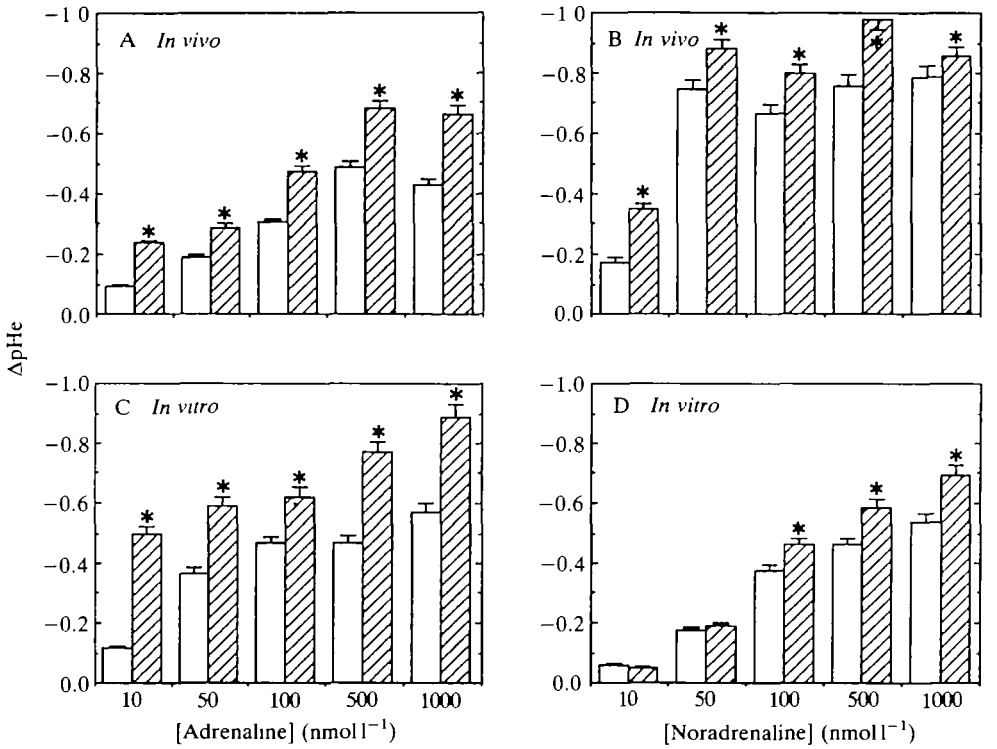

Fig. 5. Maximal reductions in whole-blood pH (ΔpHe) measured under hypoxic conditions (P_{O_2}=4.13±0.15 kPa, P_{CO_2}=0.43±0.01 kPa) after additions of adrenaline (A,C) or noradrenaline (B,D) to blood in which erythroctyes were chronically (*in vivo*, 10–13 days; A,B) or acutely (*in vitro*, 24 h; C,D) exposed to elevated cortisol levels. All other details as in Fig. 4 legend.

mal reductions in pHe in cortisol-treated blood (*in vivo* or *in vitro*) were not significantly different from their respective shams.

The results were altered considerably when these experiments were repeated under hypoxic conditions (P_{O_2}=4.13±0.15 kPa, P_{CO_2}=0.43±0.01 kPa; Fig. 5). In contrast to the responses measured in normoxia, it was observed that ΔpHe was, in the majority of cases, significantly greater when cortisol was administered to the blood, either chronically (Fig. 5A,B) or acutely (Fig. 5C,D). Significant differences in ΔpHe between the cortisol-treated erythrocytes and the shams were consistently observed between 100 and 1000 nmol l^{-1} adrenaline or noradrenaline. Significant modifications in ΔpHe, however, did occur at the lowest concentration of catecholamine. For example, ΔpHe was approximately four times greater when erythrocytes were exposed to cortisol *in vitro* following the addition of 10 nmol l^{-1} adrenaline, than in the shams (Fig. 5C).

The specific hypoxia-dependent modification in the responsiveness of erythrocytes is more clearly illustrated in Fig. 6, in which ΔpHe was calculated as the difference between ΔpHe under hypoxic and normoxic conditions. Apparent in

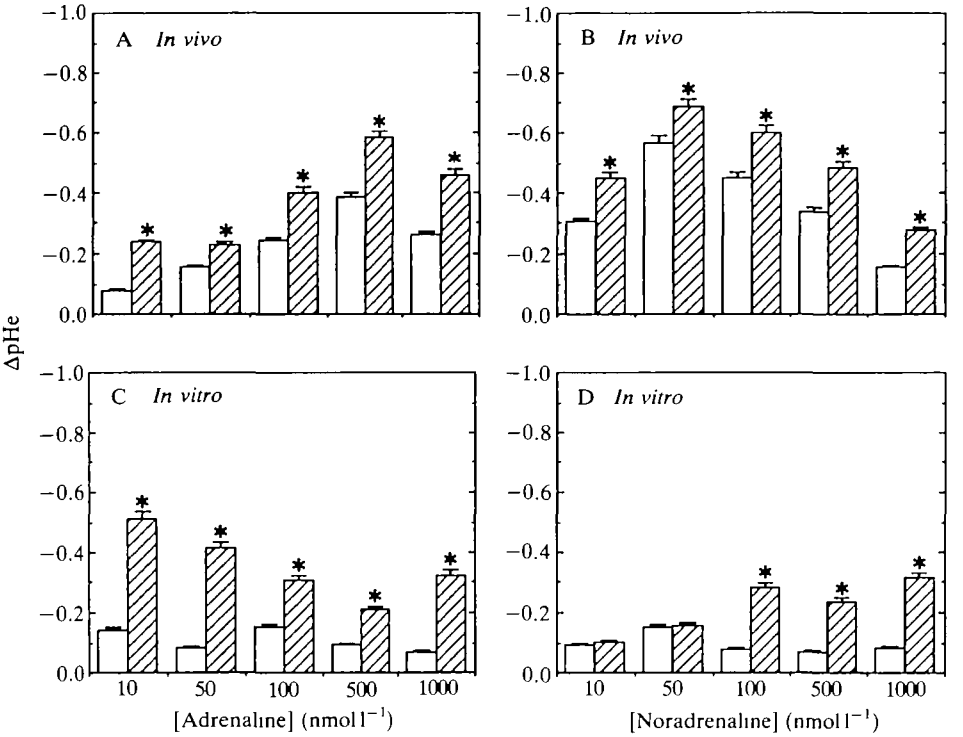

Fig. 6. Hypoxia-specific maximal reductions in whole-blood pH (ΔpHe) after additions of adrenaline (A,C) or noradrenaline (B,D) to blood in which erythrocytes were chronically (*in vivo*, 10–13 days; A, B) or acutely (*in vitro*, 24 h; C,D) exposed to elevated cortisol levels. Data were calculated as the difference between the ΔpHe measured under hypoxic and under normoxic conditions. All other details as detailed in Fig. 4 legend.

this figure is the finding that hypoxia alone increased the catecholamine-stimulated maximal reduction in pHe, as the differences between hypoxia and normoxia ΔpHe were positive in all groups. Furthermore, the additional influence of cortisol on the responsiveness of erythrocytes to adrenaline or noradrenaline is emphasized.

The dose-dependent reductions in pHe under normoxic conditions were clearly reflected by the changes in erythrocyte cyclic AMP contents under these same conditions (Fig. 7). Although not as dramatic, or as consistent, as the observed changes in pHe, intracellular cyclic AMP levels increased in response to additions of adrenaline or noradrenaline of between 10 and 1000 nmol l^{-1}. In all but one instance (Fig. 7C), cortisol had no influence on the catecholamine-stimulated increase in erythrocyte intracellular cyclic AMP content, consistent with the findings illustrated in Fig. 4. Apparently, the cyclic AMP contents of those erythrocytes sampled from fish fitted with the mini-osmotic pumps were lower than those in erythrocytes from fish lacking this interperitoneal implant (Fig. 7A,B *vs* Fig. 7C,D). However, as these differences reflect the differences between *in*

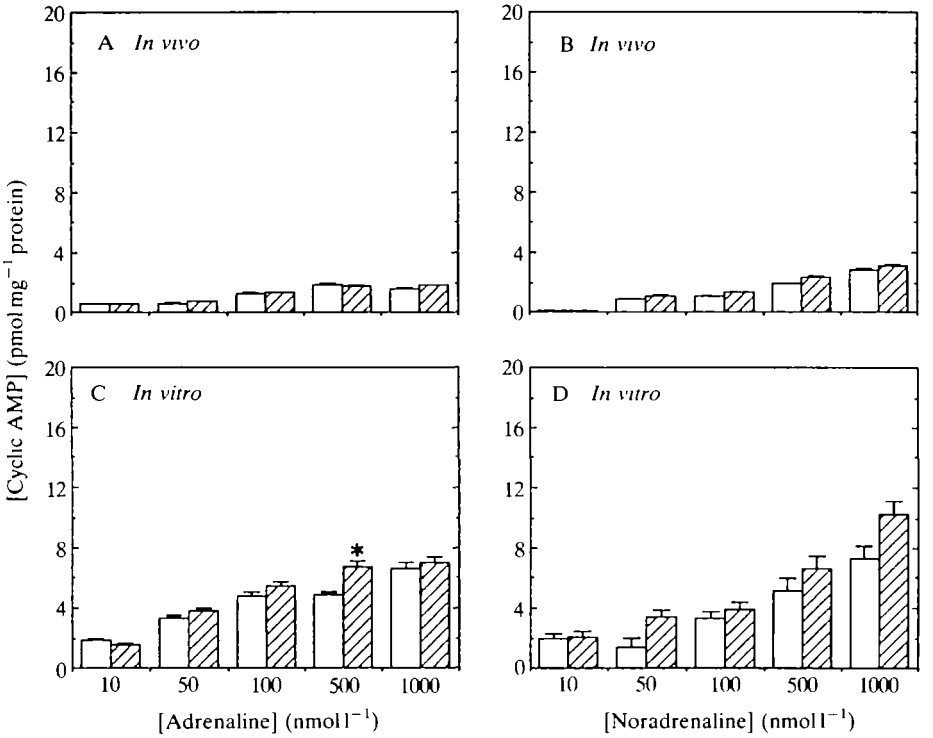

Fig. 7. Intracellular cyclic AMP concentrations of chronically (*in vivo*, 10–13 days; A, B) or acutely (*in vitro*, 24 h; C,D) cortisol-exposed erythrocytes measured under normoxic conditions (P_{O_2}=16.13±0.55 kPa, P_{CO_2}=0.41±0.01 kPa), 5 min after additions of adrenaline (A,C) or noradrenaline (B,D) to blood. Final nominal concentrations of catecholamines were 10–1000 nmol l^{-1}. All values are means ±1 standard error of the mean. Open histograms represent cyclic AMP concentrations of sham erythrocytes, while cross-hatched histograms represent cyclic AMP concentrations of erythrocytes exposed to elevated cortisol titres (N=3 for both treatments). Experiments were conducted on blood pooled from several fish (4–5). * indicates that the cortisol-treated mean is significantly different from the corresponding sham value. See Materials and methods for further details.

vivo and *in vitro* experiments, a statistical comparison of these data would not be appropriate.

Under hypoxic conditions, the dose-dependent increase in intracellular cyclic AMP levels apparent under normoxic conditions was again observed (Fig. 8), as was the difference between the cyclic AMP contents of the erythrocytes of the *in vivo* and the *in vitro* cortisol experiments (Fig. 8A *vs* Fig. 8C). However, under these conditions, the intracellular cyclic AMP contents of erythrocytes from blood with elevated cortisol titres, whether exposure was chronic (Fig. 8A,B) or acute (Fig. 8C,D), were significantly higher than in the shams. With a few exceptions, these differences were apparent across the entire range of adrenaline or noradrenaline concentrations in all treatment groups.

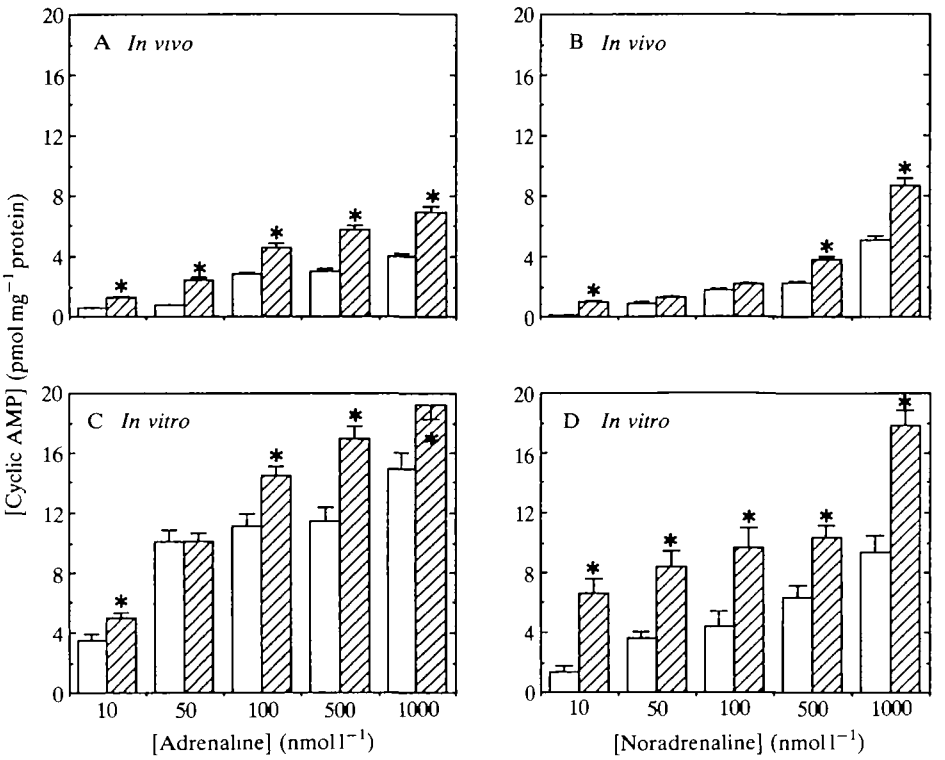

Fig. 8. Intracellular cyclic AMP concentrations of chronically (*in vivo*, 10–13 days; A,B) or acutely (*in vitro*, 24 h; C,D) cortisol-exposed erythrocytes measured under hypoxic conditions (P_{O_2}=4.13±0.15 kPa, P_{CO_2}=0.43±0.01 kPa) 5 min after additions of adrenaline (A,C) or noradrenaline (B,D) to blood. All other details as detailed in Fig. 7 legend.

Fig. 9, as did Fig. 6, illustrates that hypoxia alone alters the physiology of the erythrocytes, regardless of treatment. In most cases, and in particular in blood from the *in vitro* experiments, erythrocyte cyclic AMP levels were higher in hypoxic blood than when measured under normoxic conditions. The additional influence of cortisol on the increase in erythrocyte cyclic AMP content following catecholamine additions is also emphasized.

Under normoxic conditions, cortisol-treated erythrocytes possessed significantly greater numbers of internal β-adrenoreceptors than did shams, with no measurable difference in surface β-adrenoreceptor density regardless of the radioligand–blocker combination (isoproterenol- and propranolol-displaceable CGP binding *vs* isoproterenol-displaceable DHA binding) used (Fig. 10). As a result of the increased size of the internal β-adrenoreceptor pool, the total number of erythrocyte β-adrenoreceptors was also found to be significantly greater in cortisol-treated erythrocytes when compared to shams. The modifications in receptor numbers were apparently more reduced in erythrocytes chronically exposed to cortisol than when cortisol administration was prolonged over several

erythrocytes, whether the erythrocytes were chronically (Fig. 12A) or acutely (Fig. 12B) exposed to cortisol. When measured under hypoxic conditions, however, the surface β-adrenoreceptor K_D of cortisol-treated erythrocytes was significantly lower than that of shams, with a greater difference associated with chronic than with acute cortisol exposure (Fig. 12A *vs* Fig. 12B). Hypoxia alone

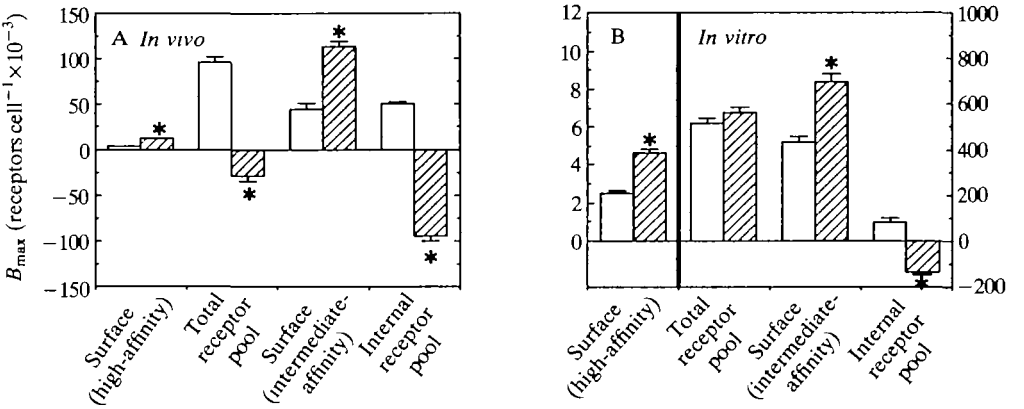

Fig. 11. Hypoxia-specific changes in erythrocyte β-adrenoreceptor pool densities of (A) chronically (*in vivo*, 10–13 days) or (B) acutely (*in vitro*, 24 h) cortisol-exposed erythrocytes. Data were calculated as the difference between β-adrenoreceptor densities measured under hypoxic and under normoxic conditions. All other details as in Fig. 10 legend.

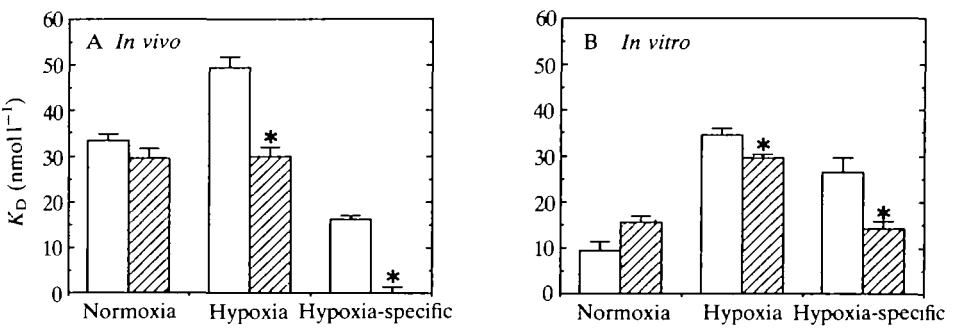

Fig. 12. Changes in the dissociation constant (K_D) of the high-affinity ([^{3}H]CGP), surface β-adrenoreceptors of erythrocytes chronically (*in vivo*, 10–13 days; (A) or acutely (*in vitro*, 24 h; (B) exposed to elevated cortisol levels. All values are means ±1 standard error of the mean. Measurements of K_D were made on erythrocytes maintained under normoxic or hypoxic conditions; the hypoxia-specific changes in K_D were calculated as the difference between the K_D measured under hypoxia and normoxia. Open histograms represent the K_D of the high-affinity surface β-adrenoreceptors of sham erythrocytes, while cross-hatched histograms represent the K_D of the high-affinity surface β-adrenoreceptors of erythrocytes exposed to elevated cortisol titres (N=3 for both treatments). Experiments were conducted on blood pooled from several fish (4–5). * indicates that the cortisol-treated mean is significantly different from the corresponding sham value. See Materials and methods for further details.

was responsible for a significant increase in the K_D of the surface β-adrenoreceptors of 16.1±0.72 and 26.3±1.05 nmol l^{-1} in the *in vivo* and *in vitro* shams, respectively. Cortisol exposure under hypoxic conditions, however, either totally eliminated the hypoxia-specific alteration in erythrocyte β-adrenoreceptor K_D (Fig. 12A) or reduced it by nearly 52 % (Fig. 12B).

Discussion

This study is the first to examine extensively modifications in the localization of β-adrenoreceptors on and within trout erythrocytes. Three studies have characterized the β-adrenoreceptors of teleost erythrocytes with two of those specific for the β-adrenoreceptors of rainbow trout erythrocytes (Marttila and Nikinmaa, 1988; Reid *et al.* 1991). Of the trout studies, Reid *et al.* (1991) most thoroughly investigated erythrocyte β-adrenoreceptor localization and determined that two distinct populations of β-adrenoreceptors were present within and on these cells. It is generally accepted that two populations of β-adrenoreceptors exist in higher vertebrates, one located on the cell surface and a second within intracellular vesicles (Waldo *et al.* 1983). Further, it has been shown in chicken aortic cells (Marsh and Sweeney, 1989) and guinea pig myocardium (Maisel *et al.* 1985) that the β-adrenoreceptors are able to move between these pools with ease in response to hypoxia or ischaemia. Our study demonstrates that the erythrocyte β-adrenoreceptors of trout are capable of similar dynamics, not only in response to hypoxia, as initially suggested for carp erythrocytes by Marttila and Nikinmaa (1988), but also in response to acute or chronic exposure to elevated cortisol levels, alone or in concert with hypoxia. In addition, it appears that the production and mobilization of the erythrocyte β-adrenoreceptors occur rapidly; exposure to elevated levels of cortisol for 24 h and for 10–15 days produced similar alterations in β-adrenoreceptor pool densities (Fig. 1B *vs* Fig. 3B).

The initial characterizations of β-adrenoreceptors in this study revealed that cortisol increases the number of internal, low-affinity β-adrenoreceptors (Figs 1 and 3) without affecting either the affinity (Fig. 2A) or the density (Fig. 2B) of high-affinity β-adrenoreceptors expressed at the erythrocyte surface. Cortisol is generally thought to penetrate cell membranes and bind to specific cytosolic and/or nuclear cortisol receptors. The receptor–ligand complex is then able to bind to chromatin, where the complex stimulates an increase in DNA-directed RNA polymerase activity and an increase in the amount of protein biosynthesis (Lehninger, 1978). Although glucocorticoid receptors have been characterized in detail in gill (eel, Sandor *et al.* 1984; brook trout, Chakraborti *et al.* 1987) and liver (brook trout, Chakraborti and Weisbart, 1987), there is no direct evidence for the existence of such receptors within trout erythrocytes. Nevertheless, indirect evidence for erythrocyte cortisol receptors emerges from the recent study of Pottinger (1990). In an investigation of the influence of stress and exogenous cortisol on receptor-like binding of cortisol in the liver of rainbow trout, Pottinger (1990) noted that plasma lacked cortisol-binding activity, while whole blood

contained high-affinity cortisol binding sites. It is likely that the high-affinity cortisol binding sites of whole blood can be attributed to erythrocyte cortisol receptors.

The physiological activity of catecholamines is dependent upon, amongst several factors, the coupling of the β-adrenoreceptor with adenylate cyclase. Since it is unlikely that the internal β-adrenoreceptors are functionally coupled to adenlyate cyclase, it is difficult to envisage the advantage of packaging additional low-affinity β-adrenoreceptors within vesicles in response to elevated plasma cortisol levels. In fact, under normoxic conditions, there was no physiological gain because the activity of the catecholamine-stimulated Na^+/H^+ antiporter did not differ between sham and cortisol-treated erythrocytes (Fig. 4). Further, cortisol had no influence on erythrocyte cyclic AMP content (Fig. 7) or on the number of high-affinity erythrocyte surface β-adrenoreceptors (Figs 1 and 10). The lack of any difference between the cyclic AMP concentration of the sham and cortisol-treated erythrocytes is probably due to the absence of any modification in the number of surface β-adrenoreceptors. Therefore, these data support our hypothesis that the internal, low-affinity β-adrenoreceptors are functionally uncoupled from adenylate cyclase and only the high-affinity surface β-adrenoreceptors are physiologically active.

In the absence of raised cortisol levels, hypoxia stimulated the production of additional internal β-adrenoreceptors and the mobilization of these receptors to the erythrocyte membrane surface (Fig. 11), where they presumably become physiologically active. Our assertion that the new surface β-adrenoreceptors are functionally coupled to adenylate cyclase is based on the observation that cyclic AMP levels, measured 5 min after addition of 10–1000 nmol l^{-1} catecholamine, were significantly enhanced in response to hypoxia (Fig. 9), concurrent with an increase in the number of β-adrenoreceptors expressed at the erythrocyte surface. Hypoxia was also shown to increase the density of high-affinity surface β-adrenoreceptors (Marttila and Nikinmaa, 1988) and catecholamine-stimulated cyclic AMP production (Salama and Nikinmaa, 1990) in carp erythrocytes.

The exact mechanism by which β-adrenoreceptor mobilization is transduced is unknown. Motais *et al.* (1987) suggested that haemoglobin can act as a signal transducer in trout erythrocytes, according to its degree of oxygenation. It is well established that haemoglobin binds to the cytoplasmic side of the erythrocyte membrane in a reversible manner, the predominant site being the cytoplasmic segment of the band 3 protein (Salhany and Shaklai, 1978; Shaklai *et al.* 1977; Salhany *et al.* 1980; Cassoly, 1983) Moreover, deoxygenated haemoglobin has a higher affinity for band 3 protein than does oxygenated haemoglobin (Walder *et al.* 1984; Chetrite and Cassoly, 1985). If haemoglobin acts as a signal transducer for β-adrenoreceptor mobilization, it would have to be associated with factors responsible for the control of erythrocyte membrane turnover and it is tempting to speculate that, depending on its degree of oxygenation, haemoglobin controls β-adrenoreceptor mobilization in erythrocytes by regulating membrane turnover. In any case, membrane turnover is likely and it may explain the reduction in surface

β-adrenoreceptor affinity (increased K_D) observed during hypoxia (Fig. 12). In the cells of higher vertebrates, it has been shown that β-adrenoreceptors may be present, at least transiently, on the cell surface in a low-affinity, uncoupled state while *en route* to sequestration in intracellular vesicles (Strasser *et al.* 1984). If hypoxia were able to increase membrane turnover directly in trout erythrocytes, then the probability of detecting surface β-adrenoreceptors in the uncoupled state would be greatly enhanced and would be reflected in the radioreceptor assay results as an apparent reduction in surface β-adrenoreceptor affinity.

The packaging of a greater number of β-adrenoreceptors within intracellular vesicles becomes a distinct physiological advantage when conditions of hypoxia and elevated cortisol titre coincide. Cortisol was found to enhance the observed hypoxia-specific increase in surface β-adrenoreceptor density by over 60% (Fig. 11B). Therefore, cortisol appears to increase the number of β-adrenoreceptors available for mobilization to the surface in response to hypoxia. Furthermore, hypoxia-stimulated membrane turnover, concurrent with increased intracellular vesicle β-adrenoreceptor density, would result in erythrocytes with a significantly enhanced ability to respond to both noradrenaline and adrenaline. This was evident in the Na^+/H^+ antiporter activities (Fig. 6) and cyclic AMP contents (Fig. 9) of these erythrocytes after the addition of catecholamine.

Cortisol treatment also inhibited the hypoxia-specific reduction in surface β-adrenoreceptor affinity (increased K_D). In erythrocytes chronically exposed to elevated plasma cortisol levels, virtually no change in K_D was detected between normoxic and hypoxic erythrocytes, while in the shams, the K_D increased from 33.3 ± 1.49 to 49.2 ± 1.06 nmol l^{-1} in response to the reduction in P_{O_2} (Fig. 12). The hypoxia-mediated modification in surface β-adrenoreceptor K_D in sham erythrocytes was somewhat greater *in vitro* than *in vivo*. Further, although the acute exposure to elevated cortisol levels was unable to inhibit the hypoxia-specific change in K_D completely, a 46.5% inhibition did result. If the increase in K_D was caused by an increase in the number of uncoupled surface β-adrenoreceptors, resulting from hypoxia-stimulated membrane turnover, it is conceivable that the reduction in K_D can be attributed directly to a cortisol-mediated enhancement in surface β-adrenoreceptor/adenylate cyclase coupling. The efficiency of coupling between the β-adrenoreceptor and G_s (the G protein that mediates stimulation of adenylate cyclase) has been reported to be modulated by glucocorticoids (Davies and Lefkowitz, 1984). As a consequence, β-adrenoreceptor-stimulated adenylate cyclase activity and cyclic AMP accumulation increase following glucocorticoid treatment (Davies and Lefkowitz, 1984). This is in agreement with the observations from this study: trout erythrocyte Na^+/H^+ antiporter activity (Fig. 6) and erythrocyte cyclic AMP concentration (Fig. 9) were significantly increased after exposure to elevated cortisol levels, although only in concert with hypoxic conditions.

An interesting observation in terms of β-adrenoreceptor pool density, hypoxia and cortisol treatment was the observation that a hypoxia-specific significant increase in the internal β-adrenoreceptor pool occurred in the shams, whereas this

pool of β-adrenoreceptors was significantly reduced within cortisol-treated erythrocytes (Fig. 12). These findings suggest that the rate of hypoxia-mediated removal of β-adrenoreceptors from internal stores is enhanced in the presence of elevated cortisol levels. In the absence of cortisol, the mobilization of β-adrenoreceptors to the erythrocyte surface may occur at a rate slower than that of β-adrenoreceptor production and thereby explain the observed increase in the size of the internal β-adrenoreceptor pool of the hypoxia shams (Fig. 11). If cortisol greatly enhances the speed at which the hypoxia-mediated β-adrenoreceptor mobilization occurs, β-adrenoreceptor mobilization might proceed at a rate exceeding that of the cortisol-mediated internal β-adrenoreceptor production. Such a situation would lead to an apparent decrease in the number of internal β-adrenoreceptors and would help to explain our observed hypoxia-specific alterations in internal β-adrenoreceptor numbers.

In summary, this study has identified that *in vitro* hypoxia by itself initiates simultaneously both the movement of β-adrenoreceptors from an internal pool to the erythrocyte surface and the replenishment of the internal receptor pool. In addition, exposure of trout erythrocytes to elevated cortisol concentrations increases β-adrenoreceptor availability by increasing the size of the internal β-adrenoreceptor pool. This increase in internal β-adrenoreceptor numbers occurs in the absence of any stimulus for β-adrenoreceptor mobilization and does not alter the ability of the erythrocytes to respond to catecholamines. However, in the presence of a cue for β-adrenoreceptor mobilization, the cortisol-mediated enhancement of the internal β-adrenoreceptor pool greatly amplifies the pool of high-affinity, erythrocyte surface β-adrenoreceptors as well as the catecholamine responsiveness of these erythrocytes. Thus, we suggest that, under conditions of chronic stress, cortisol may pre-adapt the erythrocytes to receive additional physiological inputs that can ultimately enhance respiratory performance beyond that which would be possible in the absence of chronically elevated levels of cortisol.

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