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# Nitric oxide and the control of catecholamine secretion in rainbow trout *Oncorhynchus mykiss*

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## Summary

**An *in situ* saline-perfused posterior cardinal vein preparation was used to assess the role of nitric oxide (NO) in the regulation of basal and stimulus-evoked catecholamine secretion from rainbow trout *Oncorhynchus mykiss* chromaffin cells. Addition of the NO donor, sodium nitroprusside (SNP) to the inflowing perfusate abolished catecholamine secretion during electrical field stimulation, thereby establishing the potential for NO to act as a potent inhibitor of catecholamine release. A possible role for endogenously produced NO was established by demonstrating that stimulus-evoked (depolarizing levels of KCl or electrical field stimulation) catecholamine secretion was markedly stimulated in the presence of the nitric oxide synthase (NOS) inhibitors L-NAME and 7-NI. Although *in vitro* experiments demonstrated that catecholamine degradation was enhanced by NO in a dose-dependent manner, the dominant factor contributing to the reduction in catecholamine appearance in the perfusate was specific inhibition of catecholamine secretion. Subsequent experiments were performed to identify the NOS isoform(s) contributing to the inhibition of stimulus-evoked catecholamine secretion. Inducible NOS (iNOS; an enzyme that can be activated in the absence of $Ca^{2+}$), although present in the vicinity of the chromaffin cells (based on mRNA measurements), does not appear to play a role because stimulus-evoked NO production was eliminated during perfusion with $Ca^{2+}$-free saline. The potential involvement of endothelial NOS (eNOS) was revealed by showing that hypoxic perfusate evoked NO production and corresponded with an inhibition of stimulus-evoked catecholamine secretion; chemical removal of the endothelium (using saponin) prevented the production of NO during hypoxia. However, because removal of the endothelium did not affect NO production during electrical field stimulation, it would appear that the neuronal form of NOS (nNOS) is the key isoform modulating catecholamine secretion from trout chromaffin cells.**

Key words: adrenaline, noradrenaline, catecholamine, nitric oxide synthase, stress, chromaffin cell, rainbow trout, *Oncorhynchus mykiss*.

## Introduction

In teleost fish, the catecholamine hormones, adrenaline and noradrenaline, are synthesized, stored and released from the chromaffin cells that line the posterior cardinal vein (PCV; Nandi, 1967). In numerous species, circulating catecholamines are known to play crucial roles in the acute stress response by initiating pathways that diminish the often harmful effects that may accompany stress (Wendelaar Bonga, 1997). The beneficial effects of catecholamines are achieved, in part, by modulation of the cardiovascular and respiratory systems to maintain adequate levels of oxygen in the blood (Perry and Gilmour, 1999) as well as by mobilization of energy stores to match the increased metabolic demands that may be associated with stress (Fabbri et al., 1998; Reid et al., 1998).

The current model for catecholamine release incorporates a number of cholinergic and non-cholinergic neurotransmitters and/or neuromodulators that interact either directly or indirectly with the chromaffin cells to influence secretion (Reid et al., 1998). The primary mechanism of catecholamine secretion, as in other vertebrates, is believed to be cholinergic and involves the interaction of acetylcholine (ACh) with nicotinic or muscarinic receptors (Nilsson et al., 1976; Guo and Wakade, 1994). Non-cholinergic mechanisms of catecholamine secretion in fish include activation of the rennin–angiotensin system (RAS; Bernier and Perry, 1999), direct action of elevated levels of adrenocorticotropic hormone (ACTH; Reid et al., 1998) or serotonin (Fritsche et al., 1993), and neuronal release of vasoactive intestinal polypeptide (VIP) and/or pituitary adenylyl cyclase activating polypeptide (PACAP; Montpetit and Perry, 2000).

Nitric oxide (NO) is a relatively short lived, highly reactive gas molecule that was first recognized as an endothelium-derived relaxing factor (EDRF) implicated in blood vessel dilation (Moncada et al., 1989). Subsequently, NO has been identified as an endogenous mediator of numerous

physiological processes ranging from vascular regulation to immunological responses (Kuo et al., 2003; Mungrue et al., 2003). NO is produced in various tissues by the nitric oxide synthase (NOS) family of enzymes. Of the three isoforms of NOS, neuronal NOS (nNOS) has received the most attention as a potential modulator of catecholamine secretion. In mammals, nNOS is present in chromaffin cells (Schwarz et al., 1998; Oset-Gasque et al., 1994) as well as in cholinergic fibers (Bredt et al., 1990; Dun et al., 1992; Holgert et al., 1995), suggesting that NO may be released along with ACh (Marley et al., 1995). In rainbow trout *Oncorhynchus mykiss*, nNOS was localized in the head kidney tissue (Jimenez et al., 2001) but, unlike in mammals, it appears to be only sparsely present in chromaffin cells (Gallo and Civinini, 2001). Several studies have implicated nNOS in catecholamine regulation in mammals (Schwarz et al., 1998; Vicente et al., 2002; Barnes et al., 2001) whereas fewer studies have implicated endothelial NOS (eNOS) (Barnes et al., 2001; Torres et al., 1994); there is no evidence for a role for inducible NOS (iNOS).

To date, all previous studies investigating the role of NO on basal and stimulus-evoked catecholamine secretion from chromaffin cells have used mammalian systems. Results from these studies were obtained using cultured chromaffin cells (Torres et al., 1994; Oset-Gasque et al., 1994; Rodriguez-Pascual et al., 1995; Vicente et al., 2002) or perfused adrenal glands (Marley et al., 1995; Nagayama et al., 1998; Barnes et al., 2001). These prior studies have relied mainly on pharmacological approaches including the use of NO itself (Oset-Gasque et al., 1994), NO donors, SNP and/or SNAP (Marley et al., 1995; Schwarz et al., 1998) and/or NOS inhibitors (Torres et al., 1994; Nagayama et al., 1998; Schwarz et al., 1998; Barnes et al., 2001; Vicente et al., 2002). Surprisingly, there are no published studies that have incorporated simultaneous measurements of NO and catecholamine levels.

Previous research using rainbow trout has led to the development of a well-characterized *in situ* perfusion technique in which catecholamine secretion can be studied in whole animal preparations without major disturbances to the chromaffin tissue (Reid and Perry, 1995; Montpetit and Perry, 2000). This, along with a field stimulation technique which allows stimulation of the nerves that innervate the main population of chromaffin cells (Montpetit and Perry, 1999), forms a model with which *in vivo* catecholamine secretion can be simulated. The goal of the present study was to investigate the effects of NO on both basal and stimulus-evoked catecholamine secretion in rainbow trout using this model. Experiments incorporating simultaneous measurements of NO (estimated by analysis of nitrate and nitrite levels) and catecholamine levels were designed specifically to test the hypothesis that NO derived predominantly from nNOS is a negative modulator of catecholamine secretion.

## Materials and methods

### *Experimental animals*

Rainbow trout *Oncorhynchus mykiss* Walbaum of both sexes were obtained from Linwood Acres Trout farm (Campbellcroft, Ontario, Canada). The fish were held at the University of Ottawa in large fiberglass tanks supplied with flowing, aerated and dechloraminated city of Ottawa tapwater. The fish (mean mass 233±10.9 g; $N$=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 2 weeks prior to experimentation.

### In situ *saline-perfused posterior cardinal vein 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 brass 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, VWR International, Mississuaga, ON, Canada) was inserted into the posterior cardinal vein (PCV) in the mid kidney area (~10 cm posterior to the heart) and an outflow cannula (PE 160) was inserted into the ventricle through the bulbus arteriosus. Prior to beginning 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 $CaCl_2$, and 1.25 mmol $l^{-1}$ $KH_2PO_4$, final pH 7.8) to allow catecholamine levels to stabilize (Julio et al., 1998). Preliminary experiments established that NO levels in the outflowing perfusate were also stable after 20 min. 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. In the control group perfusion with saline was continued, whereas in the experimental group, perfusion media were switched rapidly using a three-way valve. Perfusion media were identical except for the addition of specific antagonists, or NO donors. In other experiments, the preparation either received a bolus injection of an agonist *via* a three-way valve fitted to the infusion line or was electrically stimulated for 2 min using a previously validated field stimulation technique (Montpetit and Perry, 1999). Although the stimulation voltages and frequencies varied between experiments (see below), the pulse duration was kept constant at 1 ms.

During the experimental procedure, the perfusate was collected continuously for 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.

*Series 1: Assessing the potential for NO to modulate catecholamine secretion*

Following the collection of pre-samples, the preparations were administered unmodified control saline or saline containing the NO donor sodium nitroprusside (SNP; $5\times10^{-3}$ mol $l^{-1}$). Samples were collected for 6 min, at which point the preparations were electrically stimulated at 60 V at a frequency of 20 Hz.

*Series 2: Catecholamine secretion and NO production during non-specific chromaffin cell depolarization*

A previous study by Mendizabal et al. (2000) showed that a depolarizing level of KCl was able to elicit NO production and that this production could be inhibited using a NOS inhibitor. To determine if KCl-induced NO production could be inhibited in the present study, a cocktail containing the NOS inhibitors 7-nitroindazole (7-NI; $10^{-4}$ mol $l^{-1}$) and *N*-nitro L-arginine methyl ester (L-NAME; $5\times10^{-3}$ mol $l^{-1}$) was used. Preparations were either perfused with saline containing the combination of the inhibitors or with control saline. While L-NAME could be added directly to saline, 7-NI was prepared in methanol prior to the 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. Following the collection of pre-samples, preparations received a bolus injection of 10 mmol $l^{-1}$ KCl (1 ml $kg^{-1}$).

*Series 3: Catecholamine secretion and NO production during electrical field stimulation*

*In situ* preparations were continuously perfused with saline for 20 min, at which point the pre-samples were collected. The preparations were then stimulated at 30 V at either 1, 8 or 20 Hz.

To confirm the role of NOS in the generation of NO, preparations were perfused for the entire experiment with a cocktail of the NOS inhibitors, 7-NI and L-NAME as described above. Following the pre-sample collections, preparations received an electrical stimulus of 30 V and 8 Hz.

*Series 4: Assessing the mechanisms of NO*

*Increased catecholamine degradation* vs *decreased catecholamine secretion*

To determine the effect of NO on catecholamine degradation, noradrenaline and adrenaline ($5\times10^{-7}$ mol $l^{-1}$) prepared in 0.1 mol $l^{-1}$ HCl were incubated separately in freshly prepared saline containing a range of SNP concentrations ($10^{-8}$–$10^{-3}$ mol $l^{-1}$). 0.5 ml of SNP solution was added to 0.5 ml of catecholamine solution and incubated for 5 min in a glass test tube. Reaction was stopped by the addition of 0.5 ml of 0.1 mol $l^{-1}$ perchloric acid and 0.1% cysteine and the solutions were then placed on ice. Catecholamines were extracted and analyzed by high pressure liquid chromatography (HPLC), while a colorimetric assay was used to measure NO levels.

To distinguish between the effects of NO on catecholamine degradation *vs* cellular catecholamine secretion, *per se*, experiments were performed in which the cellular effects of NO, at least those known to influence catecholamine secretion, were blocked. Experimental preparations received saline containing the selective guanylyl cyclase (sGC) inhibitor, 1H-(1,2,4)oxadiazole(4,3-alpha)quinoxaline-1-one (ODQ; $10^{-5}$ mol $l^{-1}$) for the entire experiment while controls received saline. Pre-samples were collected and the preparations were stimulated at 30 V and 8 Hz.

*Series 5: Assessing the potential roles of the three NOS isoforms*

To assess whether iNOS could potentially contribute to NO generation during electrical stimulation of the chromaffin cells, experiments were performed to localize iNOS mRNA to the PCV and anterior kidney, regions known to contain high concentrations of chromaffin cells.

*Tissue collection and RNA extraction*

Fish were killed by a sharp blow to the head and tissues (brain, PCV and kidney) were collected and frozen immediately in liquid $N_2$ and stored at –80°C. Total RNA was extracted using Stratagene Absolute RNA RT-PCR miniprep kit (Stratagene, Cedar Creek, TX, USA) according to the instructions of the manufacturer. RNA concentrations were verified using spectrophotometry (Eppendorf BioPhotometer, VWR International).

*cDNA synthesis and mRNA assessment*

cDNA was synthesized from 5 μg total RNA using StrataScript reverse transcriptase (Stratagene) and random hexamer primers. iNOS mRNA levels were assessed by real-time PCR on duplicate samples of cDNA using Brilliant® SYBR® Green 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. Gene-specific primers for rainbow trout iNOS (AJ295230) and β-actin (AF550583) were designed using DNAMAN (version 4.0, Lynnon Biosoft, Vaudreuil-Dorion, Quebec, Canada) from the cDNA sequences obtained from GenBank. Relative expression of mRNA levels was determined (using actin as a standard) using the delta-delta Ct method (Pfaffl, 2001). For iNOS, the forward primer 5′-GAAGTGCAGAGGTCA-3′ was used with the reverse primer 5′-GGTATTCCAGTCGTAGGCA-3′ to yield a 134 bp product. The cycle threshold (Ct) values for actin varied little between the tissues examined (Ct=19.2±0.05 (mean ± S.E.M.; *N*=45) so it is unlikely that the data were skewed by using actin (rather than ribosomal RNA) as a standard.

To evaluate the extent of NO production attributable to iNOS activation during electrical stimulation, eNOS and nNOS induction were prevented by perfusing with $Ca^{2+}$-free saline containing the $Ca^{2+}$ chelator, EGTA (1 mmol $l^{-1}$) for 20 min prior to the collection of pre-samples; control samples were perfused with normal saline. Following the collection of the

pre-samples, both groups were electrically stimulated at 30 V and 8 Hz.

Hypoxia specifically induces eNOS to produce NO (Yamamoto et al., 2003). Thus, experiments were performed to evaluate whether hypoxia could directly affect NO production and if so, whether the NO produced during hypoxia could regulate basal and stimulus-evoked catecholamine secretion. Following the collection of the two pre-samples, fish were perfused with saline bubbled with $N_2$ to render the saline hypoxic. The $P_{O_2}$ of the hypoxic saline solution was measured using a Foxy-AL300 fiber-optic probe and associated hardware and software (Ocean Optics, Dunedin, FL, USA). In all cases, the $P_{O_2}$ of the saline was allowed to fall to 10 mmHg prior to use. Control preparations continued to receive normoxic saline following the collection of the pre-samples. Both groups were perfused for 10 min following the collection of pre-samples, with samples collected every 2 min over that time. Both groups were then electrically stimulated at 30 V and 8 Hz.

Subsequent experiments were performed to determine if the combined presence of both NOS inhibitors could inhibit hypoxia-induced NO production from eNOS. Preparations were perfused with either 7-NI ($1\times10^{-4}$ mol $l^{-1}$) and L-NAME ($5\times10^{-3}$ mol $l^{-1}$), or regular saline. Following the collection of the pre-samples, fish were switched to hypoxic saline ($P_{O_2}$<10 mmHg). The same protocol was performed on the control group, except that they were not treated with inhibitors. The preparations were perfused with hypoxic saline for 10 min, with saline being collected over 2 min intervals, after which they received an electrical stimulus of 30 V and 8 Hz.

To further assess the role of eNOS, the endothelium was removed by perfusion of the PCV with saponin as previously described (Donoso et al., 1996; Cortes et al., 1999). In brief, following establishment of perfusion flow, one group of fish received a bolus injection of 0.1% saponin for 60 s, while two groups received a saline injection. Following the collection of the pre-samples, a saline-injected group and the saponin-treated group were rapidly switched to hypoxic saline ($P_{O_2}$<10 mmHg, as described above), while the remaining group continued to receive control saline. All preparations were perfused for an additional 10 min, with collections every 2 min. The preparations were then electrically stimulated at 30 V and 8 Hz.

### *Analytical procedures*

#### *Catecholamine determination*

Catecholamine levels in perfusate were determined on alumina-extracted samples (100 µl) using 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.

#### *Nitric oxide assay*

Quantification of NO is problematic because of its short lifetime. Therefore, NO production was evaluated indirectly by measuring the concentration of nitrite and nitrate, stable metabolites of NO in biological fluids. This method demonstrates high accuracy and reproducibility and adequately reflects actual NO production (Gilliam et al., 1993; Manukhina et al., 1999). The NO assay was performed as described by Gilliam et al. (1994), with modifications. In short, a stock solution of magnesium nitrate (Sigma) was prepared in saline at a final concentration of 1 mmol $l^{-1}$. 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*; E.C.1.6.6.2; Sigma). The plate was sealed, placed in the dark and incubated at room temperature (~21°C) for 45 min. 40 µl of Griess reagents I and II (Cayman Chemicals, 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, Sunnyvale, CA, USA) micro-plate reader at a wavelength of 540 nmol $l^{-1}$.

### *Statistical analysis*

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).

### *Data presentation*

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 were performed, and all showed similar trends within each experiment. Therefore, for clarity, only the statistical analysis of total catecholamine secretion rates are presented on 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: Assessing the potential for NO to affect catecholamine secretion*

Basal noradrenaline, adrenaline and total catecholamine secretion rates were unaffected in the presence of the NO donor SNP. Upon electrical stimulation, the control preparations showed an ~12-fold increase in noradrenaline, sevenfold increase in adrenaline and an 8-fold increase in total catecholamine secretion rates over the pre values (Fig. 1A). The preparations receiving SNP displayed a 14-fold increase in NO levels in the perfusate; these preparations were unresponsive to the electrical stimulus. In control preparations, electrical stimulation evoked a threefold increase in perfusate NO levels (Fig. 1B).

### *Series 2: Catecholamine secretion and NO production during non-specific chromaffin cell depolarization*

The addition of 10 mmol $l^{-1}$ KCl to the perfusate caused significant increases in catecholamine and NO secretion (Fig. 2). However, in the presence of the NOS inhibitors, 7-NI and L-NAME, the increase in NO was prevented and there was a significantly greater increase in catecholamine secretion (Fig. 2A). The inhibitor combination reduced KCl-induced NO secretion by approximately 48% (Fig. 2B), while increasing catecholamine secretion rates by 2.3-fold (Fig. 2A).

### *Series 3: Catecholamine secretion and NO production during electrical field stimulation*

The frequency dependency of the catecholamine secretion response to electrical stimulation (30 V) is depicted in Fig. 3A. Catecholamine secretion was lowest at 1 Hz (~sixfold increase), intermediate at 8 Hz (~eightfold increase) and greatest at 20 Hz (~28-fold increase). The highest NO production in response to electrical stimulation was observed at intermediate frequency (8 Hz) with an ~ninefold increase in NO levels when compared to the pre-value (Fig. 3B). Low frequency (1 Hz) stimulation also evoked a significant response (fivefold increase), whereas high frequency (20 Hz) stimulation failed to elicit a NO response (Fig. 3B). All subsequent experiments were performed using 30 V and 8 Hz.

Upon electrical stimulation, preparations treated with the combination of 7-NI and L-NAME showed an ~twofold increase in adrenaline and total catecholamine secretion rates when compared to controls (Fig. 4A). Noradrenaline levels were not different between the two groups (Fig. 4A). Concurrently, the preparations treated with 7-NI and L-NAME showed an approximate 50% decrease in NO production (Fig. 4B).

### *Series 4: Assessing the mechanisms of NO*

*Increased catecholamine degradation* vs *decreased catecholamine secretion*

*In vitro*, total catecholamine levels exhibited a dose-dependent decrease in concentration in response to SNP. The extent of catecholamine degradation as a function of SNP concentration is depicted in Fig. 5. Percentage degradation was calculated by comparing the catecholamine levels between the sample containing SNP and the saline control. The extent of degradation was maximal at $10^{-3}$ mol $l^{-1}$ SNP and therefore was set to 100%.

ODQ treatment resulted in ~8-, 5.6- and a 5.4-fold increases in noradrenaline, adrenaline and total catecholamine secretion rates, respectively, when compared to saline treated fish (Fig. 6A). NO secretion rates for ODQ treated preparations showed an ~1.5-fold increase over saline treated fish (Fig. 6B).

### *Series 5: Assessing the potential roles of the three NOS isoforms*

Fig. 7 illustrates that mRNA for the iNOS isoform is present in tissues in close proximity to the chromaffin cells associated with the posterior cardinal vein (PCV). The anterior kidney showed the highest relative mRNA levels, while the PCV showed the lowest relative mRNA levels.

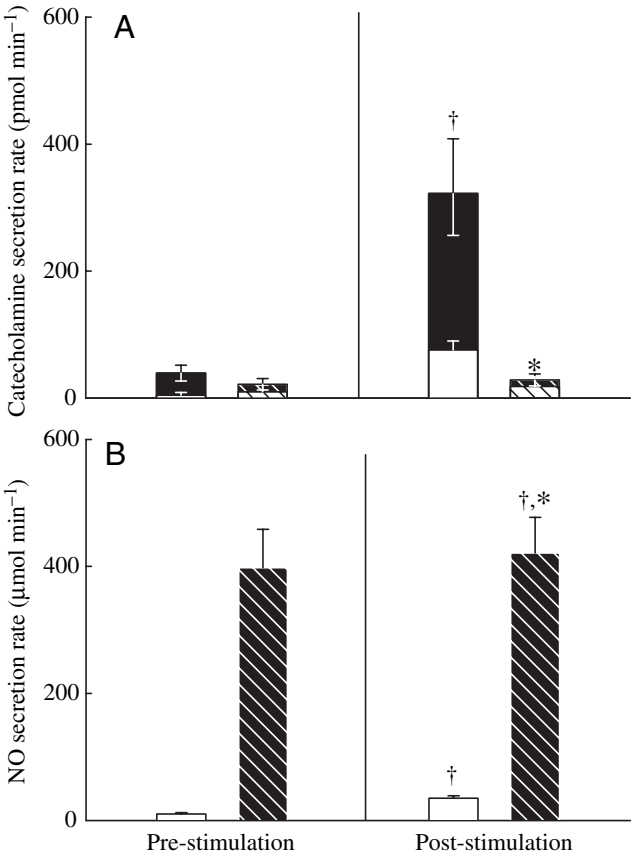


Fig. 1. The effects of the NO donor SNP ($5\times10^{-3}$ mol $l^{-1}$; cross-hatched bars; *N*=6) on (A) noradrenaline (unfilled component of bars), adrenaline (filled component of bars) and total catecholamine (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion rates over a 10 min period in response to electrical stimulation (60 V, 20 Hz) of a perfused posterior cardinal vein preparation of rainbow trout. Values are means ± 1 S.E.M. A dagger denotes a significant difference ($P$<0.5) between pre-stimulated and stimulated samples. An asterisk denotes a significant difference ($P$<0.5) between the control (*N*=6) and the SNP treated group.

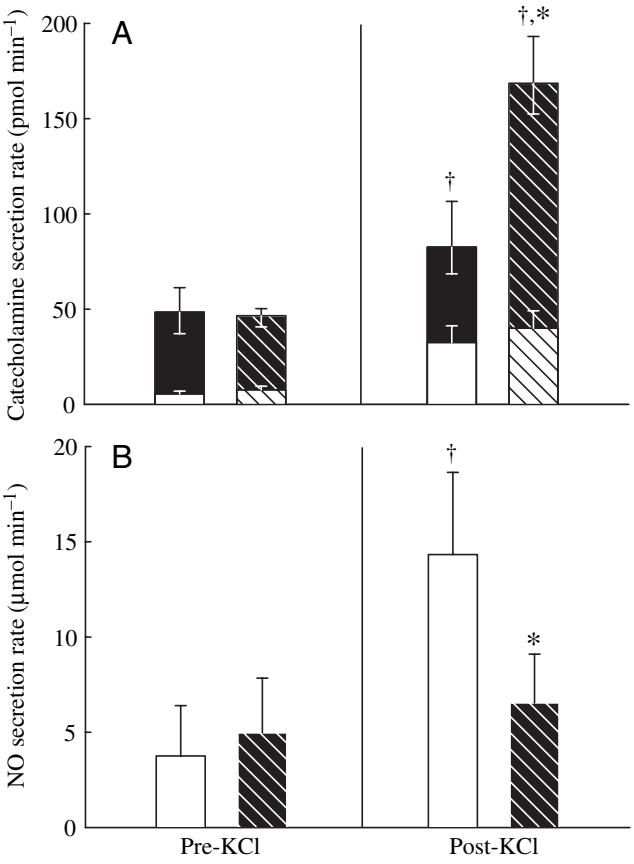


Fig. 2. The effects of nitric oxide synthase (NOS) inhibitors on (A) noradrenaline (unfilled bars), adrenaline (filled bars) and total catecholamine (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion rates over a 10 min period in response to a bolus injection of 10 mmol $l^{-1}$ KCl in a perfused posterior cardinal vein preparation of rainbow trout. Preparations were either pre-treated with saline ($N$=6) or with saline containing the combination of the NOS inhibitors 7-NI ($10^{-4}$ mol $l^{-1}$) and L-NAME ($5\times10^{-3}$ mol $l^{-1}$; cross-hatched bars; $N$=6). Values are means ± 1 S.E.M. A dagger denotes a significant difference ($P$<0.5) between pre-KCl and post-KCl samples. An asterisk denotes a significant difference ($P$<0.5) between the control and the inhibitor treated group (cross-hatched).

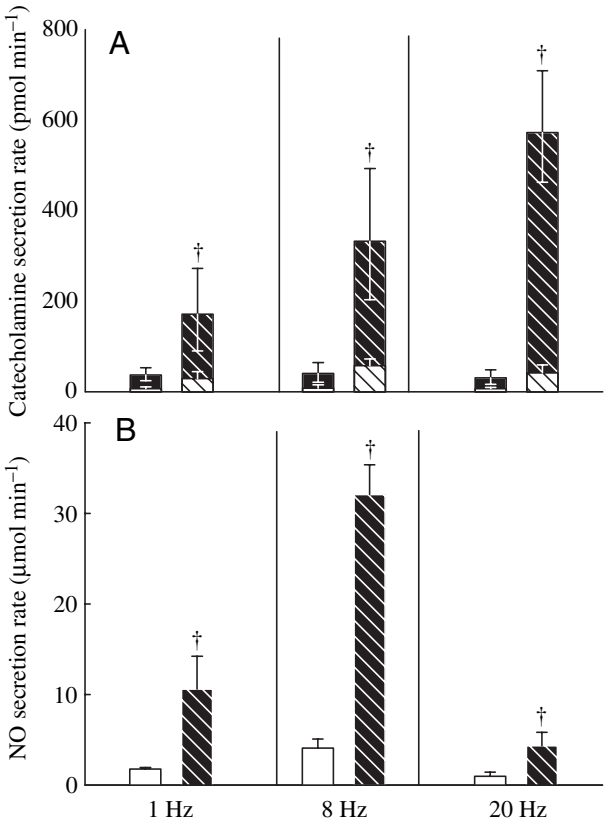


Fig. 3. The effects of field stimulation for 2 min at 30 V and either 1 Hz ($N$=5), 8 Hz ($N$=14) or 20 Hz ($N$=8) on (A) noradrenaline (unfilled component of bars), adrenaline (filled component of bars) and total catecholamine (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion rates in a perfused posterior cardinal vein preparation of rainbow rout. Values are means ± 1 S.E.M. A dagger denotes a significant difference ($P$<0.5) between pre-stimulated and stimulated samples (cross-hatched bars).

Perfusion with $Ca^{2+}$-free saline was used as a tool to specifically prevent the activation of iNOS and nNOS during electrical stimulation. The control preparations responded to electrical stimulation with an ~11-fold increase in total catecholamine secretion and a fivefold increase in NO production. Preparations perfused with $Ca^{2+}$-free saline did not exhibit an increase in catecholamine secretion (Fig. 8A) or NO production (Fig. 8B) in response to electrical stimulation.

Perfusion with hypoxic saline was used as a tool to activate eNOS. Preparations perfused with hypoxic saline showed no difference in basal catecholamine secretion (Fig. 9A). In response to hypoxia, the preparations with an intact endothelium showed an ~fivefold increase in NO levels whereas the saponin treated group was unaffected; levels of NO remained constant in the normoxic control group (data not shown). In response to electrical stimulation, the control preparations subjected to hypoxia showed an ~41% decrease in total catecholamine secretion rates in comparison to normoxic preparations (340.9±139.5 *vs* 829.4±159.1 pmol $min^{-1}$). This inhibitory effect of hypoxia on electrically evoked catecholamine secretion was prevented by the addition of saponin to the perfusate (Fig. 9A). After electrical stimulation, NO secretion rates were similar in all treatment groups.

## Discussion

In agreement with some previous studies on mammals (Oset-Gasque et al., 1994; Torres et al., 1994; Schwarz et al., 1998; Nagayama et al., 1998), the current results clearly indicate that elevated NO levels are able to inhibit stimulus-evoked catecholamine secretion from chromaffin cells of a lower vertebrate, the rainbow trout *Oncorhynchus mykiss*. The NO donor, SNP, completely inhibited the stimulus-evoked secretion of catecholamines. However, as in mammals

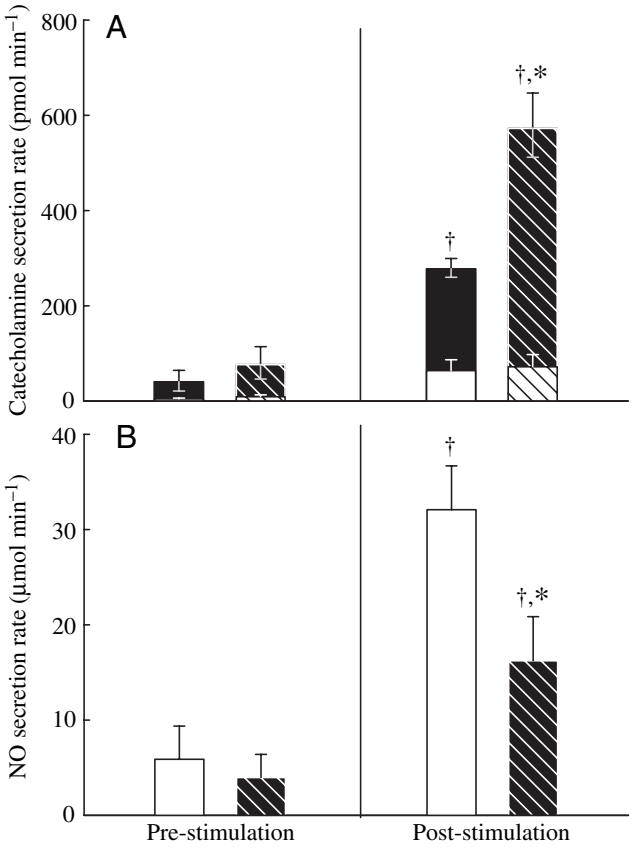


Fig. 4. The effects of nitric oxide synthase (NOS) inhibition (using a cocktail of 7-NI and L-NAME; *N*=9; cross-hatched bars) on (A) noradrenaline (unfilled component of bars), adrenaline (filled component of bars) and total catecholamine secretion (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion in response to electrical stimulation (30 V, 8 Hz) of a perfused posterior cardinal vein preparation of rainbow trout. Values are means ± 1 S.E.M. A dagger denotes a significant difference ($P$<0.5) between pre-stimulated and stimulated groups. An asterisk denotes a significant difference ($P$<0.5) between the control ($N$=6) and inhibitor treated group (cross-hatched).

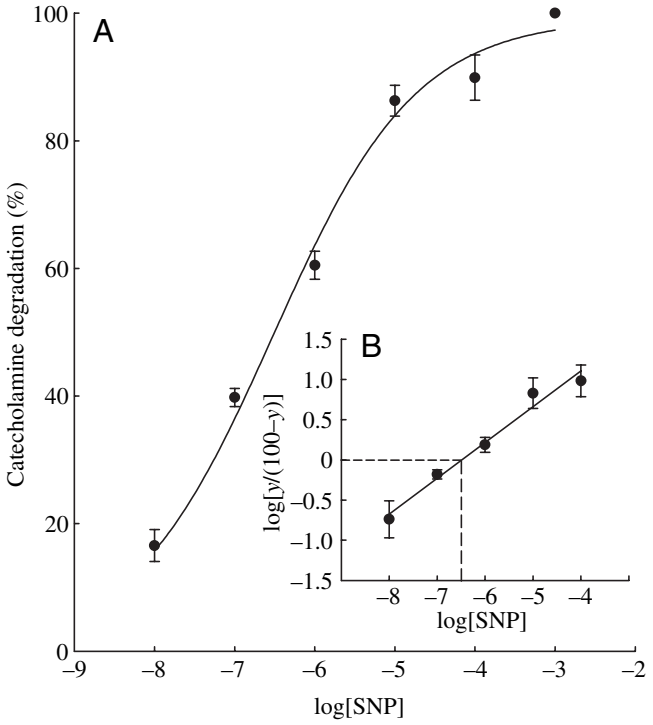


Fig. 5. The dose-dependent effects of sodium nitroprusside (SNP) on catecholamine degradation *in vitro*. (A) The sigmoidal dose–response curve depicting catecholamine degradation over a 5 min period as a function of SNP concentration was drawn using iterative curve-fitting software (Sigmaplot). (B) The data constituting the dose–response curve in A was transformed to generate the Hill plot. The following linear regression was calculated: $y$=0.45$x$+2.89; $r^2$=0.97. The $EC_{50}$ for the Hill plot was calculated to be $2.63\times10^{-7}$ mol l$^{-1}$.

(Rodriguez-Pascual et al., 1995; Marley et al., 1995), a rise in NO levels does not appear to influence basal catecholamine secretion. As a first approach to determine if endogenously produced NO could influence stimulus evoked catecholamine secretion, a cocktail of NOS inhibitors (L-NAME and 7-NI) was used in the presence of depolarizing levels of KCl. The data clearly demonstrated that KCl-induced catecholamine secretion was accompanied by a significant increase in NO production that was prevented during inhibition of NOS. Because catecholamine secretion was markedly increased in the absence of NO production, it would suggest that endogenously produced NO acts to inhibit catecholamine secretion during chromaffin cell stimulation.

*Mechanism of inhibition of catecholamine secretion by NO*

This study addressed two possible mechanisms to explain the reduction of catecholamine appearance in the perfusate in the presence of NO. The possibilities tested were an effect of NO on reducing catecholamine stability after their secretion from chromaffin cells and/or specific inhibition of catecholamines secretion caused by intracellular signaling events linked to activation of sGC. In concurrence with the study of Kolo et al. (2004) the results of the *in vitro* experiments clearly demonstrate that NO has the capability to rapidly degrade catecholamines. It has been suggested that the underlying explanation for the effect of NO on catecholamine degradation involves the conversion of catecholamines by NO to their 6-nitro derivatives (Kolo et al., 2004).

In mammals, studies suggest that NO inhibits catecholamine secretion by promoting a cascade of events beginning with activation of sGC and leading to phosphorylation of $Ca^{2+}$ channels and an attenuation of the inward $Ca^{2+}$ flux in response to stimulation (Schwarz et al., 1998; Ferrero et al., 2000; Hirooka et al., 2000; Vicente et al., 2002). In the present study, the sGC inhibitor, ODQ, was used to prevent $Ca^{2+}$ channel phosphorylation during electrical stimulation. In the presence of ODQ, there was a pronounced increase in stimulus-evoked catecholamine secretion, suggesting that the activation of sGC and the downstream events are important factors leading to the decrease in catecholamine secretion. Of the two mechanisms leading to the NO-induced decrease in catecholamine outflow

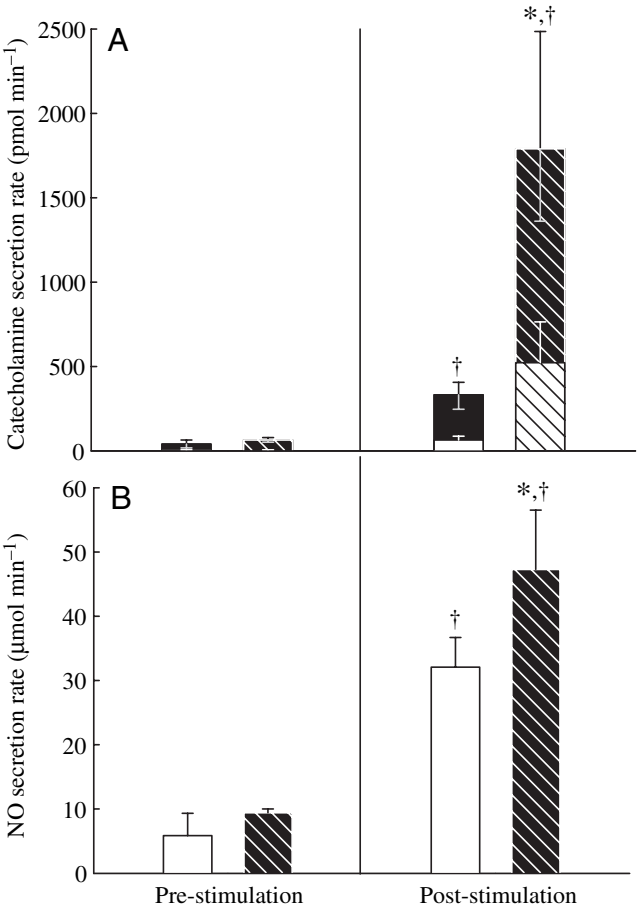


Fig. 6. The effects of the sGC inhibitor ODQ ($10^{-4}$ mol $l^{-1}$; cross-hatched bars; *N*=6) on (A) noradrenaline (unfilled component of bars), adrenaline (filled component of bars) and total catecholamine (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion rates in response to field stimulation of 30 V and 8 Hz. Values are means ± 1 S.E.M. A dagger denotes a significant difference between pre-stimulation and stimulation. An asterisk denotes a significant difference between the control (*N*=6) and ODQ (cross-hatched bars) treated group.

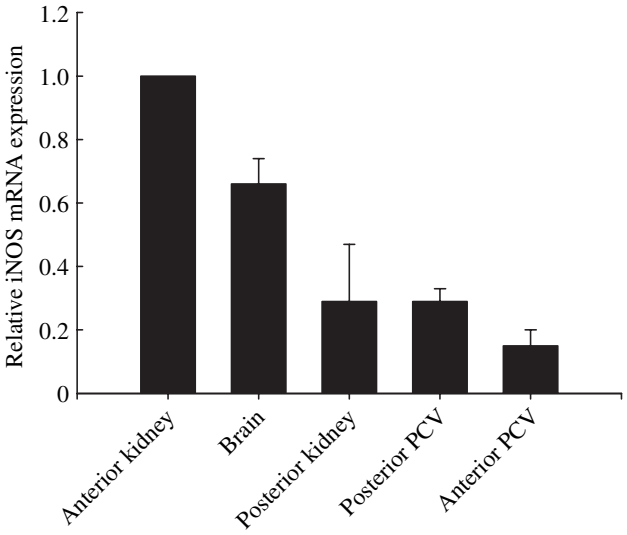


Fig. 7. Real-time PCR results showing the expression of rainbow trout iNOS mRNA relative to anterior kidney. The highest expression of this NOS isoform was detected in the anterior kidney and the lowest in the anterior posterior cardinal vein (PCV). Values are means ± 1 S.E.M.; *N*=6.

from the perfused PCV preparation, the activation of sGC would appear to be the primary mechanism. In the presence of ODQ, NO levels were elevated and catecholamine secretion was significantly increased above control levels. If the effect of NO on accelerating catecholamine degradation was the predominant factor, one would have expected to observe a decrease in catecholamine outflow during this experiment.

### *NO production is frequency dependent*

The predominant mechanism causing catecholamine secretion release from vertebrate chromaffin cells is the activation of nicotinic receptors by ACh released from pre-ganglionic sympathetic nerve fibers (Montpetit and Perry, 1999; Carrasco and Van de Kar, 2003). There are two main pathways that could lead to the increased production of NO and its subsequent regulation of catecholamine secretion. One involves the interaction of ACh with the cholinergic receptors. For example, Moro et al. (1993) showed that cholinergic receptor (nicotinic or muscarinic) stimulation was accompanied by an increase in cGMP levels in the chromaffin cells. Because the interaction of ACh with the cholinergic receptor results in an increase in intracellular [$Ca^{2+}$], $Ca^{2+}$-dependent NOS enzymes could be activated, resulting in an increased production of NO within chromaffin cells.

Another possibility involves the release of NO from the pre-ganglionic sympathetic nerve fibers. Previous studies have demonstrated that the specific type of neurotransmitter (e.g. ACh *vs* VIP) released during electrical stimulation of these fibers is related to the action potential frequency (Montpetit and Perry, 2000; McNeill et al., 2003). Because NOS has been identified in the pre-ganglionic nerve fibers (Bredt et al., 1990; Dun et al., 1993) and NO production during electrical stimulation is frequency dependent (Fig. 3B), it would appear that a similar situation may exist for NO production and release. Interestingly, the frequency dependency of NO production was markedly different than the frequency dependency of catecholamine secretion. Catecholamine secretion increased linearly with increasing frequencies whereas NO production appeared to peak at an intermediate frequency (8 Hz) and was absent entirely at the highest frequency (20 Hz). The lack of a tight correlation between NO production and catecholamine secretion is consistent with the view that there are numerous mechanisms acting in concert to regulate catecholamine secretion, all or some of which may be frequency dependent.

### *NOS isoforms*

On the basis of previous studies on mammals, nNOS is believed to be the main isoform regulating of catecholamine secretion (Schwarz et al., 1998; Vicente et al., 2002). However,

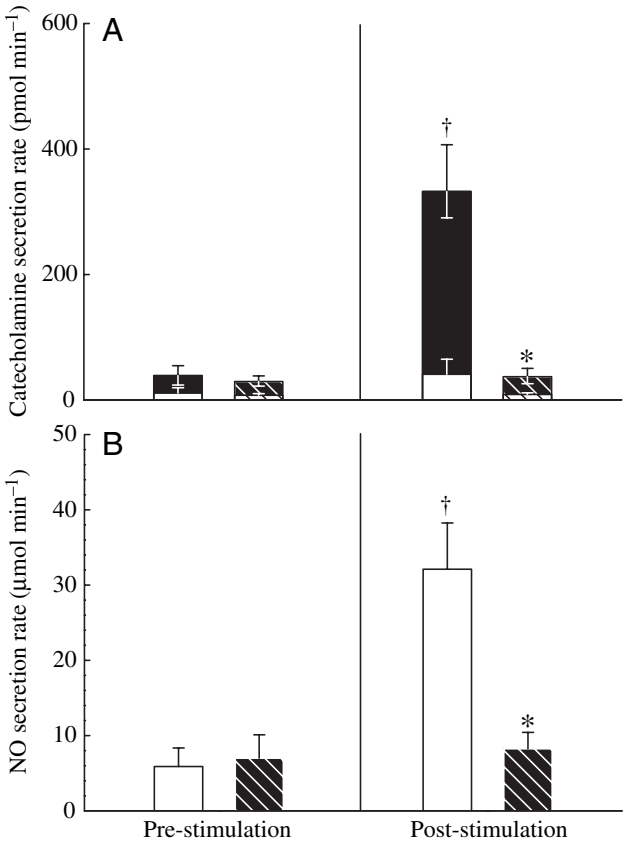


Fig. 8. The effects of calcium free saline (*N*=6; cross-hatched bars) on *in situ* (A) noradrenaline (filled bar), adrenaline (unfilled bar) and total catecholamine (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion rates in response to electrical stimulation at 30 V and 8 Hz. Values are means ± 1 S.E.M. A dagger denotes a significant difference ($P$<0.5) between pre-stimulated and stimulated groups. An asterisk denotes a significant difference ($P$<0.5) between the control (*N*=6) and the $Ca^{2+}$-free treated group (cross-hatched).

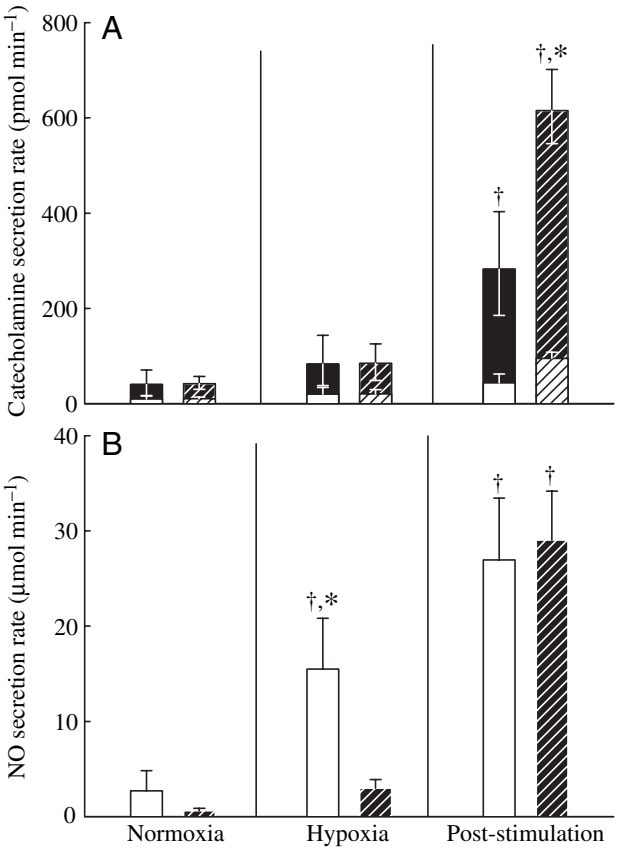


Fig. 9. The effects of hypoxia followed by electrical stimulation (30 V and 8 Hz) on (A) noradrenaline (unfilled bars), adrenaline (filled bars) and total catecholamine (sum of noradrenaline + adrenaline) and (B) nitric oxide (NO) secretion rates in untreated preparations (*N*=6; no cross-hatching) and in preparation treated with saponin (*N*=6; cross-hatched bars). Values are means ± 1 S.E.M. A dagger denotes a significant effect ($P$<0.5) of hypoxia or electrical stimulation. An asterisk denotes a significant difference ($P$<0.5) between the control and the saponin-treated group.

there is also evidence implicating eNOS (Torres et al., 1994; Barnes et al., 2001). A number of NOS inhibitors have been identified and used as experimental tools to investigate the biological significance of NO (Bland-Ward and Moore, 1995). The use of specific inhibitors in differentiating the contribution of the different isoforms in the production of NO is proving to be difficult. In mammals, the NOS homodimers show high homology between isoforms. In humans, the overall amino acid sequence identity is ~55%, with particularly strong sequence conservation in regions of the proteins involved in catalysis (Michel and Feron, 1997). For these reasons, the production of selective NOS inhibitors has been difficult. Currently, most inhibitors show a lack of selectivity on isolated enzymes (Moncada et al., 1997; Mayer and Andrew, 1998). Not surprisingly, therefore, 7-NI, a compound often used as a selective nNOS inhibitor (Barnes et al., 2001; Xu et al., 2001), was shown to inhibit the other isoforms with equal potency (Bland-Ward and Moore, 1995; Dick and Lefebvre, 1997; Moncada et al., 1997). For this reason, the use of 'selective' NOS inhibitors may not be an appropriate method to assess the involvement of the different isoforms. Therefore, in the current study, we used several alternative approaches to assess the contributions of the various NOS isoforms to the regulation of catecholamine secretion.

On the basis of its mRNA expression, iNOS is apparently present in tissues known to contain high densities of chromaffin cells (including the posterior cardinal vein and head kidney) and thus could potentially contribute to the regulation of catecholamine secretion. Unlike eNOS and nNOS, iNOS does not require an elevation of intracellular [$Ca^{2+}$] for its activation because of its high binding affinity for calmodulin (Oset-Gasque et al., 1994). Therefore, under $Ca^{2+}$-free conditions it is expected that only the iNOS isoform would be activated during electrical stimulation. Because NO was not elevated under $Ca^{2+}$-free conditions (Fig. 8) it would appear that iNOS does not contribute to NO production during electrical stimulation. The fact that catecholamines were not released in preparations perfused with $Ca^{2+}$-free saline is consistent with previous studies (Burgoyne, 1991). Based on

these results, it would appear that during electrical stimulation, the two NOS isoforms that are potentially regulating catecholamine secretion are nNOS and/or eNOS.

In an attempt to differentiate between nNOS and eNOS, experiments were performed without further use of inhibitors. Yamamoto et al. (2003) demonstrated that hypoxia treatment is able to induce NO production from eNOS in blood vessels. Similar findings were obtained in the present study, and moreover the production of NO during hypoxia was associated with a marked decrease in stimulus-evoked catecholamine secretion. Several previous studies have demonstrated that saponin treatment destroys the vascular endothelium (Donoso et al., 1996; Cortes et al., 1999) and theoretically this would eliminate the contribution of eNOS to NO production. Because saponin was able to prevent the increase in NO production in response to hypoxia treatment, it suggests that the sole source of NO production during hypoxia was *via* eNOS. Thus, the results of these experiments suggest a possible role for eNOS in regulating catecholamine secretion during hypoxia. However because saponin had no effect on stimulus-evoked NO production, it is apparent that nNOS rather than eNOS is the principal producer of NO during electrical stimulation of trout chromaffin cells.

### *Conclusion*

This is the first study to assess the role of NO in the regulation of catecholamine secretion in a non-mammalian vertebrate. Using the rainbow trout *Oncorhynchus mykiss* as a model, the results demonstrate that NO, produced during stimulation of chromaffin cells, is able to inhibit catecholamine secretion, confirming the results of previous studies on mammals (Oset-Gasque et al., 1994; Torres et al., 1994; Schwarz et al., 1998; Nagayama et al., 1998). The results suggest that of the three NOS isoforms potentially contributing to NO production and catecholamine regulation, nNOS is likely to be most important, although eNOS may play an important role during hypoxia.

## List of abbreviations

| | |
|---|---|
| 7-NI | 7-nitroindazole |
| ACh | acetylcholine |
| ACTH | adrenocorticotropic hormone |
| Ct | cycle threshold |
| DHBA | 3,4-dihydroxybenzalamine hydrobromide |
| EDRF | endothelium-derived relaxing factor |
| eNOS | endothelial NOS |
| HPLC | high-pressure liquid chromatography |
| iNOS | inducible NOS |
| L-NAME | *N*-nitro L-arginine methyl ester |
| nNOS | neuronal NOS |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| ODQ | 1H-(1,2,4)oxadiazole(4,3-alpha)quinoxaline-1-one |
| PACAP | pituitary adenylyl cyclase activating polypeptide |
| PCR | polymerase chain reaction |
| PCV | posterior cardinal vein |
| RAS | rennin–angiotensin system |
| sGC | selective guanylyl cyclase inhibitor |
| SNP | sodium nitroprusside |
| VIP | vasoactive intestinal polypeptide |

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