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THE EFFECTS OF ACUTE HYPOXIA ON CHEMICALLY OR NEURONALLY INDUCED CATECHOLAMINE SECRETION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) *IN SITU* AND *IN VIVO*

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Summary

The potential direct and modulating effects of acute hypoxia on catecholamine secretion in rainbow trout (*Oncorhynchus mykiss*) were assessed *in situ*, using a perfused cardinal vein preparation, and *in vivo*, using chronically cannulated fish. Acute (10 min) perfusion with hypoxic (P_{O_2}≤10 mmHg) saline or homologous hypoxic blood did not have a statistically significant effect on basal (non-stimulated) catecholamine secretion. A field stimulation technique was used to excite the sympathetic nerves innervating the chromaffin cells electrically *in situ* under conditions of high-P_{O_2} (saline P_{O_2}=152 mmHg; 1 mmHg=0.133 kPa) or low-P_{O_2} (saline P_{O_2}≤10 mmHg) perfusion at constant P_{CO_2} (2.3 mmHg). The results demonstrated that neuronally evoked catecholamine secretion was significantly lowered by 50 % during perfusion with hypoxic saline. To assess whether the inhibitory effect of hypoxia during neuronal stimulation *in situ* resulted from modulation of nicotinic and/or muscarinic receptor-linked pathways, perfused posterior cardinal vein preparations were injected with selective nicotinic (10^{-7} or 10^{-6} mol kg^{-1} nicotine) or muscarinic (10^{-3} mol kg^{-1} methacholine) receptor agonists. For both doses of nicotine, catecholamine secretion was significantly lowered during hypoxia by 55 %. During muscarinic receptor stimulation, perfusion with hypoxic saline caused a 42 % reduction in the rate of catecholamine secretion. In contrast, catecholamine secretion elicited by depolarising levels of KCl (60 mmol l^{-1}) was unaffected by the oxygen status of the perfusate. *In vivo*, intra-arterial injections of nicotine (300–600 nmol kg^{-1}) into normoxic (water P_{O_2}=155 mmHg) or moderately hypoxic fish (water P_{O_2}=80 mmHg) caused a dose-dependent elevation of circulating catecholamine levels. However, despite the inhibitory influence of localised hypoxia on chromaffin cell responsiveness previously demonstrated *in situ*, the increase in plasma catecholamine levels after intra-arterial injection of nicotine was significantly enhanced in the hypoxic fish. The differences between the results from the *in vivo* and *in situ* experiments may reflect the contribution of higher control centres and modulating factors *in vivo* that are absent *in situ*.

Key words: adrenaline, noradrenaline, chromaffin cell, stress, hypoxia, nicotine, methacholine, catecholamine secretion, rainbow trout, *Oncorhynchus mykiss*.

Introduction

During acute severe stress, rainbow trout (*Oncorhynchus mykiss*) secrete the catecholamine hormones adrenaline and noradrenaline into the circulatory system (for reviews, see Randall and Perry, 1992; Gamperl et al., 1994; Wendelaar Bonga, 1997; Reid et al., 1998; Perry and Bernier, 1999). The catecholamines are derived from chromaffin cells lining the posterior cardinal vein within the head kidney (Nandi, 1961). Upon release into the circulation, catecholamines are thought to initiate a series of responses aimed at alleviating the disruptive effects of stressors on physiological and metabolic function (Perry and Wood, 1989; Thomas and Perry, 1992; Randall and Perry, 1992; Fabbri et al., 1998). In teleosts, the chromaffin tissue is innervated by preganglionic sympathetic nerve fibres (Nilsson, 1984). Stimulation of these nerves during acute stress followed by the subsequent secretion of cholinergic (Nilsson et al., 1976; Reid and Perry, 1995; Al-Kharrat et al., 1997; Gfell et al., 1997; Abele et al., 1998; Montpetit and Perry, 1999) and non-cholinergic (Fritsche et al., 1993; Reid et al., 1995, 1998) neurotransmitters is believed to be the predominant mechanism promoting catecholamine secretion in teleost fish.

Exposure to acute hypoxia is exploited routinely to investigate the afferent and efferent limbs of the acute adrenergic stress response in fish (e.g. Fievet et al., 1987; Boutilier et al., 1988). Although the pathways and mechanisms promoting catecholamine release during hypoxia are unknown, it is clear that severe reductions in blood P_{O_2} must occur prior to secretion. In trout, plasma catecholamine

levels remain stable during exposure to graded hypoxia until arterial P_{O_2} falls below a critical value that corresponds roughly to the P_{50} of haemoglobin (Perry and Reid, 1992; Perry and Bernier, 1999). At this time, plasma catecholamine levels rise abruptly. Although not measured in these previous studies, venous P_{O_2} and O_2 content surely are significantly reduced at the moment of catecholamine secretion. Thus, the chromaffin cells lining the posterior cardinal vein are likely to be bathed in hypoxic interstitial fluid during the secretory event. On the basis of studies performed using mammalian chromaffin cells (Cheung, 1989; Lee et al., 1990; Dry et al., 1991) or carotid body glomus (Type 1) cells (Donnelly and Doyle, 1994), such localised changes in oxygen status could potentially influence (inhibit or stimulate) the catecholamine secretion process. Only a single study, however, has assessed the direct effects of altered blood oxygen status on basal catecholamine secretion in fish (Atlantic cod *Gadus morhua*; Perry et al., 1991), while no previous study has examined the possibility that acute hypoxia modulates neuronally or chemically evoked secretion.

The goals of this study were, therefore, to evaluate the direct effects of alterations in oxygen status on catecholamine secretion and to determine whether the response of chromaffin cells to cholinergic stimulation is modified by hypoxia. The experiments were performed on rainbow trout *in situ* using a perfused posterior cardinal vein preparation (Fritsche et al., 1993) and *in vivo* using serial blood sampling techniques.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of both sexes were obtained from Linwood Acres Trout Farm. All fish were kept in large fibreglass tanks supplied with flowing, aerated and dechlorinated, city of Ottawa tap water (14 °C). Fish were maintained on a 12 h:12 h light:dark photoperiod and were fed *ad libitum* on alternate days using a commercial pelleted fish diet; they were not fed for 48 h prior to experimentation. Two groups of fish were used: for *in vivo* experiments and to obtain donor blood for *in situ* experiments, large fish (N=48) weighing between 400 and 700 g were used. Smaller fish were used for *in situ* perfusion experiments (250–400 g; N=96).

Surgical procedures

Fish were anaesthetised in a 1:12 000 (mass/volume) solution of benzocaine (ethyl-*p*-aminobenzoate) cooled to 10 °C. After cessation of breathing movements, the fish were transferred to an operating table and the gills were irrigated with the same anaesthetic solution throughout surgery. An indwelling polyethylene cannula (Clay-Adams PE 50) was inserted into the dorsal aorta according to the basic method of Soivio et al. (1975). After surgery, fish were placed into individual opaque acrylic boxes supplied with flowing, aerated water, where they were allowed to recover for 24 h before experimentation.

In situ *experiments*

Fish were killed by a sharp blow to the head and placed ventral side up on a bed of ice. An incision was made along the length of the animal, starting at the anus and ending just anterior to the pectoral girdle. The heart was exposed using blunt dissection, and a small incision was made in the bulbus arteriosus. A cannula (Clay-Adams PE 160 tubing; i.d. 1.14 mm; o.d. 1.57 mm) was inserted through the bulbus into the ventricle and secured using surgical silk at the junction between the two chambers. This served as the outflow for the perfusate, while a posterior cardinal vein cannula (Clay-Adams PE 160 tubing pre-filled with heparinised saline, 25 i.u. ml^{-1}) served as the inflow (Fritsche et al., 1993). The preparation was pre-perfused with control Cortland saline (Wolf, 1963), adjusted to pH 7.8, for 20 min using positive pressure differences (approximately 15 cm vertical difference between the surface of the saline and the inflow cannula) to maintain constant flow (1.5 ml min^{-1}) through the posterior cardinal vein. All preparations were perfused with either high-P_{O_2} saline (gassed with a mixture of humidified air and CO_2; P_{O_2}=152 mmHg; 1 mmHg=0.133 kPa), normoxic saline (gassed with a mixture of humidified N_2, air and CO_2; P_{O_2}=32 mmHg) to simulate normal venous blood gas O_2 tensions, or hypoxic saline (gassed with humidified N_2 and CO_2; P_{O_2}<10 mmHg) for 20 min prior to experimentation. In all cases, P_{CO_2} was kept constant at 2.3 mmHg.

Effects of hypoxia on basal rates of catecholamine release

Because of the possibility that catecholamine secretion might be triggered by a reduction in O_2 concentration rather than P_{O_2} *per se*, experiments were performed using blood or saline as perfusion fluids. Two types of experiments were performed. In one series, the posterior cardinal vein was perfused initially with perfusate (saline or blood) gassed with air to achieve a P_{O_2} of between 138 and 152 mmHg (high-P_{O_2} group). In a second series, the posterior cardinal vein was perfused initially with perfusate (saline or blood) gassed with a mixture of air and N_2 to achieve a P_{O_2} of 32 mmHg. In all

Table 1. *The blood oxygen status of the various perfusion fluids used to perfuse the* in situ *posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*)*

	Saline		Blood	
	P_{O_2} (mmHg)	$[O_2]$ (mmol l^{-1})*	P_{O_2} (mmHg)	$[O_2]$ (mmol l^{-1})
High-P_{O_2}	152	0.317	138	1.34
Normoxia	32	0.067	32	1.00
Hypoxia	8	0.004	8	0.19

These data were obtained by analysing (in triplicate) blood or saline that had been pooled to achieve sufficient volumes for 6 perfusion experiments.

*The $[O_2]$ of the saline was calculated using an O_2 solubility coefficient for human plasma at 8 °C (Boutilier et al., 1984).

1 mmHg=0.133 kPa.

saline-perfusion experiments, two samples of outflowing perfusate were taken at 1 min intervals after the 20 min stabilisation period as pre-treatment (pre) samples. In control experiments, preparations were then perfused for a further 10 min with unaltered saline, and samples were taken at 1, 2, 3, 4, 5 and 10 min. In hypoxia experiments, perfusion was switched abruptly from the initial saline to hypoxic saline for a further 10 min, and samples were taken as above. Samples were frozen in liquid N_2 after collection and stored at −80 °C until determination of catecholamine levels. All perfusate samples were collected into preweighed micro-centrifuge tubes and were reweighed prior to catecholamine analysis to permit the estimation of perfusion flow rates and thus allow the calculation of catecholamine secretion rates.

For blood-perfusion experiments, preparations were perfused with high-P_{O_2} or normoxic blood after the 20 min stabilisation period of saline perfusion had ended. After a further 10 min, two samples of outflowing blood were collected as pre-treatment (pre) samples. Preparations were then perfused for an additional 10 min with unaltered (control) or hypoxic blood, and samples were taken at 1, 2, 3, 4, 5 and 10 min. Blood samples were weighed and then centrifuged (12 000 ***g*** for 1 min) to obtain plasma. The plasma was frozen in liquid N_2 after collection and stored at −80 °C until subsequent determination of catecholamine levels. Samples were also removed from the inflowing blood to determine the inflowing catecholamine levels. These values were subtracted from the outflowing values when calculating catecholamine secretion rates in blood-perfused preparations. Plasma cortisol levels in the inflowing blood were not measured; a previous study (Reid et al., 1996) demonstrated that the chromaffin tissue of rainbow trout is unresponsive to acute elevations of cortisol levels. The P_{O_2} values and O_2 concentrations of the various perfusates are summarised in Table 1.

To obtain blood for the blood-perfused preparations, blood from donor fish was removed *via* the dorsal aortic cannula and pooled in a round-bottomed flask (on ice) containing ammonium heparin (50 i.u. ml^{-1} final concentration). Generally, 5 ml of blood was removed from each fish, although blood sampling was stopped prematurely in a few instances when fish became agitated. Typically, blood from two fish was combined to yield 10 ml, and this pool was then diluted with Cortland saline (Wolf, 1963) to yield a haematocrit of approximately 12 %. In this way, a sufficient volume of blood (25–30 ml) could be obtained from two fish for a single *in situ* perfusion experiment (see above).

Effects of hypoxia on neuronally mediated catecholamine release

Fish were electrically stimulated using a pair of electrodes (connected to a Grass SD-9 stimulator) sutured to the body wall on either side of the fish in the anterior region of the posterior cardinal vein (Montpetit and Perry, 1999). Separate groups were perfused with high-P_{O_2} or hypoxic saline. After collection of the two pre-samples (see above), each preparation was stimulated for 30 s at 60 V using a frequency of 20 pulses s^{-1} (20 Hz) and 1 ms duration. Collection of post-treatment samples began immediately at the onset of stimulation and continued for 5 min. A previous study demonstrated that this protocol caused specific neuronally mediated secretion of catecholamines (Montpetit and Perry, 1999).

Effects of hypoxia on agonist-evoked catecholamine release

After perfusion for 20 min with high-P_{O_2} or hypoxic saline, two pre-samples were collected. Nicotine (10^{-7} and 10^{-6} mol kg^{-1}) or methacholine (10^{-3} mol kg^{-1}) was then delivered (0.3 ml delivery volume) to the chromaffin tissue *via* a three-way valve attached to the inflow cannula. A period of 1 min was allowed for the agonists to reach the chromaffin tissue. Five outflowing perfusate samples were collected in pre-weighed micro-centrifuge tubes (1.5 ml) at 1 min intervals after agonist administration. Generally, maximal catecholamine secretion rates were achieved in the first 1 min sampling period. However, because of differences in perfusion flow rates and variation in the rate of agonist delivery and diffusion to the chromaffin cells, the period of maximal catecholamine secretion varied among the preparations. Thus, for statistical analyses and presentation of data, maximal catecholamines secretion rates were recorded from each preparation regardless of when it was achieved (first, second or third minute). Note that control experiments (saline injection after 20 min pre-perfusion) were not performed in this study. Previous experiments (e.g. Reid et al., 1996) have shown that saline injection is without effect on catecholamine secretion.

Effects of hypoxia on KCl-evoked catecholamine release

In a separate group of experiments, preparations were perfused with saline containing 60 mmol l^{-1} KCl. These experiments were designed to assess the effects of perfusate oxygen status on catecholamine secretion evoked by non-specific (receptor-independent) membrane depolarisation. After perfusion for 20 min, two pre-samples were collected, and the preparation was then perfused with high-P_{O_2} or hypoxic saline containing 60 mmol l^{-1} KCl. Samples were collected every minute for 5 min to determine maximal catecholamine secretion rates (see above).

In vivo *experiments*

On the basis of a previous study (Julio et al., 1998), doses of nicotine of 300 and 600 nmol kg^{-1} (volume 1 ml kg^{-1}) or a saline injection (control) were administered to separate groups of fish (normoxic and hypoxic). Three blood samples (volume 0.5 ml) were taken from each fish for subsequent analysis of plasma catecholamine levels; an initial sample, followed by samples at 2 and 5 min post-injection. With few exceptions, maximal levels of circulating catecholamines were achieved 2 min after injection. Hypoxic fish were exposed to 20 min of external hypoxia (P_{wO_2}=80 mmHg) before injection/sampling, whereas normoxic fish were exposed to air-saturated water (P_{wO_2}=155 mmHg). The desired level of hypoxia was achieved by regulating the flows of water and nitrogen through

a gas-equilibration column. Pilot experiments demonstrated that this level and duration of hypoxia would not cause plasma catecholamine levels to increase significantly.

Analytical procedures

For *in vivo* experiments, $P_{W_{O_2}}$ was monitored continuously by siphoning water from the outflow tube of the gas equilibration column through a thermostatted chamber (14 °C) containing a P_{O_2} electrode (Cameron Instruments Inc.) connected to a blood gas analyser (Radiometer PHM 71). The P_{O_2} of the saline or blood used in the *in situ* perfusion experiments was measured by pumping (using a peristaltic pump) saline/blood through a P_{O_2} electrode chamber until stable readings were achieved (approximately 3 min). Blood O_2 concentration was determined on 50 µl samples using a blood O_2 content meter (Oxycon, Cameron Instruments Inc.).

To determine the haematocrit of donor blood for perfusion experiments, duplicate samples of approximately 80 µl were centrifuged at 5000 ***g*** for 10 min.

Perfusate (saline or blood) or plasma catecholamine levels were determined on alumina-extracted samples using high-pressure liquid chromatography (HPLC) with electrochemical detection according to the basic method of Woodward (1982). The voltage applied to the glassy carbon electrode was +650 mV. 3,4-Dihydroxybenzalamine hydrobromide was used as an internal standard in all determinations.

Statistical analyses

For *in vivo* experiments, statistical differences within a group (i.e. normoxic or hypoxic) were determined by repeated-measures one-way analysis of variance (ANOVA). Differences between the normoxic and hypoxic groups were determined using unpaired Student's *t*-tests. For *in situ* experiments, data were analysed by repeated-measures one-way ANOVA followed by a *post-hoc* multiple-comparison test (Dunnett's) that compared all post-treatment values with the final pre-treatment value. Differences between normoxic and hypoxic preparations were determined using unpaired Student's *t*-tests. In all cases, 5 % was used as the confidence limit.

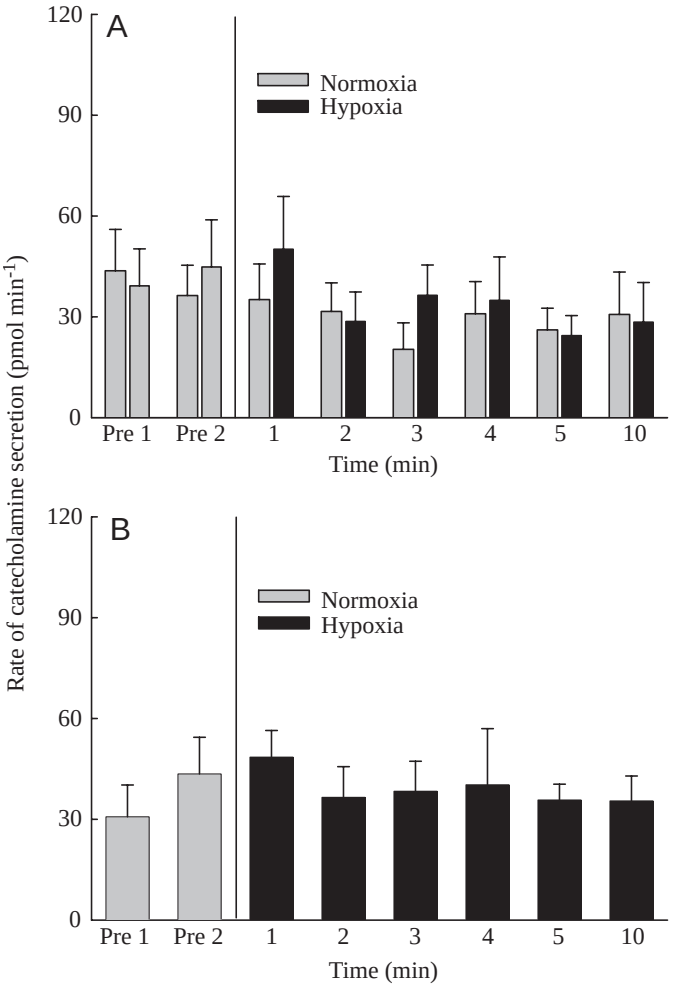

Fig. 1. The effects of an acute reduction in perfusate oxygenation levels on rates of catecholamine (adrenaline plus noradrenaline) secretion in a saline-perfused posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*). The vertical line indicates a switch from (A) high-P_{O_2} saline (152 mmHg; shaded columns; N=6) or (B) normoxic saline (32 mmHg; shaded columns; N=6) to hypoxic saline (<10 mmHg; black columns). In control experiments (A), preparations were perfused continuously with high-P_{O_2} saline (N=6). All values are shown as means +1 S.E.M. Pre 1, 2, pre-treatment samples.

Results

The effects of hypoxic perfusate on *in situ* basal catecholamine secretion rates are illustrated in Figs 1 and 2. An acute switch from either high-P_{O_2} saline (Fig. 1A) or normoxic saline (Fig. 1B) to hypoxic perfusion medium was without effect on rates of catecholamine secretion within the 10 min period examined. Similarly, an abrupt change to hypoxic blood did not alter the rates of catecholamine secretion regardless of whether the initial P_{O_2} of the blood was high (Fig. 2A) or normal (Fig. 2B).

A field stimulation technique (Montpetit and Perry, 1999) was used to induce neuronally mediated catecholamine secretion *in situ*. Catecholamine secretion was activated transiently following electrical stimulation (Fig. 3). In contrast to the results obtained *in vivo* (see below), neuronally evoked catecholamine secretion *in situ* was significantly lowered by 50 % during perfusion with hypoxic saline. This was the result of a reduction in the rates of secretion of both adrenaline and noradrenaline.

To determine whether the inhibitory effect of hypoxia on neuronally mediated catecholamine release *in situ* could potentially result from modulation of cholinergic receptor-linked signal-transduction pathways, preparations were injected with nicotinic or muscarinic receptor agonists. Although not demonstrated in this study, previous work (Montpetit and Perry, 1999) has shown that nicotine (10^{-7} mol kg^{-1}) and methacholine (10^{-3} mol kg^{-1}) act as selective nicotinic and muscarinic receptor agonists, respectively, in the perfused posterior cardinal vein preparation

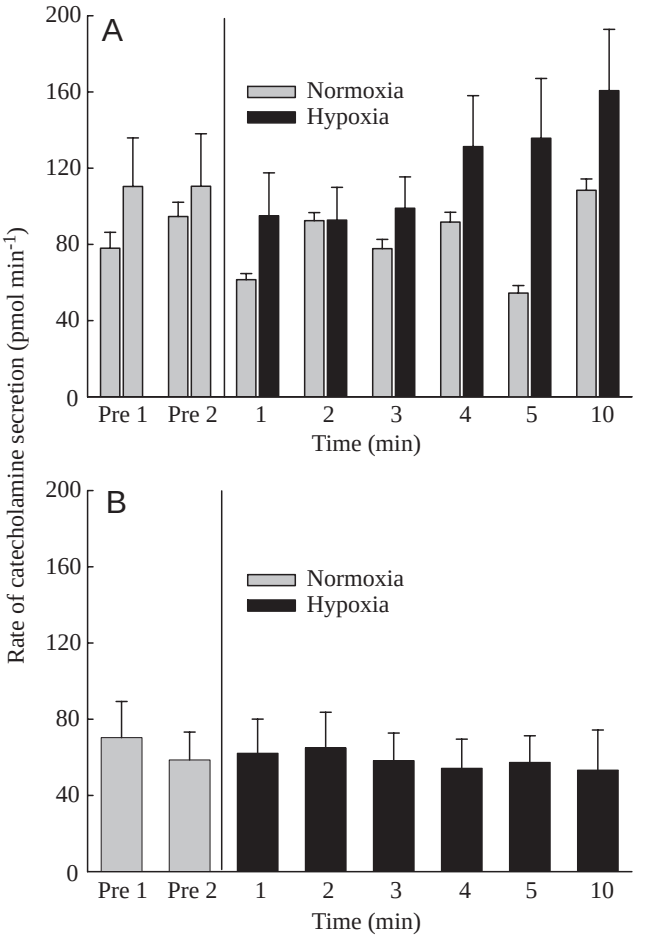

Fig. 2. The effects of an acute reduction in perfusate oxygenation levels on rates of catecholamine (adrenaline plus noradrenaline) secretion in a blood-perfused posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*). The vertical line indicates a switch from (A) high-P_{O_2} blood (138 mmHg; shaded columns; N=6) or (B) normoxic blood (32 mmHg; shaded columns; N=6) to hypoxic blood (8 mmHg; black columns). In control experiments (A), preparations were perfused continuously with high-P_{O_2} blood (N=6). All values are shown as means +1 S.E.M. Pre 1, 2, pre-treatment samples.

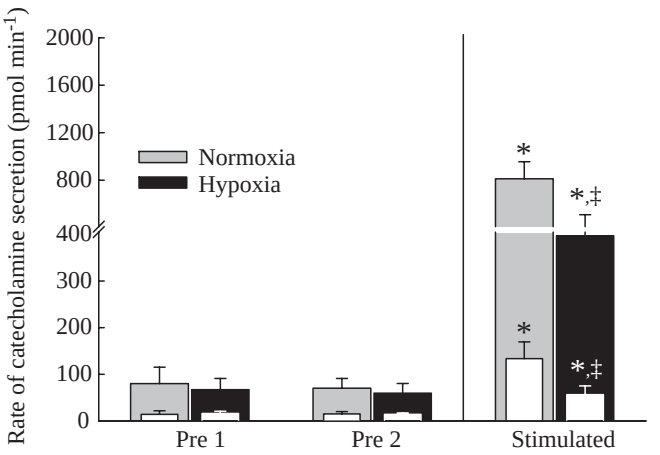

Fig. 3. The *in situ* effects of perfusate oxygen status on neuronally evoked (using electrical field stimulation) maximal rates of catecholamine (adrenaline plus noradrenaline) secretion in a perfused posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*). Preparations were stimulated while being perfused with high-P_{O_2} (shaded columns; N=6) or hypoxic (black columns; N=6) saline. The thinner white columns within each larger column represent noradrenaline secretion rates. An asterisk indicates a significant difference (P<0.05) from the corresponding final pre-stimulation (Pre 2) value; A double dagger indicates a significant difference (P<0.05) from the corresponding high-P_{O_2} value. All values are shown as means +1 S.E.M. Pre 1, pre-treatment sample.

of rainbow trout. Perfusion with hypoxic saline caused a pronounced reduction in nicotine-evoked (10^{-7} and 10^{-6} mol kg^{-1}) secretion of both adrenaline and noradrenaline (Fig. 4). For methacholine, however, only the rate of adrenaline secretion was significantly lowered during perfusion with hypoxic saline (Fig. 5).

Unlike the situation for specific cholinergic receptor agonists, the oxygen status of the perfusate did not influence rates of catecholamine secretion evoked by non-selective membrane depolarisation using 60 mmol l^{-1} KCl (Fig. 6).

The influence of acute moderate hypoxia (P_{wO_2}=80 mmHg) on nicotine-evoked catecholamine release *in vivo* is depicted in Fig. 7. Regardless of ambient oxygen status, nicotine injection caused a dose-dependent increase in plasma catecholamine levels. However, the increases in plasma catecholamine concentrations after nicotine injection were significantly larger in fish concurrently experiencing moderate hypoxia. Under normoxic conditions, catecholamine levels were increased after nicotine treatment by 30–75 nmol l^{-1}, whereas under hypoxic conditions, catecholamine levels were increased by 235–1244 nmol l^{-1}. Hypoxia itself did not cause a significant increase in plasma catecholamine levels (P>0.05; one-way ANOVA).

Discussion

Hypoxia is arguably the most widely employed environmental stressor in studies examining the acute adrenergic stress response in fish (Randall and Perry, 1992). Surprisingly, however, this is the first study to examine the potential modulating effects of acute external and/or internal hypoxia on the catecholamine secretion process. The results demonstrated that, *in vivo*, external hypoxia enhances the responsiveness of the chromaffin cells to nicotinic receptor stimulation. Because of an obvious inhibitory effect of internal hypoxia on neuronally evoked or cholinergic receptor agonist-evoked catecholamine secretion *in situ*, the stimulatory effects of hypoxia observed *in vivo* are probably unrelated to any local influence of hypoxia on the chromaffin cells. Instead, the results suggest that the presence of functional higher control centres *in vivo* and/or other modulating factors serve to override the inhibitory actions of local hypoxia on agonist-evoked catecholamine secretion.

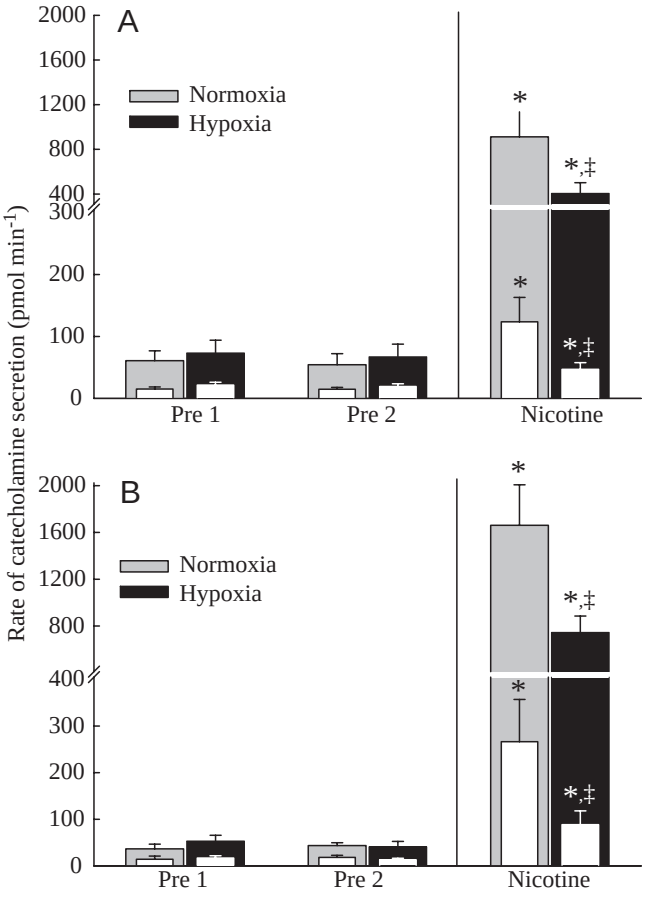

Fig. 4. The *in situ* effects of perfusate oxygen status on maximal rates of catecholamine (adrenaline plus noradrenaline) secretion in response to (A) 10^{-7} mol kg^{-1} nicotine (N=14) or (B) 10^{-6} mol kg^{-1} nicotine (N=16) in a perfused posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*). For each dose, nicotine was administered to 7/8 preparations under conditions of high P_{O_2} (shaded columns) or 7/8 different preparations under conditions of hypoxia (black columns). The thinner white columns within each larger column represent noradrenaline secretion rates. An asterisk indicates a significant difference (P<0.05) from the corresponding final pre-stimulation (Pre 2) value; a double dagger indicates a significant difference (P<0.05) from the corresponding high-P_{O_2} value. All values are shown as means +1 S.E.M. Pre 1, pre-treatment sample.

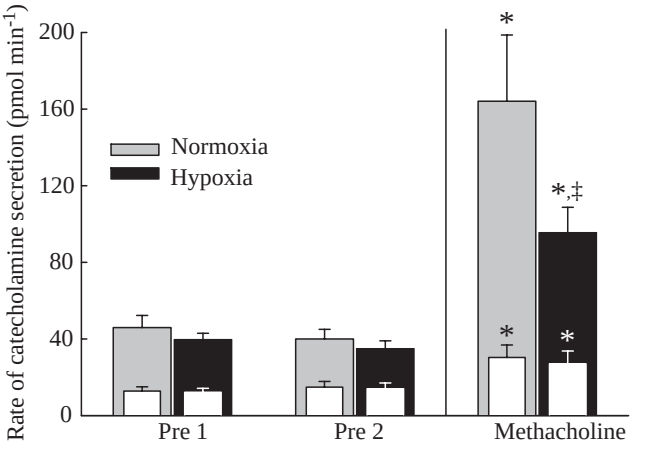

Fig. 5. The *in situ* effects of perfusate oxygen status on maximal catecholamine (adrenaline plus noradrenaline) secretion in response to 10^{-3} mol kg^{-1} methacholine in a perfused posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*). Methacholine was administered to preparations under conditions of high P_{O_2} (shaded columns; N=5) or hypoxia (black columns; N=6). The thinner white columns within each larger column represent noradrenaline secretion rates. An asterisk indicates a significant difference (P<0.05) from the corresponding final pre-stimulation (Pre 2) value; a double dagger indicates a significant difference (P<0.05) from the corresponding high-P_{O_2} value. All values are shown as means +1 S.E.M. Pre 1, pre-treatment sample.

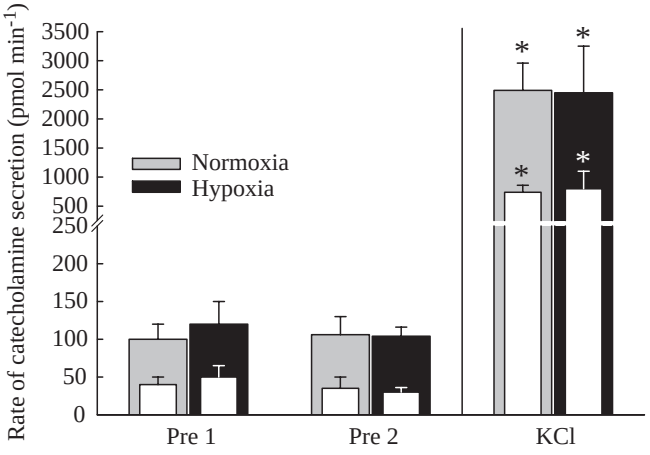

Fig. 6. The *in situ* effects of perfusate oxygen status on maximal rates of catecholamine (adrenaline plus noradrenaline) secretion in response to 60 mmol l^{-1} KCl in a perfused posterior cardinal vein preparation of rainbow trout (*Oncorhynchus mykiss*). KCl was administered to preparations under conditions of high P_{O_2} (shaded columns; N=6) or hypoxia (black columns; N=6). The thinner white columns within each larger column represent noradrenaline secretion rates. An asterisk indicates a significant difference (P<0.05) from the corresponding final pre-stimulation (Pre 2) value. All values are shown as means +1 S.E.M. Pre 1, pre-treatment sample.

The direct and modulating effects of hypoxia on catecholamine release in situ

The majority of the experiments performed in the present study utilised saline as the perfusion fluid, a medium with a low capacitance coefficient for O_2. Because changes in P_{O_2} and O_2 concentration are both potential modulators of catecholamine secretion, there are obvious limitations associated with using saline as a perfusate. Thus, equilibration of saline with gases that simulate normoxic venous blood O_2 tensions (approximately 30 mmHg) results in a saline with a very low O_2 concentration (Table 1). Conversely, equilibration of saline with a higher P_{O_2} yields an O_2 concentration that is more representative of venous values yet, at such high P_{O_2} values, the saline must be considered as hyperoxic. For these reasons, experiments designed to assess the direct effects of hypoxia on catecholamine secretion utilised both saline and

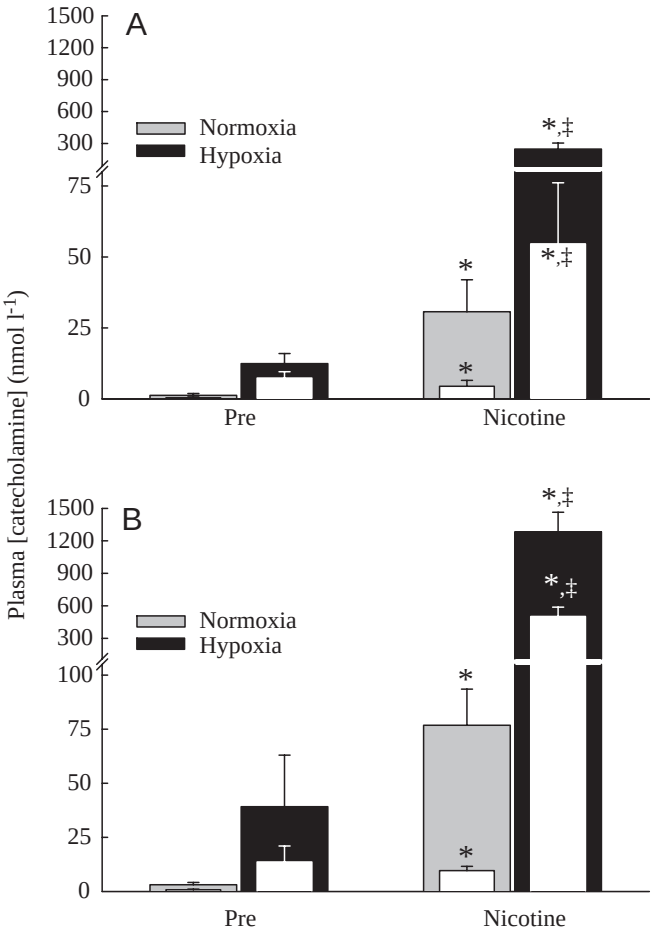

Fig. 7. The effects of intra-arterial injections of (A) 300 nmol kg^{-1} nicotine (N=12) or (B) 600 nmol kg^{-1} nicotine (N=12) on total plasma catecholamine (adrenaline plus noradrenaline) levels in rainbow trout (*Oncorhynchus mykiss*). For each dose, nicotine was administered to six fish under conditions of normoxia (shaded columns) or to six different fish under conditions of hypoxia (black columns). The thinner white columns within each larger column represent plasma noradrenaline levels. An asterisk indicates a significant difference (P<0.05) from the corresponding pre-injection (Pre) value; a double dagger indicates a significant difference (P<0.05) from the corresponding normoxic value. All values are shown as means +1 S.E.M.

blood as perfusion fluids. Moreover, these experiments were performed using abnormally high P_{O_2} (140–150 mmHg) or normal P_{O_2} (32 mmHg) during the pre-hypoxia period; similar results were obtained in both situations. Thus, to conserve animals, all subsequent experiments employed high-P_{O_2} saline (rather than normoxic blood) as the pre-hypoxic perfusate.

In contrast to the effect in Atlantic cod (*Gadus morhua*; Perry et al., 1991), perfusion of trout chromaffin cells with hypoxic saline or blood did not affect basal (spontaneous) rates of catecholamine secretion. Thus, localised hypoxaemia in the vicinity of the chromaffin tissue probably does not contribute to the release of catecholamines in this species during exposure to environmental hypoxia. The P_{O_2} of the venous blood perfusing the chromaffin tissue at the onset of catecholamine release during hypoxia is unknown. However, during severe hypoxia (water P_{O_2} ⩽35 mmHg), the P_{O_2} of venous blood may be lowered to 5–6 mmHg (Holeton and Randall, 1967; Thomas et al., 1994). In the present study, the P_{O_2} of the saline or blood was lowered to ⩽10 mmHg. Although the perfusate (blood or saline) was gassed with pure N_2, it was not possible to achieve P_{O_2} values less than 8 mmHg. Thus, it is conceivable that the degree of hypoxia imposed in the present *in situ* study was insufficient to evoke a secretory response. In comparison, previous studies have demonstrated significant secretory responses of mammalian chromaffin cells exposed to similar or even milder levels of hypoxia (15–40 mmHg; Dry et al., 1991; Thompson et al., 1997; Mochizuki-Oda et al., 1997; Rychkov et al., 1998). In mammals, the chromaffin cells contained within the adrenal medulla are perfused by arterial blood, and this may explain their responsiveness to relatively mild hypoxia. Regardless, the direct effects of hypoxia on catecholamine secretion in mammalian chromaffin cells are highly variable. In cells derived from foetal animals, there is an obvious and consistent stimulatory effect of hypoxia on catecholamine secretion (Thompson et al., 1997; Rychkov et al., 1998; Mojet et al., 1999). In cells derived from juveniles or adults, the direct response to hypoxia may be absent (Lee et al., 1990; Lee and Sekine, 1993; Thompson et al., 1997), attenuated (Mojet et al., 1999) or retained (Cheung, 1989; Mochizuki-Oda et al., 1997; Inoue et al., 1998). Given this variability, it is not surprising, therefore, that trout and cod chromaffin cells respond differently to hypoxia.

The direct stimulatory effect of hypoxia on mammalian foetal chromaffin cells is of obvious physiological significance because they lack sympathetic nerve fibre innervation. In the mammalian foetus, the non-neurogenic release of catecholamines from the adrenal medulla *via* regional hypoxaemia is an essential element of foetal development. While less important in the innervated adult adrenal medulla, a direct O_2-sensing mechanism in chromaffin cells may be advantageous in mammals because of the inhibitory effects of hypoxia on sympathetic neurotransmission. It is possible that the absence of a direct response of trout chromaffin cells to hypoxia reflects a high degree of tolerance of the sympathetic nervous system to reduced oxygen levels.

Although the chromaffin tissue was insensitive to any direct effects of lowered perfusate oxygen levels, localised hypoxia did cause a significant reduction in neuronally evoked and cholinergic agonist-evoked catecholamine secretion. Because the rate of catecholamine secretion elicited by non-specific membrane depolarisation (using KCl) was unaffected by hypoxia, it is likely that the inhibitory influence of reduced P_{O_2} on secretion was mediated at the level of the cholinergic receptors. In support of this idea, previous studies using cultured bovine chromaffin cells have demonstrated that hypoxia lowers the ligand-binding affinity of nicotinic receptors (Lee et al., 1995) and reduces the inward flux of Na^+ during nicotinic receptor stimulation (Lee et al., 1990). Ultimately, an attenuation of the intracellular Ca^{2+} spike and a resultant inhibition of Ca^{2+}-dependent exocytosis are thought to explain the reduced

secretion of catecholamines during cholinergic receptor stimulation under hypoxic conditions (Lee et al., 1990).

Catecholamine secretion evoked by cholinergic receptor stimulation is generally attributed predominantly to nicotinic receptor activation (Reid et al., 1998). However, in rainbow trout (Julio et al., 1998; Montpetit and Perry, 1999) and other teleosts (Gfell et al., 1997; Abele et al., 1998), muscarinic receptors have also been implicated in the secretory process. In the present study, hypoxia reduced the release of catecholamines elicited by both nicotinic and muscarinic receptor agonists. Thus, the trout chromaffin cell appears to differ from the bovine model that exhibits hypoxia-insensitive muscarinic receptors (Lee et al., 1995). Another difference is that, unlike in bovine chromaffin cells (Lee et al., 1990), hypoxia does not inhibit KCl-evoked release in trout. Thus, the apparent inhibitory effect of hypoxia on voltage-gated Ca^{2+} channels suggested for bovine chromaffin cells is absent from trout.

The significance of a negative modulating influence of localised hypoxia on neuronally evoked catecholamine secretion from trout chromaffin cells is unclear and without any obvious physiological benefit. Indeed, the implication of such negative modulation is that, during acute environmental hypoxia, trout may exhibit an impaired ability to exploit the adrenergic stress response in comparison with their response to stressors occurring under normoxic conditions.

The direct and modulating effects of hypoxia on catecholamine release in vivo

Exposure of fish to moderate hypoxia ($P_{W}O_2$=80 mmHg), in itself, did not cause significant release of catecholamines. This observation is consistent with the prevailing view that substantial catecholamine release does not occur until blood O_2 content is reduced by approximately 50 % (Thomas and Perry, 1992; Perry and Reid, 1992). In contrast to the results obtained *in situ*, moderate hypoxia markedly increased the levels of plasma catecholamines achieved in fish after injections of nicotine. Considering the large differences between the normoxic and hypoxic fish and the rapidity of blood withdrawal after injection, the most likely explanation is that nicotine-evoked catecholamine secretion from chromaffin cells was enhanced (rather than catecholamine degradation being reduced) by environmental hypoxia. Although venous P_{O_2} was not measured in the present study, it probably fell to below 15 mmHg during moderate hypoxia ($P_{W}O_2$=80 mmHg) on the basis of previous experiments (Holeton and Randall, 1967; Thomas et al., 1994). Thus, catecholamine secretion was enhanced during environmental hypoxia despite the likelihood of localised hypoxaemia reducing the responsiveness of the chromaffin cells to nicotinic stimulation (see above). The mechanisms underlying the positive modulating effect of hypoxia on nicotine-evoked catecholamine release *in vivo* have not been examined in this study. Although blood acidosis is known to stimulate nicotine-evoked secretion *in vivo* (Julio et al., 1998), it is unlikely to be responsible for the stimulatory effect of hypoxia because blood pH is not generally lowered during moderate hypoxia; indeed, trout typically experience respiratory alkalosis at such levels of $P_{W}O_2$. It would appear that activation of one or more neural or humoral factors during hypoxia sensitises the chromaffin cells to nicotinic receptor stimulation and counteracts the negative influence of localised hypoxia itself. The increased sensitivity to cholinergic stimulation in hypoxic trout is not restricted to acute exposures; Montpetit and Perry (1998) demonstrated an increased responsiveness of chromaffin cells *in situ* after 5 days of prior exposure to moderate hypoxia (60–80 mmHg). An obvious difference between the *in situ* and *in vivo* experiments is the potential for catecholaminotropic effects *in vivo*, as demonstrated for American eel *Anguilla rostrata* (Epple and Nibbio, 1985). Although there is no evidence for catecholaminotropic effects in rainbow trout under normoxic conditions (Perry and Vermette, 1987), it is possible that this type of positive feedback is able to modify catecholamine release under hypoxic conditions. In American eel, both α- and β-adrenergic receptors have been implicated in mediating catecholaminotropic effects (Abele et al., 1998). In trout erythrocytes, the numbers of cell surface β-adrenergic receptors is increased by acute hypoxia (Reid et al., 1993). If a similar mechanism is operative in trout chromaffin cells, catecholaminotropic effects *via* stimulation of β-receptors may become significant in hypoxic fish. The differential effects of hypoxia on nicotine-evoked catecholamine secretion *in vivo* and *in situ* warrants further investigation.

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