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The interactive effects of hypoxia and nitric oxide on catecholamine secretion in rainbow trout (*Oncorhynchus mykiss*)

Brian McNeill and Steve F. Perry*
Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, ON K1N 6N5, Canada
*Author for correspondence (e-mail: sfperry@science.uottawa.ca)

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Summary

Experiments were performed to test the hypothesis that exposure of rainbow trout to repetitive hypoxia would result in a decreased capacity of chromaffin cells to secrete catecholamines owing to increased production of nitric oxide (NO), a potent inhibitor of catecholamine secretion. A partial sequence of trout neuronal nitric oxide synthase (nNOS) was cloned and its mRNA was found to be present in the posterior cardinal vein (PCV), the predominant site of chromaffin cells in trout. Using heterologous antibodies, nNOS and endothelial NOS (eNOS) were localized in close proximity to the chromaffin cells of the PCV.

Exposure of trout to acute hypoxia (5.33 kPa for 30 min) *in vivo* resulted in significant increases in plasma catecholamine and NO levels. However, after 4 days of twice-daily exposures to hypoxia, the elevation of plasma catecholamine levels during hypoxia was markedly reduced. Associated with the reduction in plasma catecholamine levels during acute hypoxia was a marked increase in basal and hypoxia-evoked circulating levels of NO that became apparent after 2–4 days of repetitive hypoxia. The capacity of the chromaffin cells of the hypoxia-exposed fish to secrete catecholamine was assessed by electrical stimulation of an *in situ* saline-perfused PCV preparation. Compared with control (normoxic) fish, the PCV preparations derived from fish exposed to repeated hypoxia displayed a significant reduction in electrically evoked catecholamine secretion that was concomitant with a marked increased in NO production. This additional rise in NO secretion in preparations derived from hypoxic fish was prevented after adding NOS inhibitors to the perfusate; concomitantly, the reduction in catecholamine secretion was prevented. The increased production of NO during hypoxia *in vivo* and during electrical stimulation *in situ* was consistent with significant elevations of nNOS mRNA and protein; eNOS protein was unaffected. These results suggest that the reduced capacity of trout chromaffin cells to secrete catecholamines after repeated hypoxia reflects an increase in the expression of nNOS and a subsequent increase in NO production during chromaffin-cell activation.

Key words: hypoxia, catecholamines, nitric oxide, nNOS, eNOS.

Introduction

In response to severe acute stressors, the catecholamine hormones, noradrenaline and adrenaline, are released into the circulatory system (for a review, see Reid et al., 1998). The predominant source of catecholamines in teleost fish is from a cluster of chromaffin cells within the walls of the posterior cardinal vein (PCV) in the vicinity of the head kidney (Nandi, 1961). Once in the circulation, catecholamines serve to reduce the detrimental effects that are often associated with stress (Wendelaar Bonga, 1997). The beneficial effects of catecholamines are achieved, in part, by modulation of the cardiovascular and respiratory systems (reviewed by Perry and Gilmour, 1999). In particular, the rise in catecholamine levels is thought to initiate a series of compensatory physiological processes directed towards the enhancement of branchial O_2 transfer and blood O_2 transport (Perry and Wood, 1989; Thomas and Perry, 1992; Randall and Perry, 1992; Nikinmaa, 1992; Nikinmaa and Boutilier, 1995).

The primary mediator of catecholamine secretion from the chromaffin cells in rainbow trout is the activation of nicotinic cholinergic receptors by acetylcholine (Ach) (Montpetit and Perry, 1999). This leads to an influx of extracellular Ca^{2+} into the chromaffin cell, which initiates a series of events culminating in catecholamine secretion through exocytosis (Livett and Marley, 1993; Furimsky et al., 1996). In addition to Ach, the preganglionic nerve fibers release a range of other transmitters, including serotonin, adenosine, pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) (Montpetit and Perry, 2000; Shioda et al., 2000; Wong et al., 2002). In general, these neurotransmitters or modulators function by increasing intracellular Ca^{2+} levels within chromaffin cells, thereby augmenting catecholamine secretion.

Nitric oxide (NO) is another such neurotransmitter (Furchgott, 1999) that, in addition to being implicated in cardiovascular control (Donald and Broughton, 2005; Tota et al., 2005; Agnisola, 2005; Eddy, 2005) and osmoregulation (Evans et al., 2004; Ebbesson et al., 2005), also plays an important role in the regulation of catecholamine secretion in fish (McNeill and Perry, 2005). As in mammals (Schwarz et al., 1998; Nagayama et al., 1998; Barnes et al., 2001; Kolo et al., 2004), NO profoundly inhibits agonist-evoked catecholamine secretion in rainbow trout (McNeill and Perry, 2005).

NO is a relatively short-lived, highly reactive gas molecule that is produced in various tissues by the nitric oxide synthase (NOS; EC 1.14.13.39) family of enzymes. There are three known isoforms of NOS: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Currently, there is considerable debate as to which NOS isoform(s) (in particular eNOS *versus* nNOS) contribute to cardiovascular regulation in fish. At least in major systemic blood vessels, NO derived from eNOS would appear to be less important than neuronally produced NO (Donald and Broughton, 2005). Similarly, although NO derived from both eNOS and nNOS can potentially regulate catecholamine secretion in trout, nNOS is probably the more important contributor (McNeill and Perry, 2005).

In many fish species, including rainbow trout, acute hypoxia is a potent stimulus for the secretion of catecholamines into the circulation (Ristori and Laurent, 1989). In mammals, hypoxia is known to increase NO production in various tissues including skeletal muscle (Javeshghani et al., 2000), brain (Prabhakar et al., 1996) and lung (Vaughan et al., 2003). Although less well-studied, there is emerging evidence that hypoxia also evokes NO production in fish (McNeill and Perry, 2005; Agnisola, 2005; Swenson et al., 2005). Thus, although plasma catecholamine and NO levels presumably increase concurrently during hypoxia in rainbow trout, the consequences of NO production on catecholamine secretion during hypoxia *in vivo* have not been investigated. Because of the inhibitory influence of NO on catecholamine secretion (McNeill and Perry, 2005), it was hypothesized that repeated bouts of hypoxia would serve to decrease the capacity of trout chromaffin cells to secrete catecholamines owing to induction of NOS and an associated increase in NO production during chromaffin-cell activation. To test this idea, plasma catecholamine and NO levels were measured in trout that were exposed to acute hypoxia twice-daily for 4 days. After 4 days, the capacity of chromaffin cells to secrete catecholamines and NO was evaluated in an *in situ* saline-perfused PCV preparation and correlated with the expression of eNOS and nNOS mRNA and protein in the PCV.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of both sexes were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). The fish were held at the University of Ottawa in large fiberglass tanks supplied with flowing, aerated and dechloraminated City of Ottawa tap water. The fish [mean mass of 233±10.9 g (s.e.m.); N=102] were maintained at a temperature of 13°C on a 12 h:12 h light:dark photoperiod. They were fed daily with a commercial trout diet. Fish were allowed to acclimate to the holding facility for at least two weeks prior to experimentation.

Series 1: cloning of nNOS and its tissue distribution

Fish were killed by a sharp blow to the head and the PCV was dissected and immediately frozen in liquid N_2 and stored at –80°C. Total RNA was extracted from rainbow trout PCV by using Trizol (Invitrogen, Burlington, ON, Canada) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primers. A single 2285-bp segment was generated (GenBank Accession No. DQ640498) by PCR using the following primers:

nNOS FWD 3′-TCACCACNCACCTGGAGAC-5′
nNOS REV 3′-TACAAGGTGCGKTTYAASNGCG-5′.

Gene-specific primers were then designed to obtain sufficient overlapping sequences to obtain a consensus sequence. All PCR reactions involved an initial denaturation at 94°C for 30 s, followed by 35 cycles of: 94°C for 30 s; annealing temperature for 60 s; 72°C for 90 s; and ending with a final extension for 10 min at 72°C. PCR products were cloned using TOPO TA cloning kits (Invitrogen) and sequenced (University of Ottawa Core Sequencing Facility).

A separate group of fish was killed by a sharp blow to the head, and tissues (brain, anterior PCV and kidney, posterior PCV and kidney, white muscle, blood, spleen and intestine) were collected and frozen immediately in liquid N_2 and stored at –80°C. Total RNA was extracted using Trizol according to the manufacturer's instructions. To remove any remaining genomic DNA, the RNA was treated with DNAse (eight units per sample; Invitrogen). RNA quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf BioPhotometer, VWR, Mississauga, ON, Canada). cDNA was synthesized from 5 μg total RNA using StrataScript reverse transcriptase (Stratagene, Cedar Creek, TX, USA) and random hexamer primers.

nNOS mRNA levels were assessed by real-time PCR on samples of cDNA using Brilliant® SYBR® Green quantitative polymerase chain reaction (QPCR) (Stratagene) and a Stratagene MX-4000 multiplex QPCR system. PCR conditions were as instructed by the manufacturer, except scaled down from a 50 μl to a 25 μl final reaction volume. The following primer pairs were designed using DNAMAN software (version 4.0; Lynnon Biosoft, Vaudreuil-Dorian, QC, Canada): β-actin forward (5′-CCA ACA GAT GTG GAT CAG CAA-3′), β-actin reverse (5′-GGT GGC ACA GAG CTG AAG TGG TA-3′), nNOS forward (5′-TGG AGA GAA ATT CGG AGC TG-3′), nNOS reverse (5′-CGG GTG TCA GAA TAG GAG GA-3′), 18S forward (5′-TCT CGA TTC TGT GGG TGG T-3′), 18S reverse (5′-CTC AAT CTC GTG TGG CTG A-3′).

The specificity of the primers was verified by the cloning (TA cloning kit; Invitrogen) and sequencing of amplified products. Relative expression of nNOS mRNA levels was determined [using *actin* and *18S* as reference genes; the average of the two Ct-values (threshold cycle) was used in calculations] by the Δ–ΔCt method (Pfaffl, 2001).

Series 2: immunocytochemistry of nNOS and eNOS within the PCV

PCVs, in the anterior region of the head kidney, were dissected and collected in phosphate-buffered saline (PBS; adjusted to pH 7.4). The catecholamine-containing cell fraction of the PCV was identified by detection of catecholamines using aldehyde-induced green fluorescence (Furness et al., 1977; Lacoste et al., 2001). Each PCV was cut into 5-mm pieces and incubated for 24 h at 4°C in a solution of 4% paraformaldehyde and 0.55% glutaraldehyde (prepared in PBS). Tissues were washed with PBS and cryoprotected by immersion in a series of PBS solutions containing 15% and 30% sucrose (w/v) for at least 2 h each. Tissues were embedded in Cryomatrix (OCT-compound; Shandon, Pittsburg, PA, USA) and 10 μm cross-sections were collected at –15°C using a Leica CM 1900 cryostat (Leica Microsystems). Sections were collected and thaw-mounted on Superfrost Plus slides (VWR).

Tissue sections were prepared by creating a hydrophobic barrier around each section with a PAP pen (Cedarlane, Mississauga, ON, Canada) and were rehydrated (2×5 min) in PBS. Sections were then incubated in 3% H_2O_2 (1×10 min) followed by PBS (2×5 min). Sections were then incubated with either eNOS (AB16301; 1:200; Chemicon, Temecula, CA, USA) or nNOS (SC-648; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at room temperature. Sections were washed (3×5 min) with PBS before incubating for 1 h with Alexa-546 anti-rabbit (Molecular Probes, Burlington, ON, Canada). The slides were then washed again (3×5 min) in PBS and mounted with a mounting medium (Vector Laboratories, Burlington, ON, Canada) containing 4′,6′-diamidino-2-phenylindole (DAPI) for the visualization of the nuclei. The sections were viewed using a conventional epifluorescence microscope (Zeiss Axiophot) and CCD camera (Hamamatsu C5985). Images were captured using Metamorph v. 4.01 imaging software.

Series 3: effects of repeated hypoxia exposure in vivo

Animal preparation

Rainbow trout were anaesthetized in an aerated solution of ethyl-*P*-amino-benzoate (benzocaine; 2.4×10^{-4} mol l^{-1}; Sigma, St Louis, MO, USA). Fish were placed onto an operating table where the gills were continuously irrigated with aerated water containing anaesthetic. To permit periodic blood sampling, an indwelling polyethylene cannula (Clay-Adams PE 50; VWR) was implanted into the dorsal aorta through percutaneous puncture of the roof of the buccal cavity (Soivio et al., 1975). Trout were then placed individually in opaque Perspex boxes supplied with aerated flowing water, where they were allowed to recover for 24 h prior to experimentation.

Experimental protocol

Trout were exposed to acute hypoxia for 30 min twice-daily for a period of 4 days. Acute hypoxia was achieved by replacing the air supplying a water/gas equilibration column with N_2. The partial oxygen pressure (P_{O_2}) was reduced to approximately 5.33 kPa, at which point the 30-min period began. This degree of hypoxia was chosen on the basis of a previous study (Perry and Reid, 1992), showing significant catecholamine release at this P_{O_2}. The water P_{O_2} was monitored using a Foxy-AL300 O_2 fiber-optic probe and associated hardware (Ocean Optics, Dunedin, FL, USA). Blood samples were collected prior to, and following, each exposure to hypoxia and analyzed for catecholamine and NO levels. On the morning of the fifth day, the fish were removed, and *in situ* perfused PCV preparations, as described above, were derived from these fish. Control fish were placed in the Perspex boxes and received normoxic water for 4 days.

To determine whether the effects observed were because of the repeated hypoxia exposure or because of the collection of multiple blood samples, a group of fish were treated as above, although blood samples only were taken prior to and following the first and final hypoxia exposures.

Series 4: the effect of repeated hypoxia on in-situ catecholamine secretion, NO production and NOS expression

Uncannulated fish were placed into opaque Perspex boxes supplied with aerated flowing water and allowed to recover for 24 h prior to experimentation. After 24 h, the water P_{O_2} was decreased, as described above, to approximately 5.33 kPa. Fish were exposed twice-daily to 30 min of hypoxia for a total of 4 days. On the morning of the fifth day, the fish were removed and *in situ* perfused PCV preparations were derived from these fish as outlined below. Control fish were placed in the Perspex boxes and received normoxic water for 4 days. Prior to terminating the experiment, tissues were collected, frozen in liquid N_2 and stored at –80°C until processed. The tissues included the brain, PCV and kidney. Tissues for reverse transcriptase-polymerase chain reaction (RT-PCR) were processed as described above. Proteins for western blots were prepared from frozen tissue samples (0.4 g ml^{-1} homogenization buffer) as described in detail by Georgalis et al. (Georgalis et al., 2006).

In situ saline-perfused PCV preparation

The fish were killed by a sharp blow to the head, weighed and placed on ice. To electrically stimulate the nerves innervating the chromaffin cells, a field-stimulation technique was used whereby electrodes were sutured to the skin on each side of the fish immediately behind the operculum at the level of the lateral line (Montpetit and Perry, 1999). A ventral incision was made from the anus to the pectoral girdle, and the tissues overlying the heart were removed by blunt dissection to expose the ventricle and the bulbus arteriosus. An inflow cannula (PE-160 polyethylene tubing; Clay-Adams) was inserted into the PCV, and an outflow cannula (PE-160) was inserted into the ventricle through the bulbus arteriosus. Prior

to the experiments, the preparations were perfused for 20 min with modified aerated Cortland saline (Wolf, 1963) (125 mmol l^{-1} NaCl, 2.0 mmol l^{-1} KCl, 2.0 mmol l^{-1} $MgSO_4$, 5.0 mmol l^{-1} $NaHCO_3$, 7.5 mmol l^{-1} glucose, 2.0 mmol l^{-1} $CaCl_2$ and 1.25 mmol l^{-1} KH_2PO_4, final pH 7.8) to allow catecholamine and NO levels to stabilize. Perfusion was accomplished using positive pressure differences between the surface of the saline and the outflow cannula, resulting in a relatively constant flow (approximately 0.3 ml min^{-1}).

Following the stabilization period, two samples were collected in pre-weighed microcentrifuge tubes to assess basal-catecholamine- and NO-secretion rates prior to any experimental procedure. Fish were then subjected to an electrical stimulation of 30 V and 8 Hz using a previously validated field-stimulation technique (Montpetit and Perry, 1999). During the experimental procedure, the perfusate was collected continuously at 2-min intervals over a 10-min period. All samples were immediately centrifuged for 20 s at 7500 ***g*** and the perfusate was quickly frozen in liquid N_2 and stored at –80°C until subsequent determination of catecholamine and NO levels. In one series of experiments, preparations were electrically stimulated during NOS inhibition. This was achieved by adding the NOS inhibitors 7-nitroindazole (7-NI; 10^{-4} mol l^{-1}) and N-nitro L-arginine methyl ester (L-NAME; 5×10^{-3} mol l^{-1}) to the perfusion fluid (McNeill and Perry, 2005). Preparations were either perfused with saline containing the combination of the inhibitors or with control saline. Whereas L-NAME could be added directly to saline, 7-NI was prepared in methanol prior to addition to the saline (final concentration in the perfusate was 0.2%). Preliminary experiments showed that 0.2% methanol was without effect on basal- or stimulus-evoked catecholamine secretion.

Determination of catecholamine levels

Catecholamine levels in perfusate were determined on alumina-extracted samples (100 μl) using high-performance liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). The HPLC incorporated a Varian ProStar 410 solvent-delivery system (Varian Chromatography systems, Walnut Creek, CA, USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ, USA). Concentrations were calculated relative to appropriate standards, using 3,4-dihydroxybenzalamine hydrobromide (DHBA) as an internal standard.

NO assay

Quantification of NO is problematic because of its short lifetime. Therefore, NO production was evaluated by measuring the concentration of nitrite and nitrate, stable metabolites of NO in biological fluids. Thus, NO levels will be reported as NO_X. In mammals, this method demonstrates high accuracy and reproducibility and adequately reflects actual NO production (Gilliam et al., 1993; Manukhina et al., 1999). It is possible that NO levels could be underestimated, however, owing to the exit of nitrite (formed from NO) from the plasma or perfusate through Cl^- channels; this potential underestimation, however, would be expected to be constant among the treatment groups. The NO assay was performed as described by Gilliam et al. (Gilliam et al., 1993), with modifications. Briefly, a stock solution (1 mmol l^{-1}) of magnesium nitrate was prepared in saline. The stock solution was serially diluted in 0.14 mol l^{-1} $KHPO_4$ to prepare standard curves. The assay procedure consisted of adding 50 μl of standard or sample along with 15 μl of NADPH (0.8 mmol l^{-1}; Sigma) to a 96-well plate. Next, 2.5 μl of FAD (100 μmol l^{-1}; Sigma) was added followed by 0.01 units of nitrate reductase (from *Aspergillus niger*; EC1.6.6.2; Sigma). The plate was sealed, placed in the dark and incubated at room temperature (~21°C) for 45 min. Griess reagents I and II (40 μl) (Cayman Chemical, Ann Arbor, MI, USA) were then added and allowed to incubate for 5 min. Color development was assessed using a Spectra Max Plus 384 (Molecular Devices VWR, Mississauga, ON, Canada) microplate reader at a wavelength of 540 nmol l^{-1}.

Western blotting

Samples (50 μg protein) were size-fractionated by reducing SDS-PAGE using 10% separating and 6% stacking polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) using a Trans-Blot electrophoretic-transfer cell (Bio-Rad) according to the manufacturer's instructions. After transfer, each membrane was blocked for 1 h in phosphate-buffered, 1% Tween 20 (PBST) containing 5% milk powder. Membranes were probed first with either eNOS (1:250) or nNOS (1:250) antibodies (see above) overnight at 4°C and then with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000; Pierce, Fisher Scientific, Ottawa, ON, Canada). After each incubation, the membranes were washed for 3×5 min in PBST. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce SuperSignal West Pico Chemiluminescent Substrate; Fisher Scientific) and exposure to imaging film (Blue XB-1; Kodak). The protein-size marker used was obtained from Fermentas Life Sciences (Burlington, ON, Canada). To demonstrate specificity of the NOS antibodies, primary antisera were preabsorbed using the peptide (nNOS blocking peptide # SC-648; Santa Cruz Biotechnology; eNOS blocking peptide # AG593; Chemicon) against which the antibody was raised. To assess for equal loading, blots were stripped by soaking them for 30 min in PBS or TBS containing 2% SDS and 7 μl ml^{-1} β-mercaptoethanol at room temperature. After rinsing for 30 min with PBS or TBS, the blot was blocked twice in 5% PBST-milk powder for 10 min each. The blot was then probed with an anti-β-tubulin antibody (1:1000; Sigma-Aldrich Canada) for 1 h at 37°C. The blot was then incubated in anti-mouse Ig, horseradish peroxidase (1:5000) for 1 h at room temperature. Following additional washings, the proteins were visualized using ECL (Pierce; SuperSignal West Pico Chemiluminescent Substrate) as above.

The density of the antigenic bands was determined by scanning the films and then analyzing the digital images

using commercial software (Quantity One v4.1.1; Biorad, Mississauga, ON, Canada). The results are presented as the ratio of NOS to tubulin-band density.

Statistical analysis and data presentation

The data are presented as means ± 1 standard error of the mean (s.e.m.). All data sets were analyzed using two-way repeated measures analysis of variance (ANOVA). If a statistical difference was identified, a *post-hoc* multiple ('all pair-wise') comparison test (Bonferroni's *t*-test) was applied. All statistical tests were performed using a commercial statistical software package (SigmaStat version 2.03; Point Richmond, CA, USA).

Owing to a high degree of temporal variability, peak catecholamine-secretion rates, generally obtained 2 or 4 min after stimulation/agonist addition, were calculated by taking the mean of the maximal noradrenaline and adrenaline secretion rates in response to stimulation for each fish within a given group. For total catecholamine-secretion rates, the sum of adrenaline and noradrenaline was determined at each time point and the resultant maximum values were used. Statistical analysis of noradrenaline, adrenaline and total catecholamines was performed, and all showed similar trends within each experiment. Therefore, for clarity, only the statistical analysis of total catecholamine-secretion rates is presented in the figures.

NO peak levels, generally obtained 2 or 4 min after stimulation/agonist addition, were calculated by taking the mean of the maximal NO levels in response to stimulation for each fish within a given group.

Results

Series 1: cloning of nNOS and its tissue distribution

A 2285 bp consensus sequence was generated for rainbow trout nNOS; multiple attempts to complete the sequence using RACE were unsuccessful. Conceptual translation yielded a protein segment of 761 amino acids. Multiple sequence alignment with other vertebrate NOS isoforms yielded a phylogeny tree (Fig. 1) in which the trout NOS groups closely with nNOS of other vertebrates and is most similar to the nNOS of other fishes. Because the trout NOS grouped closely with vertebrate nNOS and did not group with eNOS or iNOS, we tentatively conclude that the trout isoform is nNOS.

The results of real-time PCR indicated that spleen, intestine and brain contained relatively high levels of nNOS mRNA, whereas PCV, kidney, liver and white muscle contained detectable, yet lower mRNA levels (Fig. 2).

Series 2: immunocytochemistry of nNOS and eNOS within the PCV

The chromaffin cell-containing fraction of the PCV was identified using aldehyde-induced fluorescence that is characteristic of catecholamine-containing cells (Figs 3, 4). The double-labeling of the PCV tissue using the aldehyde-induced fluorescence of catecholamines and immunostaining for either nNOS (Fig. 3) or eNOS (Fig. 4) demonstrated that both of the NOS isoforms are in close proximity to the chromaffin cells. Although some chromaffin cells appeared to express nNOS (Fig. 3A), eNOS appeared to be localized exclusively to non-chromaffin cells (Fig. 4A). Because heterologous NOS antibodies were used for the immunoctyochemistry, their specificity on trout tissues was assessed by western blotting. Single immunoreactive bands were observed for nNOS and eNOS at 150 kDa and 140 kDa, respectively; these bands were eliminated after preabsorption of the primary antibodies with blocking peptides (Figs 3, 4).

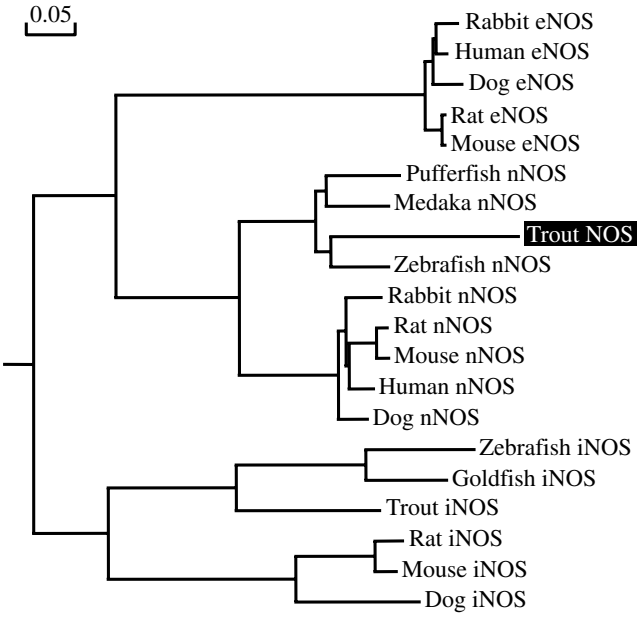

Fig. 1. Phylogenetic analysis of rainbow trout (*Oncorhynchus mykiss*) NOS protein. The phylogenetic tree was constructed with commercial software (DNAMAN version 5.2.9; Lynnon Biosoft) using maximum likelihood as the distance method. Support values for nodes (100% in all cases and therefore not shown) were determined through bootstrapping of 100 pseudo-datasets. Branches are drawn to scale as the scale represents replacement of 5% of the amino acids in the protein alignment. Sequences for eNOS were obtained from rabbit (*Oryctolagus cuniculus*; accession AAO47084), human (*Homo sapiens*; accession NP_00059), dog (*Canis familiaris*; NP_001003158), rat (*Rattus norvegicus*; NP_068610) and mouse (*Mus musculus*; P70313). Sequences for nNOS were obtained from pufferfish (*Takifugu rubripes*; accession AAL82736), Japanese medaka (*Oryzias latipes*; accession BAD11808), zebrafish (*Danio rerio*; accession AAO53340), rabbit (accession AAB68663), rat (NP_434686), mouse (NP_032738), human (NP_000611) and dog (XP_534695). Sequences for iNOS were obtained from zebrafish (XP_692103), goldfish (*Carassius auratus*), rainbow trout (CAC8306), rat (CAB46089), mouse (AAH62378) and dog (NP_001003186).

Series 3: effects of repeated hypoxia exposure in vivo

Upon exposure of fish to acute hypoxia, a significant increase in circulating catecholamine levels was observed (Fig. 5A). Plasma NO_X levels were also elevated following each exposure

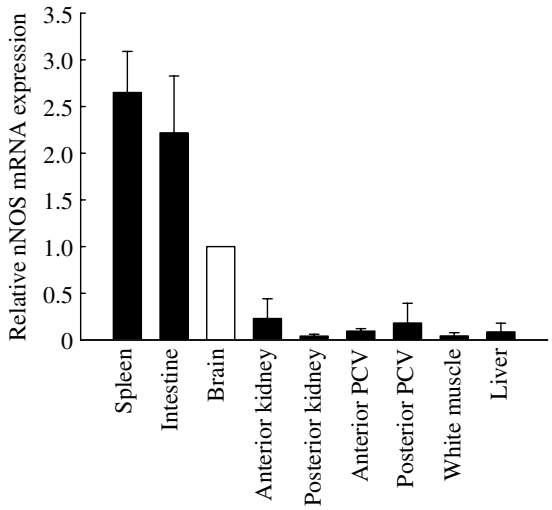

Fig. 2. Mean tissue distributions of nNOS RNA in rainbow trout (*Oncorhynchus mykiss*) as determined by real-time RT-PCR. The results are presented as the expression of nNOS relative to β-actin and standardized to nNOS expression in the brain (*N*=5).

to hypoxia (Fig. 5C). Basal levels of NO_X were increased by approximately 2-fold prior to the final two exposures on the fourth day (Fig. 5C). By contrast, basal catecholamine levels were unaffected, and hypoxia-evoked catecholamine concentrations were decreased markedly during the final two exposures on day 4 (Fig. 5A). Similar results were observed when fish were exposed to an identical regimen of repeated hypoxia but when paired blood samples (pre- and post-hypoxic) were collected only once on each of days 1 and 4 (Fig. 5B,D).

Series 4: the effect of repeated hypoxia on in-situ catecholamine secretion, NO production and NOS expression

This series was performed to determine if repeated hypoxia exposure was associated with any effects on NO enzyme activity or NOS mRNA and protein levels, and if there were any accompanying effects on stimulus-evoked catecholamine secretion. *In situ* preparations derived from fish exposed to repeated hypoxia showed an increase in basal catecholamine

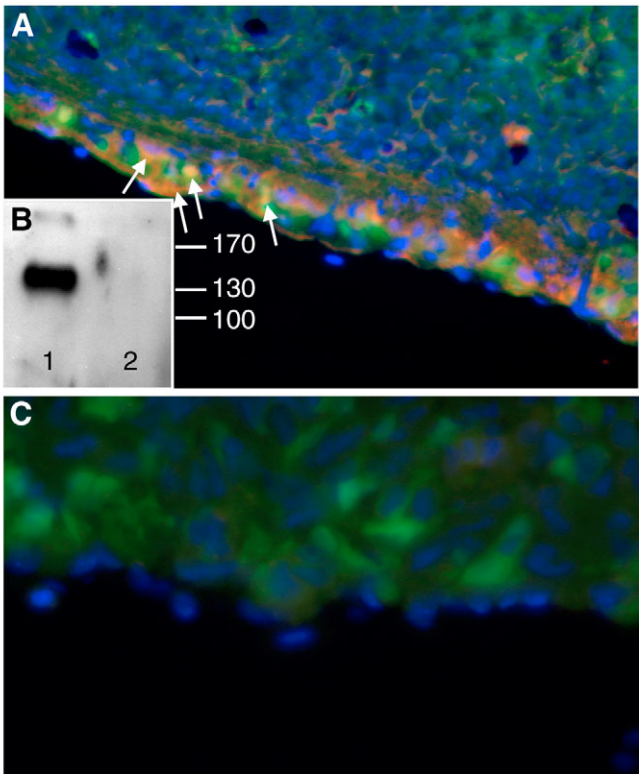

Fig. 3. Representative fluorescence photomicrographs of rainbow trout PCV cross-sections demonstrating (A) the presence of nNOS immuno-positive cells (red) in chromaffin cells [green; areas of co-localization (arrows) appear as orange/yellow] or in close proximity to the chromaffin cells as identified by aldehyde-induced fluorescence. Cell nuclei are indicated by blue fluorescence (DAPI staining). (B) A representative western blot of trout PCV tissue demonstrating the presence of a single immunoreactive band at approximately 150 kDa (lane 1) that was absent after preabsorption of the nNOS antibody with blocking peptide (lane 2). There was no detectable red fluorescence in sections where the primary antibody was omitted (C).

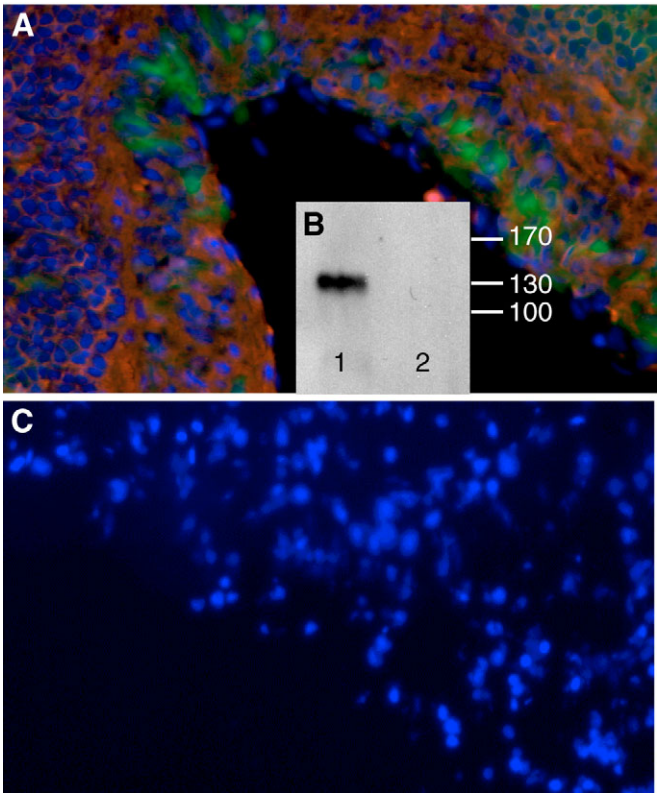

Fig. 4. Representative fluorescence photomicrographs of rainbow trout PCV cross-sections demonstrating (A) the presence of eNOS immuno-positive cells (red) in close proximity to the chromaffin cells (green) as identified by aldehyde-induced fluorescence. Cell nuclei are indicated by blue fluorescence (DAPI staining). (B) A representative western blot of trout PCV tissue demonstrating the presence of a single immunoreactive band at approximately 140 kDa (lane 1) that was absent after preabsorption of the eNOS antibody with blocking peptide (lane 2). There was no detectable red or green fluorescence in sections not treated with aldehyde and on which the primary antibody was omitted (C).

secretion but an approximate 50% decrease in stimulus-evoked secretion (Fig. 6A). *In situ* preparations derived from hypoxic fish displayed an approximate 2-fold increase in basal NO_X levels and a 1.8-fold increase in stimulus-evoked NO_X production (Fig. 6B). Addition of the NOS inhibitors, L-NAME and 7-NI, to the perfusate, prevented the additional rise in NO_X during electrical stimulation of preparations derived from hypoxic fish (Fig. 6B). Similarly, after NOS inhibition, stimulus-evoked catecholamine secretion was comparable to that observed in preparations derived from control fish (Fig. 6A).

The results of western blotting revealed that nNOS protein was significantly increased in hypoxic fish (Fig. 7A); eNOS protein was unaffected by repeated hypoxia (Fig. 7B). The real-time PCR analysis demonstrated that nNOS mRNA was significantly increased in brain and anterior PCV following the hypoxia exposures (Fig. 8).

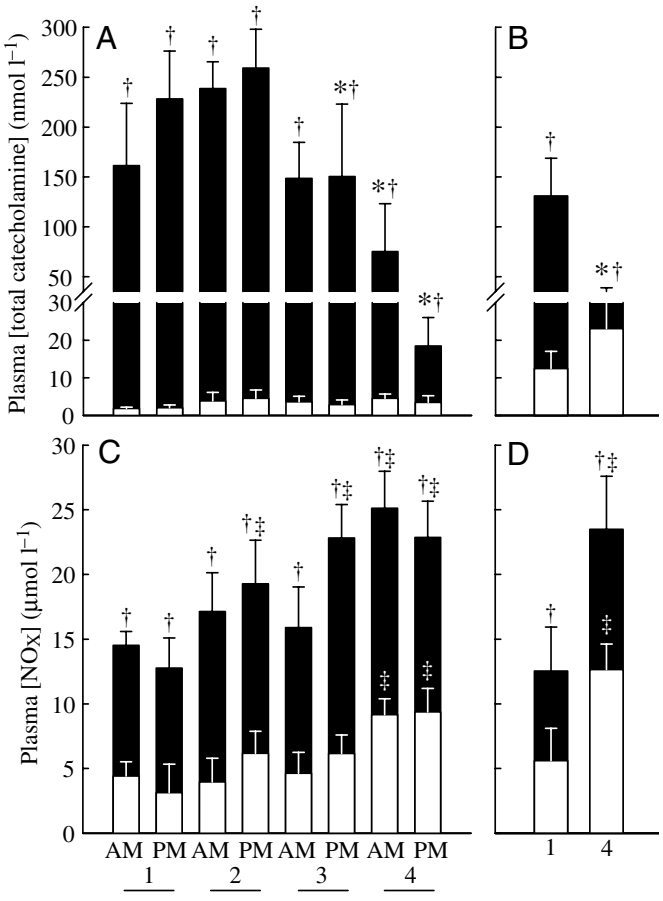

Fig. 5. The effects of 30-min exposure to acute hypoxia (P_{O_2}<5.99 kPa) on plasma (A,B) total catecholamine levels (filled bars; *N*=6) and (C,D) NO_X concentrations (filled bars; *N*=6). Unfilled bars are basal levels prior to hypoxia exposure. In all cases, fish were exposed twice-daily to acute hypoxia for a total of 4 days, but in one series (C,D) paired blood samples were withdrawn only once each on day 1 and 4. Values are shown as means ± 1 s.e.m. A dagger denotes a significant difference (*P*<0.5) between the pre-hypoxia values (unfilled bars) and hypoxia exposure (filled bars). An asterisk denotes a significant difference (*P*<0.5) in plasma catecholamine levels during hypoxia from value of the peak response (day 2, PM). A double dagger denotes a significant difference (*P*<0.5) in basal or hypoxia-evoked NO levels from the lowest values (Day 1, PM).

Discussion

Numerous studies have demonstrated that acute hypoxia is a stimulus for catecholamine secretion in fish (reviewed by Perry and Gilmour, 1999). To our knowledge, however, this is the first study to examine the effects of repetitive hypoxia on the acute adrenergic stress response. Although plasma catecholamine levels increased to similar values during the first three days of hypoxic exposure, there was a marked decrease in apparent catecholamine secretion on day 4 as indicated by significantly lower plasma catecholamine levels. Because the capacity to secrete catecholamines in response to electrical stimulation was decreased in PCV preparations derived from hypoxic fish, the attenuation of the adrenergic stress response *in vivo* was probably caused by a specific impairment of catecholamine secretion from

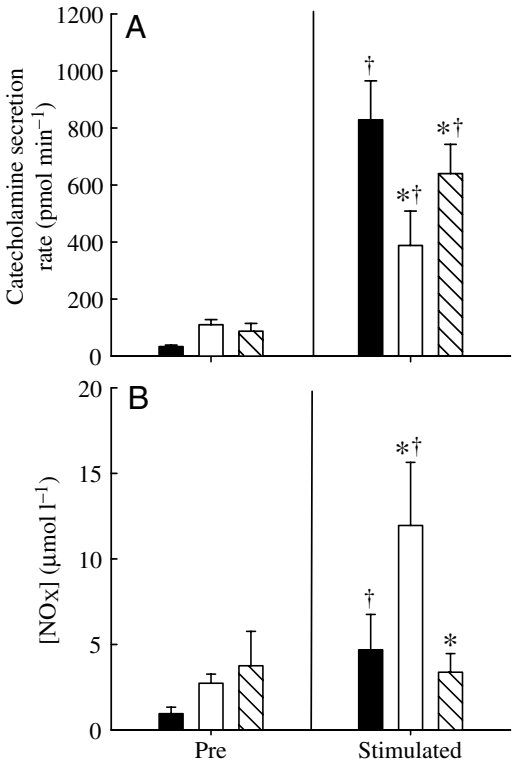

Fig. 6. The effects of *in situ* electrical stimulation on (A) total catecholamine secretion (sum of noradrenaline plus adrenaline) and (B) perfusate NO_X levels in preparations derived from fish repeatedly exposed to hypoxia (unfilled bars; *N*=9) or from control fish (filled bars; *N*=9). Following the stabilization period, the preparations were perfused with control saline or saline containing a cocktail of NOS inhibitors (cross-hatched bars) and electrically stimulated at 30 V and 8 Hz. Values are shown as means ± 1 s.e.m. A dagger denotes a significant difference (*P*<0.5) between pre-experimental (Pre) and stimulated samples. An asterisk denotes a significant difference (*P*<0.5) between the hypoxia-treated preparations with or without NOS inhibitors.

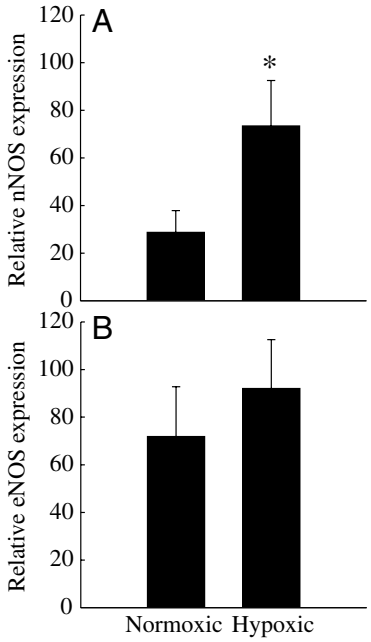

Fig. 7. The relative expression of (A) nNOS (N=10) and (B) eNOS (N=7) protein in the PCV of rainbow trout (*Oncorhynchus mykiss*) that were either subjected to 4 days of repeated hypoxia or continued normoxia. Values are shown as means ± s.e.m. An asterisk denotes a significant difference (P<0.5) between the control and hypoxia-treated groups.

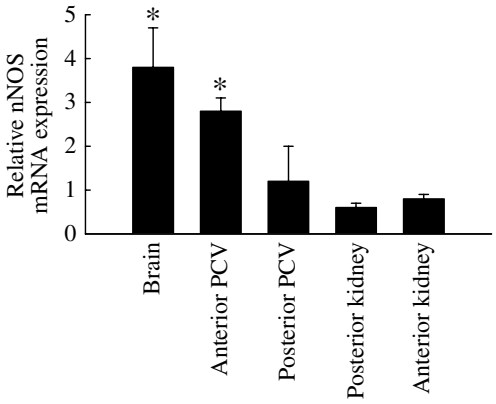

Fig. 8. The effects of repeated hypoxia exposures on the relative expression of nNOS mRNA in various tissues as determined by real-time RT-PCR. Values are shown as means ± 1 s.e.m. (N=5). In each tissue, an asterisk denotes a significant difference (P<0.5) in the relative levels of mRNA between control and hypoxia-exposed fish.

chromaffin cells rather than alterations in catecholamine metabolism (Nekvasil and Olson, 1986; Olson, 2002) or O_2-sensing mechanisms (Reid and Perry, 2003). NO, a potent inhibitor of catecholamine secretion in rainbow trout (McNeill and Perry, 2005), was also released into the circulation during hypoxia. However, in contrast to catecholamines, higher levels of NO in the blood were observed on days 3–4 of the hypoxia exposures. We believe that an increased production of NO in the vicinity of chromaffin cells, a consequence of upregulation of nNOS (see below), contributed to the reduced catecholamine release in the chronically hypoxic fish. The most compelling evidence supporting this idea was that the reduction in catecholamine secretion in PCV preparations derived from hypoxic fish was no longer observed when the production of NO during electrical stimulation was prevented by treatment with NOS inhibitors.

NOS, NO and catecholamine secretion

The results of a previous study demonstrated that in rainbow trout head kidney nNOS was predominantly localized to nerve fibers in close proximity to chromaffin cells and less frequently to the chromaffin cells themselves (Gallo and Civinini, 2001). The finding that nNOS is found in close proximity to, or within, chromaffin cells was confirmed in the present study by immunocytochemistry and extended further by demonstrating nNOS protein and mRNA in anterior PCV by western blotting and real-time PCR, respectively. As in mammals, it is likely that nNOS resides within the terminals of cholinergic nerve fibers (Holgert et al., 1995) from where it is co-released with acetylcholine during neuronal stimulation of catecholamine secretion.

Although nNOS has been identified in a range of fish tissues including kidney (Jimenez et al., 2001), head kidney (Gallo and Civinini, 2001), brain (Jadhao and Malz, 2003; Bordieri and Cioni, 2004; Bordieri et al., 2005) and gill (Ebbesson et al., 2005), there are comparatively few studies that have attempted to characterize eNOS expression in fish. Although there appears to be agreement that eNOS exists in gill tissue (Ebbesson et al., 2005; Zaccone et al., 2006), the results of studies examining cardiovascular tissues have yielded conflicting results. Thus, immunocytochemical experiments have provided evidence both for (Fritsche et al., 2000; Tota et al., 2005) and against (Jennings et al., 2004; Donald and Broughton, 2005) the presence of eNOS in the circulatory system. The results of the present study using immunocytochemistry and western blotting support the existence of eNOS in the trout cardiovascular system. Importantly, eNOS was localized to cells in close proximity to chromaffin tissue, thus suggesting its potential role in the modulation of catecholamine secretion. Although eNOS in the PCV is the predominant source of NO production in the PCV during acute hypoxia, the nNOS isoform would appear to be more important in generating NO during stimulus-evoked catecholamine secretion (McNeill and Perry, 2005). As in previous studies on fish (Fritsche et al., 2000; Tota et al., 2005; Ebbesson et al., 2005; Zaccone et al., 2006), a heterologous antibody was used to detect eNOS. Nevertheless, we are confident that the antibody was specifically recognizing trout eNOS based on the results of the control experiments that were performed. Thus, a single band of the correct molecular mass was obtained by western blotting, and pre-absorption of the antibody with blocking peptide eliminated the band. Unfortunately, we were unable to extend these studies to include measurements of eNOS mRNA levels because all

attempts to amplify trout eNOS cDNA using degenerate primers were unsuccessful.

Hypoxia, NOS and catecholamine secretion

In situ perfused preparations derived from hypoxia-treated fish displayed an increase in basal NO levels as well as increased production of NO in response to electrical stimulation. Moreover, there was a significant reduction in stimulus-evoked catecholamine secretion in preparations derived from hypoxic fish. *In vivo*, the levels of circulating NO during hypoxia increased on the final two days of the repetitive hypoxia protocol and this was associated with an attenuation of the adrenergic stress response on day 4. These results suggest that NOS is upregulated in the fish subjected to repeated hypoxia and that the net effect of the upregulation is inhibition of catecholamine secretion *in situ* and *in vivo*.

Through western blotting, it was shown that only nNOS protein was elevated and that this increase coincided with an increase in mRNA as demonstrated by real-time RT-PCR. These findings suggest that nNOS rather than eNOS is the predominant NOS isoform regulating catecholamine secretion in response to hypoxia. Because the results of a previous study (McNeill and Perry, 2005) eliminated the involvement of iNOS in regulating catecholamine secretion, no attempt was made to monitor its expression in the present experiments.

Conclusion

Catecholamine secretion in trout is controlled by a multitude of factors including neurotransmitters (Montpetit and Perry, 1999), neurohormones (Fritsche et al., 1993; McNeill et al., 2003) and hormones (Bernier et al., 1999). Recently, the gaseous signaling molecule NO was identified as an inhibitory modulator of stimulus-evoked catecholamine secretion in trout (McNeill and Perry, 2005). We propose that along with its role in regulating acute catecholamine secretion, NO is also involved in moderating catecholamine secretion during chronic or repetitive hypoxia owing to the increased expression and activity of nNOS.

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