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The role of angiotensin II in regulating catecholamine secretion during hypoxia in rainbow trout *Oncorhynchus mykiss*

Katherine N. Lapner and Steve F. Perry*

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, Canada K1N 6N5

*Author for correspondence (e-mail: sfperry@science.uottawa.ca)

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Summary

Experiments were performed *in vivo* on chronically cannulated adult rainbow trout (*Oncorhynchus mykiss*) to assess the involvement of serotonergic or muscarinic receptor stimulation or activation of the renin–angiotensin system in eliciting catecholamine release during acute hypoxia during periods of nicotinic receptor desensitisation.

Despite nicotinic receptor desensitisation induced by intravenous infusion of nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}), plasma catecholamine levels were increased to levels (adrenaline plus noradrenaline 125–200 nmol l^{-1}) similar to those in control fish during severe hypoxia (40–45 mmHg; 5.3–6.0 kPa). Blockade of serotonergic receptors using methysergide or of muscarinic receptors using atropine did not affect the ability of fish to elevate circulating catecholamine levels during hypoxia. However, selective blockade of the renin–angiotensin system, using lisinopril to inhibit angiotensin-converting enzyme, prevented the elevation of both angiotensin II and circulating catecholamine levels in acutely hypoxic fish experiencing nicotinic receptor desensitisation. In fish possessing functional nicotinic receptors, angiotensin-converting enzyme blockade attenuated but did not prevent the elevation of plasma catecholamine levels during hypoxia. The results of this study indicate that the renin–angiotensin system is activated during hypoxia and plays a role in eliciting catecholamine release that is secondary to activation of nicotinic receptors. However, under conditions of nicotinic receptor desensitisation, activation of the renin–angiotensin system during hypoxia is a prerequisite for catecholamine release.

Key words: desensitisation, rainbow trout, *Oncorhynchus mykiss*, chromaffin cell, adrenaline, noradrenaline, hypoxia, catecholamine, nicotinic receptor, angiotensin II, serotonin, muscarinic receptor.

Introduction

In teleost fish, the catecholamine hormones (noradrenaline and adrenaline) are secreted from the chromaffin tissue into the bloodstream in response to acute stress (for reviews, see Randall and Perry (1992) and Reid et al. (1998). Catecholamines are stored in chromaffin cells that line the walls of the posterior cardinal vein (PCV) in the region of the head kidney (Nandi, 1961; Nakano and Tomlinson, 1967). Once released into the circulation, these hormones serve to maintain or enhance a variety of physiological processes including cardiovascular function (Perry and Gilmour, 1999) and blood oxygen transport (Nikinmaa, 1992). Stressors capable of causing catecholamine secretion include hypoxia (Ristori and Laurent, 1989), hypercapnia (Perry et al., 1987), intensive exercise (Primmett et al., 1986), air exposure (Walhqvist and Nilsson, 1980) and hypotension (Bernier et al., 1999b).

The control of catecholamine secretion in teleosts is achieved through several cholinergic and non-cholinergic mechanisms (Reid et al., 1998). However, it has generally been accepted that the primary mechanism initiating catecholamine release in trout involves increased neuronal stimulation by preganglionic sympathetic nerve fibres that innervate chromaffin cell cholinergic receptors (Nilsson et al., 1976; Montpetit and Perry, 1999; Reid et al., 1998). The subsequent release of the neurotransmitter acetylcholine predominantly stimulates nicotinic receptors to elicit a series of Ca^{2+}-dependent events leading to catecholamine secretion (Nilsson et al., 1976; Montpetit and Perry, 1999; Furimsky et al., 1996). Recently, however, it has been demonstrated that catecholamine secretion into the circulation still occurred in hypoxic rainbow trout that possessed non-functional (desensitised) nicotinic receptors (Lapner et al., 2000). Thus, other mechanisms are clearly contributing to catecholamine secretion at times when nicotinic receptors are non-functional.

With this background, the goal of the present study was to identify the mechanism(s) promoting catecholamine release in rainbow trout subjected to acute hypoxia under conditions of nicotinic receptor desensitisation. Three mechanisms were evaluated on the basis of previous studies demonstrating their potential involvement in catecholamine secretion in trout. First, the contribution of muscarinic receptors was assessed

because it has recently been shown that muscarinic cholinergic stimulation enhances nicotinic-evoked catecholamine secretion and may, under intense stimulation, cause direct secretion (Julio et al., 1998; Montpetit and Perry, 1999). Second, the involvement of serotonin, a secretagogue of catecholamine release in trout (Fritsche et al., 1993), was assessed because of the known localisation of serotonergic cells in the vicinity of chromaffin tissue (Fritsche et al., 1993; Reid et al., 1995) and the sensitivity of similar gill serotonergic cells to oxygen (Dunel-Erb et al., 1982). Finally, the role of angiotensin II (Ang II), the biologically active product of the renin–angiotensin system (RAS), was investigated. Ang II is known to cause catecholamine release in rainbow trout (Bernier and Perry, 1997). Further, it was recently demonstrated that hypoxia is a powerful stimulant of renin secretion and renin gene expression in rats (Ritthaler et al., 1997). If a similar mechanism were operative in rainbow trout, Ang II could play an important role in eliciting catecholamine secretion during acute hypoxia.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing between 200 and 500 g (mean mass 366.5±13.6 g; N=107) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held indoors in large fibreglass tanks supplied with dechlorinated City of Ottawa tapwater maintained at 13 °C. Fish were allowed to acclimate to the aquarium for at least 3 weeks before experimentation. They were maintained on a 12 h:12 h L:D photoperiod and fed daily to satiation with a commercial salmonid diet until 24 h before experimentation.

Surgical procedures

Rainbow trout were anaesthetised in a solution of ethyl-*p*-amino-benzoate (benzocaine; Sigma; final concentration 2.4×10^{-4} mol l^{-1}) and placed onto an operating table where the gills were continuously irrigated with aerated anaesthetic solution. An indwelling polyethylene cannula (Clay-Adams PE50 polyethylene tubing; internal diameter 0.580 mm, outer diameter 0.965 mm) was implanted into the dorsal aorta (Soivio et al., 1975) to permit injections and periodic blood sampling. A second cannula (Clay-Adams PE160 polyethylene tubing; internal diameter 1.14 mm, outer diameter 1.57 mm) was inserted through the snout into the oral cavity to permit monitoring of inspired water P_{O_2}. To allow nicotine or saline infusion, a third cannula (PE50) was inserted into the caudal vein at the level of the caudal peduncle in the anterograde direction using standard surgical procedures (Axelsson and Fritsche, 1994). The caudal incision was sutured using a running stitch, and the cannula was secured to the body wall with silk ligatures.

After surgery, trout were placed into individual opaque Perspex boxes supplied with aerated flowing water and allowed to recover for 24 h prior to experimentation.

Experimental protocol

To desensitise nicotinic receptors, fish were infused (0.2 ml min^{-1}) *via* the caudal vein with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) using a syringe infusion pump (Sage Instruments) for 60 min (Lapner et al., 2000); control fish were infused with Cortland saline (Wolf, 1963). During continuing infusion, the fish were subjected to 10 min of hypoxia (beginning when the inspired P_{O_2} had fallen to 40–45 mmHg; 5.3–6.0 kPa).

Acute hypoxia was achieved by replacing the air supplying a water/gas equilibration column with N_2. The desired water P_{O_2} (P_{wO_2}) (40–45 mmHg) was pre-set and established by adjusting the rate of water and/or N_2 flow through the column. This level of hypoxia was chosen on the basis of a previous study (Perry and Reid, 1992) that demonstrated significant catecholamine release in rainbow trout using this protocol. The P_{wO_2} within the experimental box was monitored continuously using a peristaltic pump (flow rate 0.6 ml min^{-1}) that withdrew water from each individual trout box and passed it across a P_{O_2} electrode (Cameron Instruments) connected to an O_2 meter (Cameron Instruments). Generally, the desired P_{wO_2} in the experimental box was reached within 10 min and thereafter never varied more than ±5 mmHg (0.67 kPa). After experimentation, P_{wO_2} was restored to normoxic levels and the fish were allowed to recover.

The involvement of muscarinic receptors, serotonin or the RAS in promoting catecholamine release

After 40 min of infusion (nicotine or saline) under normoxic conditions, fish were injected *via* the dorsal aorta with the muscarinic receptor antagonist atropine (1 μmol kg^{-1}). Efficacy of blockade was confirmed in preliminary experiments by comparing the cardiac frequency responses of control and atropinised fish to the muscarinic receptor agonist methacholine. The infusion continued for a further 20 min prior to the commencement of hypoxia (see above). Using the same protocol, a separate group of fish was injected with the serotonin receptor antagonist methysergide (10^{-8} mol kg^{-1}) prior to hypoxia. Efficacy of blockade was not evaluated in the present study, although a previous study (Fritsche et al., 1992) demonstrated that the same dose of methysergide was effective at blocking serotonergic receptors in rainbow trout. Finally, a third group was treated (as above) with the Ang-II-converting enzyme (ACE) inhibitor lisinopril (10^{-4} mol kg^{-1}) to prevent the formation of Ang II (Bernier et al., 1999b). The effectiveness of ACE inhibition was established in preliminary experiments in which blood pressure changes were measured after injection of Ang I. Control fish were injected with saline after 40 min of infusion.

Determination of plasma catecholamine and Ang II levels

Blood samples collected for catecholamine analysis were taken immediately prior to beginning the infusion and after 10 and 60 min of infusion. Prior to hypoxia, samples withdrawn for Ang II analysis were only taken at 60 min of infusion. For measurement of plasma catecholamine and Ang II levels, four

blood samples were withdrawn during the hypoxic period: at 0, 3, 5 and 10 min after reaching the targeted 40–45 mmHg P_{WO_2}. Because of fluctuating P_{WO_2} levels during the 10 min hypoxic period (±5 mmHg; 0.67 kPa), the blood sample that was withdrawn at a P_{WO_2} level nearest to the target of 40 mmHg was used to represent levels during hypoxia. The collected blood samples were placed into micro-centrifuge tubes (1.5 ml) and centrifuged (12 000 ***g*** for 20 s). The plasma was transferred to micro-centrifuge tubes containing 10 µl (25 units) of heparin (ammonium salt). Samples were quick-frozen in liquid N_2 and then stored at –80 °C until subsequent analysis.

Extraction of plasma catecholamine samples

All plasma catecholamine samples were subjected to alumina extraction and then analysed by high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). 3,4-Dihydroxybenzylamine hydrobromide was used as an internal standard in these analyses. Detection limits for adrenaline and noradrenaline were 0.1 nmol l^{-1}.

Determination of angiotensin II levels by radioimmunoassay

Plasma samples were extracted according to the method of Phillips et al. (1991), with incorporated modifications by Bernier et al. (1999a) and by us. Just-thawed plasma (0.1 ml) was mixed with acidic acetone (volume ratio of acetone:H_2O:1 mol l^{-1} HCl=40:5:1) and vortexed vigorously for 10 s. The mixture was centrifuged for 10 min at 10 000 ***g*** and 4 °C. The supernatant was collected in a new tube, and the pellet was re-solubilised and re-extracted with 0.1 ml of acidic acetone. Once combined, the supernatants were centrifuged for 10 min as above. The supernatant was collected into a new tube and lyophilized. For the radioimmunoassay (RIA), the extracted pellet was re-suspended in 0.025 ml of RIA buffer (diluent). The recovery rate of Ang II through this extraction procedure, as measured with ^{125}I-labelled [Asn^1,Val^5]-Ang II, was 89.8 %.

A single-antibody method was used that employed an Ang II antibody designed for the detection of mammalian Ang II ([Asp^1,Ile^5]-Ang II). Recently, however, it has been shown that a similar mammalian antibody cross-reacts (approximately 70 %) with rainbow trout Ang II ([Asn^1,Val^5]-Ang II) and does not cross-react with piscine or mammalian Ang I (Bernier et al., 1999a).

96-well microtitre plates (Wallac; Rigid Plates) were prepared by coating each well with 100 µl of Protein A/G (1 µg ml^{-1} prepared in 0.1 mol l^{-1} $NaHCO_3^-$; pH 9.0). Plates were wrapped in Parafilm and stored at 4 °C overnight. The following day, the plates were washed twice with wash buffer [0.1 % Tween-20 in diluent (0.5 % bovine serum albumin, BSA, in 0.08 mol l^{-1} barbitol buffer)] for 2 min each at room temperature (20 °C). The plates were then washed once with diluent for 20 min at room temperature and were blotted dry on paper towel for 1 min. Antibody (1:200 000 dilution of rabbit anti-Ang-II serum; Amersham Pharmacia Biotech) or normal rabbit serum (to determine non-specific binding) was added to the appropriate wells. The plates were wrapped in Parafilm and incubated at 4 °C overnight. The following day, the plates were washed with wash buffer three times for 2 min each at room temperature. The plates were blotted dry on paper towel for 1 min before adding 50 µl of RIA buffer (diluent) to each well. Radiolabelled hormone {25 µl of ^{125}I-labelled Ang II ([Asp^1,Ile^5]-Ang II; Amersham Pharmacia Biotech; reconstituted to 100 µCi ml^{-1} with water, and diluted to a final concentration of 400 disints min^{-1} l^{-1} with diluent)} and 25 µl of standard (unlabelled [Asn^1,Val^5]-Ang II) or sample was added to the appropriate wells. Where required, diluent was added to cells such that each well contained a total volume of 100 µl. The plates were wrapped in Parafilm and incubated at 4 °C for 48 h with intermittent gentle shaking after 24 h. After the 48 h incubation period, the plates were washed three times with wash buffer for 2 min each at room temperature. The plates were blotted dry on paper towel before adding 100 µl of scintillation cocktail to each well. Labelled hormone (25 µl) was added to the appropriate wells for determination of 'total' radioactivity. The plates were incubated at room temperature for 2 h before counting in a MicroBeta Liquid Scintillation and Luminescence Counter, using a MicroBeta Windows Workstation. Data were analysed in MS-DOS using a WIA Level 5.M MultiCalc Advanced program.

Statistical analyses

The data are presented as means ±1 standard error of the mean (S.E.M.). Where appropriate, data were analysed statistically using one-way analysis of variance (ANOVA) followed by Dunn's multiple-comparison test. If assumptions for parametric statistics were violated, an ANOVA on ranks was performed followed by Dunn's multiple-comparison test. In other instances, data were analysed using Student's *t*-tests and, if assumptions for parametric statistics were violated, a Mann–Whitney rank sum test was performed. All statistical analyses were performed using commercial software (SigmaStat Version 2.0; SPSS). The fiducial limits of significance were set at 5 %.

Results

Continued chromaffin cell responsiveness during nicotinic receptor desensitisation

To confirm the ability of fish with desensitised nicotinic receptors to release catecholamines into the circulation upon acute hypoxia, saline or nicotine (to desensitise the nicotinic receptors) (Lapner et al., 2000) was infused continuously for 60 min prior to the onset of hypoxia and continued throughout the hypoxic period. As expected, the nicotine infusion elicited a significant increase in plasma catecholamine levels at 10 min that was not observed in saline-infused fish (Fig. 1). Owing to receptor desensitisation, the elevated plasma catecholamine levels returned to baseline levels within 60 min, despite continuous nicotine stimulation (Fig. 1B).

Upon acute hypoxia, a significant increase in plasma adrenaline and noradrenaline levels was observed in fish with

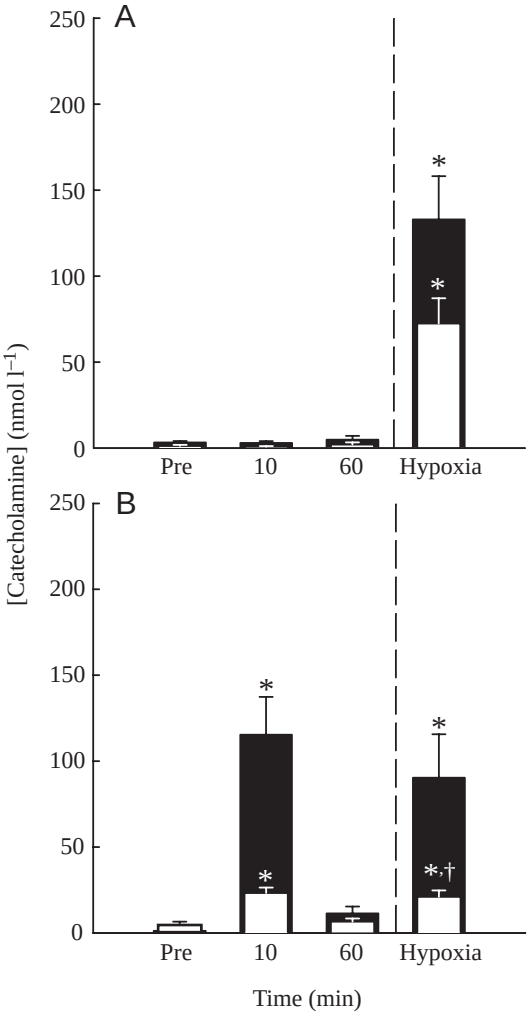

Fig. 1. The effects of acute hypoxia (40 mmHg=5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) and noradrenaline (open columns) in rainbow trout (*Oncorhynchus mykiss*) previously infused (0.2 ml min^{-1}) with (A) saline (N=9) or (B) nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}; N=17) for 60 min under conditions of normoxia. Plasma catecholamine levels were measured at 'Pre', 10 min and 60 min of infusion and during acute hypoxia. Values are shown as means + S.E.M. An asterisk denotes a statistically significant increase (P<0.05) from the corresponding 'Pre' value or '60 min' pre-hypoxia value. A dagger indicates a statistically significant difference (P<0.05) from the control (saline-infused) value.

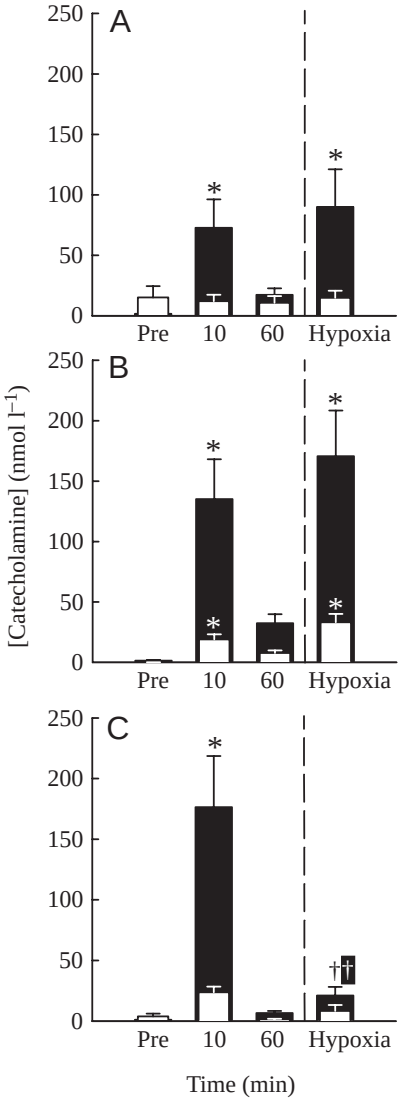

Fig. 2. The effects of acute hypoxia (40 mmHg=5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) and noradrenaline (open columns) in rainbow trout (*Oncorhynchus mykiss*) previously infused (0.2 ml min^{-1}) with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) for 60 min under conditions of normoxia. An injection of (A) atropine (1 μmol kg^{-1}; N=9), (B) methysergide (10^{-8} mol kg^{-1}; N=9) or (C) lisinopril (10^{-4} mol kg^{-1}; N=20) was administered after 40 min of nicotine infusion. Plasma catecholamine levels were measured at 'Pre', 10 min and 60 min of infusion and during acute hypoxia. Values are shown as means + S.E.M. An asterisk denotes a statistically significant increase (P<0.05) from the corresponding 'Pre' value or '60 min' pre-hypoxia value. A dagger indicates a statistically significant difference (P<0.05) from the control value (saline injection; Fig. 1B).

both functional (Fig. 1A) and non-functional/desensitised (Fig. 1B) nicotinic receptors. However, noradrenaline levels were reduced in comparison with hypoxic control fish (Fig. 1). Subsequent experiments were designed to elucidate the mechanisms causing the elevation in plasma catecholamine levels during hypoxia in fish with desensitised nicotinic receptors.

Mechanisms eliciting catecholamine release during nicotinic receptor desensitisation

All fish displayed the characteristic transient increase in plasma catecholamine levels at 10 min of nicotine infusion that is indicative of nicotinic receptor desensitisation (Fig. 2). Blockade of muscarinic (Fig. 2A) or serotonergic (Fig. 2B) receptors did not diminish plasma catecholamine levels during acute hypoxia (compare with Fig. 1A). However, blockade of the RAS abolished the ability of desensitised fish to mobilize circulating catecholamines during hypoxia (Fig. 2C). Fig. 3

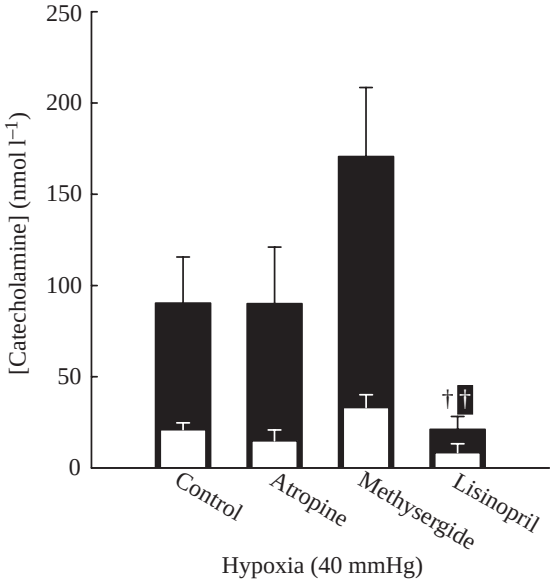

Fig. 3. Plasma levels of adrenaline (filled columns) and noradrenaline (open columns) during acute hypoxia (40 mmHg=5.3 kPa) in rainbow trout (*Oncorhynchus mykiss*) previously infused (0.2 ml min^{-1}) with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) for 60 min under conditions of normoxia. To block specific receptors or enzymes, injections were administered at 40 min (20 min prior to hypoxia) using saline (control; *N*=17), atropine (muscarinic receptor antagonist; 1 μmol kg^{-1}; *N*=9), methysergide (serotonin receptor antagonist; 10^{-8} mol kg^{-1}; *N*=9) or lisinopril (angiotensin-converting enzyme inhibitor; 10^{-4} mol kg^{-1}; *N*=20). Values are shown as means + S.E.M. A dagger indicates a statistically significant difference ($P<0.05$) from the control value.

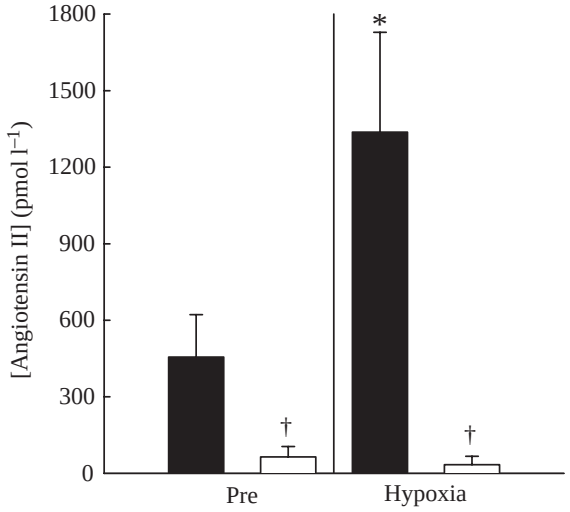

Fig. 4. The effects of acute hypoxia (40 mmHg=5.3 kPa) on plasma angiotensin II levels in rainbow trout (*Oncorhynchus mykiss*) previously infused (0.2 ml min^{-1}) with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) or saline (data were combined as there were no significant differences in [angiotensin II] between the two groups) for 60 min under conditions of normoxia. An injection of saline (filled columns; *N*=15) or lisinopril (10^{-4} mol kg^{-1}; open columns; *N*=15) was made prior to hypoxia at 40 min infusion. 'Pre' samples were taken immediately prior to the onset of hypoxia. Values are shown as means + S.E.M. An asterisk denotes a statistically significant increase ($P<0.05$) from the corresponding 'Pre' value. A dagger indicates a statistically significant difference ($P<0.05$) from the control value.

effectively illustrates the importance of the RAS in promoting catecholamine secretion during hypoxia in desensitised fish.

To reinforce the conclusion that catecholamine release during acute hypoxia in desensitised fish was indeed a consequence of activation of the RAS, plasma Ang II levels were measured before and after hypoxia (Fig. 4). Fish were infused with nicotine or saline for 60 min prior to hypoxia; however, data from these two experiments were compiled because there were no statistical differences between the two groups. A saline (control) or lisinopril injection was made after 40 min of infusion. The results demonstrated a significant increase in plasma Ang II levels (from 456±166 to 1338±391 pmol l^{-1}, *N*=15) during hypoxia in control fish possessing a functional RAS (Fig. 4). In contrast, fish experiencing RAS blockade displayed significantly lower Ang II levels during normoxia and did not exhibit an increase in plasma Ang II levels during acute hypoxia.

The involvement of the RAS in eliciting catecholamine release from non-desensitised chromaffin cells

To test further the involvement of the RAS in eliciting catecholamine release from chromaffin tissue during hypoxia, angiotensin-converting enzyme (ACE) activity was inhibited in fish possessing functional nicotinic receptors. Although plasma catecholamine levels were increased significantly upon acute hypoxia in both groups (Fig. 5), the ACE-blocked fish displayed significantly lower plasma noradrenaline levels during hypoxia than the control fish. Further, total plasma catecholamine levels (adrenaline plus noradrenaline) were significantly reduced during hypoxia in the ACE-blocked fish (93.6±33.7 *versus* 205.5±35.5 nmol l^{-1}; $P<0.05$).

Discussion

This study demonstrates that the RAS in rainbow trout is activated during hypoxia and leads to a significant elevation of plasma Ang II levels. Although Ang II is a potent activator of catecholamine release in trout (Bernier and Perry, 1997), an increase in Ang II concentration is not required to elicit catecholamine release during acute hypoxia in intact fish. However, using a protocol that we previously developed to desensitise chromaffin cell nicotinic receptors (Lapner et al., 2000), the results of the present study demonstrate that Ang II plays an essential role in eliciting catecholamine release in hypoxic fish experiencing nicotinic receptor desensitisation. Activation of muscarinic or serotonergic receptors, in contrast, does not appear to contribute to catecholamine release in desensitised fish.

Catecholamine release in desensitised fish

Upon stimulation, the nicotinic receptor of trout chromaffin cells, as in mammals (Boksa and Livett, 1984), undergoes rapid desensitisation (Lapner et al., 2000). The re-sensitisation process

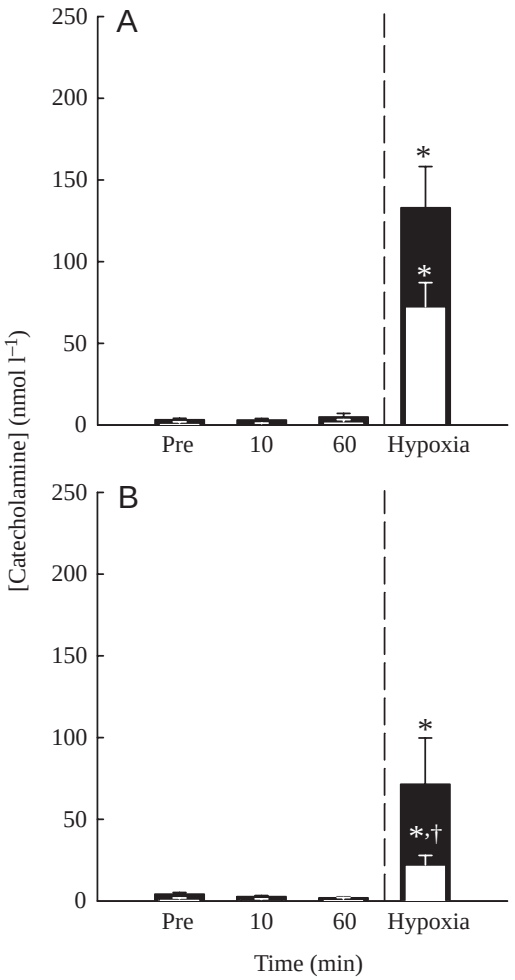

Fig. 5. The effects of acute hypoxia (40 mmHg=5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) and noradrenaline (open columns) in rainbow trout (*Oncorhynchus mykiss*) previously infused (0.2 ml min^{-1}) with saline for 60 min under conditions of normoxia. An injection of (A) saline (control; N=9) or (B) lisinopril (10^{-4} mol kg^{-1}; N=10) was made prior to hypoxia at 40 min saline infusion. Plasma catecholamine levels were measured at 'Pre', 10 min and 60 min of infusion and during acute hypoxia. Values are shown as means + S.E.M. An asterisk denotes a statistically significant increase (P<0.05) from the corresponding '60 min' pre-hypoxia value. A dagger indicates a statistically significant difference (P<0.05) from the control value.

is gradual (e.g. as long as 40 min in rainbow trout) (Lapner et al., 2000). Thus, although it is believed to be a mechanism preventing excessive catecholamine secretion, nicotinic receptor desensitisation, if prolonged, could potentially impair catecholamine release in animals experiencing repeated bouts of acute stress. The single previous study to address this issue, however, demonstrated that the ability of trout to release catecholamines during acute hypoxia was not impaired during periods of nicotinic receptor desensitisation (Lapner et al., 2000). This finding indicated that other pathways that are not reliant on nicotinic receptors were activated to elicit catecholamine secretion. The goal of this study was to attempt to identify one or more of these pathways. It was necessary, however, first to confirm that catecholamine release during hypoxia was indeed unimpaired in trout experiencing nicotinic receptor desensitisation. Thus, using a protocol of intravascular nicotine infusion to desensitise nicotinic receptors (Lapner et al., 2000), initial experiments revealed similar elevations of plasma catecholamine levels during hypoxia irrespective of the state of the nicotinic receptor. Because local hypoxia in the vicinity of chromaffin cells does not evoke catecholamine secretion in rainbow trout (Perry et al., 2000), subsequent experiments were designed to establish alternative mechanisms of catecholamine release in desensitised fish.

Potential involvement of serotonin

Although serotonergic receptors are present on rainbow trout chromaffin cells and the ability of serotonin to elicit catecholamine secretion has previously been demonstrated *in vivo* and *in situ* (Fritsche et al., 1993), serotonin does not appear to be a 'secondary' catecholamine secretagogue during hypoxia when nicotinic receptors are desensitised. Previous work (Fritsche et al., 1993) showed that injection of the serotonergic receptor antagonist methysergide blocked serotonin-induced adrenaline release *in situ* but not *in vivo*. It was suggested that this discrepancy could be attributed to serotonin acting on methysergide-insensitive receptors within higher control centres *in vivo*. Furthermore, Perry et al. (2000) showed that localised hypoxia inhibited chromaffin cell responsiveness to nicotine *in situ* but had an opposite, enhancing effect *in vivo*, further supporting the idea that higher control centres are involved in catecholamine release *in vivo*. Therefore, although our results cannot rule out the possibility that serotonin is acting indirectly on higher control centres to elicit catecholamine secretion during nicotinic receptor desensitisation, we can exclude any direct effect of serotonin on chromaffin cells.

The possible involvement of muscarinic receptors

Previous *in situ* studies have revealed that activation of chromaffin cell muscarinic receptors can enhance nicotine-evoked catecholamine secretion, and it has been suggested that, under intense stimulation, muscarinic receptors might directly cause catecholamine secretion (Montpetit and Perry, 1999). The results of the present study, however, demonstrate that muscarinic receptors do not contribute to catecholamine release during hypoxia in nicotinic receptor-desensitised fish. In contrast, stimulation of chromaffin cell muscarinic receptors in mammals can evoke significant catecholamine secretion during periods of nicotinic receptor desensitisation (Malhotra et al., 1989). In trout, it seems that chromaffin cell muscarinic receptors serve only to enhance the nicotinic-evoked catecholamine secretion (Montpetit and Perry, 1999) but are incapable of independently eliciting catecholamine release during stress (at least acute hypoxia).

Potential involvement of the renin–angiotensin system

Although chromaffin cell serotonergic and muscarinic receptors do not appear to be involved in eliciting

catecholamine secretion during hypoxia in trout experiencing nicotinic receptor desensitisation, the results of the present study indicate an essential role of the RAS. Under normal conditions, the RAS is an important regulator of cardiovascular function in fish (Olson, 1992). Recently, a link between the RAS and circulating catecholamine levels has been established in cardiovascular control whereby Ang II, mobilised during hypotension, dose-dependently stimulates catecholamine release (Bernier and Perry, 1997; Bernier et al., 1999b). Thus, typically, Ang II and catecholamines function together as vasopressive agents to regulate blood pressure during periods of hypotension (Nishimura, 1985; Oudit and Butler, 1995; Fuentes and Eddy, 1998; Bernier and Perry, 1999). The present paper provides the first evidence of RAS recruitment during acute hypoxia, a condition that is not associated with hypotension. Further, the results demonstrate that activation of the RAS is a prerequisite for catecholamine secretion in hypoxic trout possessing desensitised nicotinic receptors.

Circulating Ang II is derived *via* activation of systemic and/or regional renin–angiotensin systems (Bernier and Perry, 1997). In mammals, key components indicative of a local RAS have been demonstrated in multiple tissues of rodents (Campbell and Habener, 1986; Dzau et al., 1987; Leung et al., 1999). Interestingly, secretory granules in adrenaline-containing chromaffin cells of rat adrenal medulla contain both renin and pro-renin (Berka et al., 1996). Furthermore, chronic or acute hypoxia has recently been shown to result in the enhanced expression of local RAS component genes in rat pancreas (Chan et al., 2000) and primary cultures of renal juxtaglomerular cells (Ritthaler et al., 1997), to increase the numbers of lung Ang II receptors (Zhao et al., 1996) and to increase cardiac ACE activity (Morrell et al., 1997). In rat renal juxtaglomerular cells, renin secretion and renin gene expression were indirectly stimulated by acute hypoxia; their expression was enhanced *in vivo* but not *in situ*. The origin of the increased circulating Ang II levels was not investigated in the present study. Further experiments are required to determine whether, as in mammals, local or systemic RAS components exhibit increased expression during hypoxia.

Angiotensin II levels were elevated equally during hypoxia in control fish and in fish experiencing nicotinic receptor desensitisation. However, unlike in the desensitised fish, blockade of the RAS did not prevent catecholamine release in control fish exposed to hypoxia. This result reinforces the idea that stimulation of nicotinic receptors *via* activation of sympathetic nerve fibres is the dominant pathway controlling catecholamine secretion in trout under normal conditions. Angiotensin II, however, may be contributing to the overall response in control fish on the basis of the significant reduction in total plasma catecholamine levels after RAS blockade. Previous studies have demonstrated that Ang II preferentially stimulates adrenaline secretion from fish chromaffin cells (Bernier and Perry, 1997). Interestingly, however, RAS blockade prevented the release of both catecholamines in desensitised fish and had a greater effect on noradrenaline secretion in control fish.

Most teleosts that have been studied, including trout, exhibit pronounced cardiovascular adjustments when exposed to hypoxia including bradycardia and hypertension (for a review, see Fritsche and Nilsson, 1993). Traditionally, the hypertension has been attributed to increased systemic vascular resistance owing to increased activity of sympathetic nerve fibres as well as elevated circulating catecholamine levels. In the light of the results of the present study showing elevated Ang II levels in hypoxic trout, it seems that additional mechanisms may be contributing to the hypoxic hypertension, including direct vasoconstrictory effects of Ang II and indirect effects of Ang II in evoking catecholamine secretion.

As in mammals, stimulation of the preganglionic nerve fibres that innervate the chromaffin cells leads to the release of both cholinergic (i.e. acetylcholine) and non-cholinergic neurotransmitters (Montpetit and Perry, 2000). In particular, vasoactive intestinal polypeptide (VIP) and pituitary adenylyl-cyclase-activating peptide (PACAP) are potent catecholamine secretagogues in rainbow trout (Montpetit and Perry, 2000). Because blockade of the RAS abolished catecholamine secretion during hypoxia in fish with desensitised nicotinic receptors, it would appear that these non-cholinergic neurotransmitters were not secreted in sufficient quantities to evoke catecholamine release. The relative rates of secretion of cholinergic *versus* non-cholinergic neurotransmitters are partially dependent on the frequency of neuronal action potentials. Specifically, in trout, the secretion of non-cholinergic neurotransmitters is favoured under conditions of low-frequency nerve activity (Montpetit and Perry, 2000), a situation that might not exist during acute hypoxic stress. Thus, while catecholamine release in trout is thought to be controlled by multiple redundant pathways (see Reid et al., 1998), the specific involvement of each of these pathways may depend on the precise nature of the stressor. For example, while it is clear that serotonin, VIP, PACAP and muscarinic receptor stimulation are capable of independently eliciting catecholamine secretion (Reid et al., 1998), they appear to play no role during hypoxia. Thus, during hypoxic stress, they cannot complement or replace the nicotinic-receptor-mediated pathway of catecholamine secretion when nicotinic receptors are desensitised. Their possible role during other types of stress, however, cannot be ruled out. Ang II, usually considered to be a catecholamine secretagogue during periods of hypotension (Bernier et al., 1999a,b), also contributes to catecholamine release during hypoxia and is indeed essential to allow catecholamine release in fish experiencing nicotinic receptor desensitisation.

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References

Axelsson, M. and Fritsche, R. (1994). Cannulation techniques. In *Analytical Techniques* (ed. P. W. Hochachka and T. P. Mommsen), pp. 17–36. Amsterdam: Elsevier.

Berka, J. L., Darren, J. K., Robinson, D. B., Alcorn, D., Marley, P. D.,

Fernley, R. T. and Skinner, S. L. (1996). Adrenaline cells of the rat adrenal cortex and medulla contain renin and prorenin. *Mol. Cell. Endocrinol*. **119**, 175–184.

Bernier, N. J., Kaiya, H., Takei, Y. and Perry, S. F. (1999a). Mediation of humoral catecholamine secretion by the renin–angiotensin system in hypotensive rainbow trout (*Oncorhynchus mykiss*). *J. Endocrinol*. **160**, 351–363.

Bernier, N. J., McKendry, J. E. and Perry, S. F. (1999b). Blood pressure regulation during hypotension in two teleost species: differential involvement of the renin–angiotensin and sympathetic nervous systems. *J. Exp. Biol.* **202**, 1677–1690.

Bernier, N. J. and Perry, S. F. (1997). Angiotensins stimulate catecholamine release from the chromaffin tissue of the rainbow trout. *Am. J. Physiol*. **273**, R49–R57.

Bernier, N. J. and Perry, S. F. (1999). Cardiovascular effects of angiotensin-II-mediated adrenaline release in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol*. **202**, 55–66.

Boksa, P. and Livett, B. G. (1984). Desensitisation to nicotinic cholinergic agonists and K^+, agents that stimulate catecholamine secretion, in isolated adrenal chromaffin cells. *J. Neurochem*. **42**, 607–617.

Campbell, D. J. and Habener, J. (1986). Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. *J. Clin. Invest*. **78**, 31–39.

Chan, W. P., Fung, M. L., Nobiling, R. and Leung, P. S. (2000). Activation of local renin–angiotensin system by chronic hypoxia in rat pancreas. *Mol. Cell. Endocrinol.* **160**, 107–114.

Dunel-Erb, S., Bailly, Y. and Laurent, P. (1982). Neuroepithelial cells in fish gill primary lamellae. *J. Appl. Physiol.* **53**, 1342–1353.

Dzau, V. J., Ellison, K. E., Brody, T., Ingelfinger, J. and Pratt, E. (1987). A comparative study of the distribution of renin and angiotensin messenger ribonucleic acids in rat and mouse tissues. *Endocrinology* **120**, 358–364.

Fritsche, R. and Nilsson, S. (1993). Cardiovascular and ventilatory control during hypoxia. In *Fish Ecophysiology* (ed. J. C. Rankin and F. B. Jensen), pp. 180–206. London: Chapman & Hall.

Fritsche, R., Reid, S. G., Thomas, S. and Perry, S. F. (1993). Serotonin-mediated release of catecholamines in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **178**, 191–204.

Fritsche, R., Thomas, S. and Perry, S. F. (1992). Effects of serotonin on circulation and respiration in the rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol.* **173**, 59–73.

Fuentes, J. and Eddy, F. B. (1998). Cardiovascular responses *in vivo* to angiotensin II and the peptide antagonist saralasin in rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol*. **201**, 267–272.

Furimsky, M., Moon, T. W. and Perry, S. F. (1996). Calcium signaling in isolated single chromaffin cells of the rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B* **166**, 395–404.

Julio, A. E., Montpetit, C. and Perry, S. F. (1998). Does blood acid–base status modulate catecholamine secretion in the rainbow trout (*Oncorhynchus mykiss*)? *J. Exp. Biol.* **201**, 3085–3095.

Lapner, K. N., Montpetit, C. J. and Perry, S. F. (2000). Desensitisation of chromaffin cell nicotinic receptors does not impede catecholamine secretion during acute hypoxia in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **203**, 1589–1597.

Leung, P. S., Chan, W. P., Wong, T. P. and Sernia, C. (1999). Expression and localization of renin–angiotensin system in the rat pancreas. *J. Endocrinol*. **160**, 13–19.

Malhotra, R. K., Wakade, T. D. and Wakade, A. R. (1989). Comparison of secretion of catecholamines from the rat adrenal medulla during continuous exposure to nicotine, muscarine and excess K. *Neurosci.* **26**, 313–320.

Montpetit, C. J. and Perry, S. F. (1999). Neuronal control of catecholamine secretion from chromaffin cells in the rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **202**, 2059–2069.

Montpetit, C. J. and Perry, S. F. (2000). Vasoactive intestinal polypeptide- and pituitary adenylate cyclase activating polypeptide-mediated control of catecholamine secretion from chromaffin tissue in the rainbow trout, *Oncorhynchus mykiss*. *J. Endocrinol.* **166**, 705–714.

Morrell, N. W., Danilov, S. M., Satyan, K. B., Morris, K. G. and Stenmark, K. R. (1997). Right ventricular angiotensin converting enzyme activity and expression is increased during hypoxic pulmonary hypertension. *Cardiovasc. Res*. **34**, 393–403.

Nakano, T. and Tomlinson, N. (1967). Catecholamine and carbohydrate concentrations in rainbow trout (*Salmo gairdneri*) in relation to physical disturbances. *J. Fish. Res. Bd. Can*. **24**, 1701–1715.

Nandi, J. (1961). New arrangement of interrenal and chromaffin tissues in teleost fish. *Science* **134**, 389–390.

Nikinmaa, M. (1992). Membrane transport and control of hemoglobin-oxygen affinity in nucleated erythrocytes. *Physiol. Rev.* **72**, 301–321.

Nilsson, S., Abrahamsson, T. and Grove, D. J. (1976). Sympathetic nervous control of adrenaline release from the head kidney of the cod, *Gadus morhua*. *Comp. Biochem. Physiol*. **55C**, 123–127.

Nishimura, H. (1985). Evolution of the renin–angiotensin system and its role in control of cardiovascular functions in fishes. In *Evolutionary Biology of Primitive Fishes* (ed. R. E. Foreman, A. Gorbman, J. M. Dodd and R. Olsson), pp. 275–293. New York: Plenum Press.

Olson, K. R. (1992). Blood and extracellular fluid volume regulation: role of the renin–angiotensin system, kallikrein–kinin system and atrial natriuretic peptides. In *Fish Physiology. The Cardiovascular System*, vol. XIIB (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 136–232. San Diego, CA: Academic Press.

Oudit, G. Y. and Butler, D. G. (1995). Angiotensin II and cardiovascular regulation in a freshwater teleost, *Anguilla rostrata* LeSueur. *Am. J. Physiol.* **38**, R726–R735.

Perry, S. F. and Gilmour, K. M. (1999). Respiratory and cardiovascular systems. In *Stress Physiology* (ed. P. H. M. Balm), pp. 52–107. Sheffield: Sheffield Academic Press.

Perry, S. F., Malone, S. and Ewing, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid–base status. *Can. J. Zool.* **65**, 888–895.

Perry, S. F., Montpetit, C. J. and Borowska, M. (2000). The effects of acute hypoxia on chemically or neuronally induced catecholamine secretion in rainbow trout (*Oncorhynchus mykiss*) *in situ* and *in vivo*. *J. Exp. Biol*. **203**, 1487–1495.

Perry, S. F. and Reid, S. D. (1992). Relationships between blood oxygen content and catecholamine levels during hypoxia in rainbow trout and American eel. *Am. J. Physiol*. **263**, R240–R249.

Phillips, M. I., Kimura, B. and Riazada, M. K. (1991). Measurement of brain peptides: angiotensin and atrial natriuretic peptide in tissue and cell culture. In *Methods in Neurosciences, Neuropeptide Technology: Synthesis, Assay, Purification and Processing* vol. 6 (ed. P. M. Conn), pp. 177–206. London: Academic Press.

Primmett, D. R. N., Randall, D. J., Mazeaud, M. and Boutilier, R. G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *J. Exp. Biol*. **122**, 139–148.

Randall, D. J. and Perry, S. F. (1992). Catecholamines. In *Fish Physiology. The Cardiovascular System*, vol. XIIB (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 255–300. San Diego, CA: Academic Press.

Reid, S. G., Bernier, N. J. and Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol.* **120C**, 1–27.

Reid, S. G., Fritsche, R. and Jönsson, A.-C. (1995). Immunohistochemical localization of bioactive peptides and amines associated with the chromaffin tissue of five species of fish. *Cell Tissue Res*. **280**, 499–512.

Ristori, M. T. and Laurent, P. (1989). Plasma catecholamines in rainbow trout (*Salmo gairdneri*) during hypoxia. *Exp. Biol*. **48**, 285–290.

Ritthaler, T., Schricker, K., Kees, F., Krämer, B. and Kurtz, A. (1997). Acute hypoxia stimulates renin secretion and renin gene expression *in vivo* but not *in vitro*. *Am. J. Physiol.* **272**, R1105–R1111.

Soivio, A., Nynolm, K. and Westman, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J. Exp. Biol*. **62**, 207–217.

Walhqvist, I. and Nilsson, S. (1980). Adrenergic control of the cardiovascular system of the Atlantic cod, *Gadus morhua* during 'stress'. *J. Comp. Physiol. B* **137**, 145–150.

Wolf, K. (1963). Physiological salines for freshwater teleosts. *Progve. Fish Cult.* **25**, 135–140.

Woodward, J. J. (1982). Plasma catecholamines in resting rainbow trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography. *J. Fish Biol*. **21**, 429–432.

Zhao, L., al Tubuly, R., Sebkhi, A., Owji, A. A., Nunez, D. J. and Wilkins, M. R. (1996). Angiotensin II receptor expression and inhibition in the chronically hypoxic rat lung. *Br. J. Pharmacol*. **119**, 1217–1222.