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Nitric Oxide and the Control of Catecholamine Secretion in Rainbow Trout (*Oncorhynchus mykiss)*

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NITRIC OXIDE AND THE CONTROL OF CATECHOLAMINE SECRETION IN RAINBOW TROUT (*Oncorhynchus mykiss*)

By

Brian McNeill

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Abstract

The aim of this thesis was to investigate the effects of nitric oxide (NO) on both basal and stimulus-evoked catecholamine secretion in rainbow trout (Oncorhynchus mykiss). Experiments were performed *in vivo* and *in situ* using an electrical field stimulation technique.

Catecholamine secretion rates *in situ* were markedly reduced in response to electrical stimulation in the presence of the NO donor sodium nitroprusside (SNP; 5 X 10^{-3} M). This reduction was through the activation of soluble guanylyl cyclase (sGC) rather than via an acceleration of catecholamine degradation. Pre-treatment with the NO inhibitors 7-nitroindazole (10^{-4} M) and L-NAME (5 X 10^{-3} M) decreased NO production in response to electrical stimulation while increasing catecholamine secretion rates approximately 2-fold. Experimental evidence suggests that nNOS is the main isoform involved in the inhibition of catecholamine secretion.

Following the repeated exposure of rainbow trout to hypoxia over 4 days *in vivo*, *in situ* preparations derived from these fish showed an approximately 2- and 1.8-fold increase in basal and stimulated NO levels, respectively, while catecholamine secretion rates were reduced by approximately 50%. Expression levels for the nNOS isoform in the head kidney and anterior posterior cardinal vein (PCV) suggests that this isoform is involved in the catecholamine response.

In summary, nNOS appears to be the main isoform in producing NO under neuronal stimulation and that this NO decreases stimulus-evoked catecholamine secretion through the activation of sGC.

Résumé

Le but de cette thèse était d'étudier les effets de l'oxyde nitrique (ON) sur la sécrétion basique et évoquée de catécholamine en truite arc-en-ciel (*Oncorhynchus mykiss*). Des expériences ont été exécutées *in situ* en utilisant une technique de stimulation de champ électrique et *in vivo* employer des expositions répété d'hypoxie.

Des taux de sécrétion de catécholamine *in situ* ont été nettement réduits en réponse à la stimulation électrique en presence de nitroprusside de sodium (SNP; 5 X 10^{-3} M) une distributeur d'ON. Cette réduction était cependant l'activation du sGC plutôt puis par le degredation des catécholamines à leurs dérivés 6-nitro. Traitement avec les inhibiteurs 7-nitroindazole (10^{-4} M) et L-NAME (5 x 10^{-3} M) ont diminué la production be ON en réponse à la stimulation électrique tandis que la sécrétion croissante de catécholamine évalue approximativement 2-foix. L'évidence expérimentale suggère que les nNOS soit l'isoform principal impliqué dans l'inhibition de la sécrétion de catécholamine.

Après l'exposition répétée du truite arc-en-ciel à l'hypoxie plus de 4 jours *in vivo*, les préparations *in situ* dérivées de ces poissons ont montré une augmentation de ON approximativement de 2 et 1.8-foix plus haute que les niveauz basique et stimulé respectivement, alors que des taux de sécrétion de catécholamine étaient réduits par approximativement 50 %. L'expression nivelle pour l'isoform de nNOS dans le rein PCV antérieur suggère que cet isoform soit impliqué dans la réponse de catécholamine.

En résumé, le nNOS semble être l'isoform principal en produisant ON sous la stimulation neuronale et cette ON est responsible pour la reduction de catécholamine par l'activation du sGC.

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1H-(1, 2, 4) oxadiazole (4, 3-alpha) quinoxaline-1-one	ODQ
3,4-Dihydroxybenzylamine Hydrobromide	DHBA
7-Nitroindazole	7-NI
Acetylcholine	Ach
Analysis of Variance	ANOVA
bp	Base Pair
Calmodulin	CaM
Cyclic Guanosine 3',5' Monophosphate	cGMP
Dopamine-β-hydroxylase	DBH
Endothelial-dependent Relaxing Factor	EDRF
Endothelial Nitric Oxide Synthase	eNOS
Hemoglobin	Hb
High-Pressure liquid Chromatography	HPLC
Hz	Hertz
Inducible Nitric Oxide Synthase	iNOS
Inositol 1,4,5-Trisphosphate	IP3
kb	Kilobase
L-Aromatic Amino Acid Decarboxylase	AADC
L-Dihydroxyphenylalanine	L-DOPA
L-NG-Nitroarginine Methyl Ester	L-NAME
Messenger RNA	mRNA
Mitochondrial Nitric Oxide Synthase	mtNOS
Neuronal Nitric Oxide Synthase	nNOS
Nitric Oxide	NO
Nitric Oxide Synthase	NOS
Oncorhynchus mykiss	*O. mykiss*
Polymerase Chain Reaction	PCR
Phynylethanolamine-N-Methyltransferase	PNMT
Pituitary Adenylate-Cyclase Activating Polypeptide	PACAP
Posterior Cardinal Vein	PCV
Protein-interaction Domain	PDZ
Protein Kinase G	PKG
S-nitrosothiol	SNO
Sodium Nitroprusside	SNP
Soluble Guanylyl Cyclase	sGC
Standard Error of the Mean	SEM
Tyrosine Hydroxylate	TH
Volts	V
Vasoactive Intestinal Polypeptide	VIP

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CHAPTER 1

GENERAL INTRODUCTION

Stress is defined as a state of threatened homeostasis, to which the organism responds with a series of coordinated responses organized to enhance the probability of survival. These coordinated responses, often referred to as "stress responses," are composed of alterations in behavior, autonomic function and the secretion of multiple hormones including adrenocorticotropin hormones (ACTH) and cortisol/corticosterone, catecholamines, oxytocin, prolactin and renin (Carrasco and Van de Kar, 2003). In particular, the catecholamine hormones, adrenaline and noradrenaline, are known to play crucial roles in stimulating responses to diminish the often harmful effects that accompany stress (Wendelaar Bonga, 1997; Sabban and Kvetnansky, 2001; Carrasco and Van der Kar, 2003). The beneficial effects of catecholamines are achieved, in part, by modulation of the cardiovascular and respiratory systems in order to maintain adequate levels of oxygen in the blood (Perry and Gilmour, 1999) as well as by mobilization of energy stores to provide for the increased energy demands that often accompany stress (Fabbri et al., 1998; Reid et al., 1998).

In rainbow trout (*Oncorhynchus mykiss*), the catecholamines are synthesized by a single biosynthetic pathway (Fig 1.1) in chromaffin cells that line the posterior cardinal vein (PCV) and in adrenergic neurons. This series of enzymatic reactions is known as the "Blaschko pathway" (Nilsson, 1983; Randall and Perry, 1992). Catecholamines are synthesized by four sequential enzymatic reactions beginning with the rate-limiting enzyme tyrosine hydroxylase (TH) which produces L-dihydroxyphenylalanine (L-DOPA) from the hydroxylation of tyrosine (for review see Kumer and Vrana, 1996). L-DOPA is rapidly converted to dopamine by aromatic L-amino acid decarboxylase

(AADC; Waymire and Haycock, 2002). Dopamine is taken up into storage vesicles and is either stored as such for later release (e.g., dopaminergic neurons) or further metabolized [e.g. adrenergic neurons, chromaffin cells (Randall and Perry 1992)]. Dopamine-β-hydroxylase (DBH) is the enzyme responsible for the conversion of dopamine to noradrenaline. The final step is the formation of adrenaline from noradrenaline by phenylethanolamine-N-methyl transferase (PNMT; Tai and Wong, 2003).

Control of catecholamine release

The current model for catecholamine release contends that a number of cholinergic and non-cholinergic neurotransmitters and/or neuromodulators interact either directly or indirectly with the chromaffin cells to influence secretion (Reid et al., 1998).

Cholinergic mechanisms

Chromaffin tissue of teleosts and elasmobranches is innervated by pre-ganglionic sympathetic nerve fibers (Randall and Perry, 1992). Upon stimulation, these nerve fibers release acetylcholine (Ach) onto the chromaffin cells, where it interacts with cholinergic receptors to elicit catecholamine secretion. There are two types of cholinergic receptors, nicotinic and muscarinic. The relative involvement of nicotinic *versus* muscarinic receptor is species dependent (McNeill et al., 2003). In the majority of vertebrate species studied to date, including fish, it appears as if the nicotinic receptor is the predominant cholinergic receptor controlling catecholamine secretion (Reid et al., 1998). Stimulation of the nicotinic receptor either results in membrane depolarization via the

opening of voltage-dependent Ca^{2+} channels in the plasma membrane or Ca^{2+} entering through ligand-gated nicotinic acetylcholine receptor channels (ionotropic receptors; Zaika et al., 2004). The increase in intracellular Ca^{2+} leads to the activation of proteins necessary for the rearrangement and disassembly of the cytoskeletal matrix and thus causes the release of catecholamines via exocytosis of secretory vesicles (Burgoyne et al., 1993; Furimsky et al., 1996).

Stimulation of the muscarinic cholinergic receptors, on the other hand, leads to the release of intracellular stores of Ca^{2+} through the activation of inositol triphosphate (IP_3; Kao and Schneider, 1985; Furimsky et al., 1996). This rise in free intracellular Ca^{2+} is generally low when compared to the increase in Ca^{2+} following nicotinic receptor stimulation (Cheek and Burgoyne, 1985; Forsberg et al., 1986; Oomori et al., 1998) and could explain why muscarinic receptor stimulation is generally unable to cause catecholamine secretion. Although stimulation of the muscarinic receptor is not usually able to cause catecholamine secretion, it may be able to potentiate secretion during activation of the nicotinic receptor (Forsberg et al., 1986; Montpetit and Perry, 1999).

Non-cholinergic mechanisms

In addition to Ach, the pre-ganglionic nerve fibers release a variety of other transmitters including serotonin, adenosine, polyadenylate cyclase activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP) and neuropeptide Y (Shioda et al., 2000; Wong et al., 2002). In general, these neurotransmitters or modulators function by increasing intracellular Ca^{2+} levels within chromaffin cells leading to catecholamine secretion through exocytosis.

Nitric Oxide

Nitric oxide (NO) is a relatively short lived highly reactive gas molecule which was first recognized as an endothelium-derived relaxing factor (EDRF) and was implicated in blood-vessel relaxation (Chen et al., 1998). 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; EC 1.14.13.39) family of enzymes. NOS is a complex flavo-hemoprotein that catalyzes two sequential mixed-function oxidations, the first being L-arginine hydroxylation to N^{w}-hydroxy-L-arginine (NOHA) as a bound intermediate, and the second converting NOHA to citrulline and NO (Iwasaki et al., 1999;Santolini et al., 2001; Rettori et al., 2003). There are three known isoforms of NOS, each comprised of two distinct domains. They have an N-terminal oxygenase domain that contains iron protoporphyrin IX (heme) and the L-arginine binding site (Larsson and Phillips, 1998). The other domain, the C-terminal reductase domain, contains FMN, FAD, and NADPH binding sites (Mungrue et al., 2003). Additionally, an intervening calmodulin (CaM) binding sequence is located between the oxygenase and reductase domains [Fig 1.2 (Panda et al., 2003; Mayer and Andrew, 1998; Stuehr et al., 2001)]. To become active, two NOS polypeptides must form a homodimer (Lajoix et al., 2004). Dimerization creates high affinity binding sites for (6R)-tetrahydrobiopterin (H4B) and arginine in each oxygenase domain, and enables electrons to transfer between NOS flavin and heme groups (Panda et al., 2003).

The three isoforms of NOS are neuronal NOS, inducible NOS and endothelial NOS:

Neuronal NOS

Neuronal NOS (nNOS or NOS I) is mainly expressed in neurons but is also found in non-neuronal cell types including cardiac myocytes (Xu et al., 1999), skeletal myocytes (Kobzik et al., 1994), and the adventitia of a subset of neuronal blood vessels (Nozaki et al., 1993). nNOS is one of two isoforms that is Ca^{2+} and CaM dependent for its induction. NO is formed in the course of excitatory neurotransmission via activation of nNOS following Ca^{2+} influx through the N-methyl-D-aspartate subtype of glutamate receptors (Garthwaite et al., 1988; Mayer and Andrew, 1998) or through the activation of the $GABA_A$ receptor (Pepicelli et al., 2004). To date, nNOS is the only isoform to exist with multiple N-terminal splice variants (Panda et al., 2003). There are at least three proteins; nNOSα, nNOSβ and nNOSγ. nNOSα exhibits full enzyme activity, while the other two lack the PDZ domain that is encoded by exon 2 (Huber et al., 1998). This results in a cytosolic localization of these proteins (Panda et al., 2003). In addition, in rat three different splice forms with distinct 5'-untranslated first exons of the nNOSα mRNA (nNOSα-a, nNOSα-b, nNOSα-c) have been identified, which show a tissue-and development-specific expression (Lee et al., 1997).

Inducible NOS

Inducible NOS (iNOS or NOS II) is expressed principally in phagocytes, particularly macrophages, in response to proinflammatory cytokines and/or bacterial products such as lipopolysaccharides (Barroso et al., 2000; Chi et al., 2003). Due to a

tight non-covalent interaction with CaM , iNOS does not require additional Ca^{2+} inputs (Vicente et al., 2002), but requires an immunologic (cytokine) or inflammatory (endotoxin) stimulus for induction (Sanson and Malangoni, 2003).

Endothelial NOS

Endothelial NOS (eNOS or NOS III) is the major NOS isoform expressed in the cardiovascular system (Mungrue et al., 2003). eNOS plays a key role in regulating vascular tone (Oyan et al., 2000), but is also involved in several other functions including inhibition of platelet aggregation and adhesion, inhibition of leukocyte adhesion and modulation of smooth muscle cell proliferation (Moncada et al., 1997). The main direct action of NO produced by eNOS in the vasculature is to cause vasodilation by a series of reactions initiated in the smooth muscle cells (Barnes et al., 2001). eNOS, like nNOS requires Ca^{2+} and CaM for its activation (Moncada et al., 1997).

Mitochondrial NOS

There is much controversy surrounding the existence of mitochondrial NOS (mtNOS). It has been shown that NO is produced in the mitochondria (Kanai et al., 2004) but its mechanism of production is unknown. mtNOS is similar to eNOS and nNOS based on its Ca^{2+} dependence (Zanella et al., 2004). However it does not react with either eNOS or nNOS antibodies (Lacza et al., 2003). In contrast, it strongly binds CaM and exhibits iNOS-like immunoreactivity and a similar molecular weight (Lacza et al., 2003). Other studies suggest that mtNOS is the α splice variant of nNOS (Kanai et al., 2001; Elfering et al., 2002; Haynes et al., 2004). Lacza et al. (2003) have shown

using 12 different anti-NOS antibodies, eNOS knockout animals and classic NOS inhibitors that mtNOS may actually be another NOS isoform. Currently there is no mtNOS sequence available to differentiate mtNOS from the other isoforms.

Mode of action of NO

NO elicits most of its physiological functions by binding to iron in a heme moiety that is present in soluble guanylyl cyclase (sGC; Oset-Gasque et al., 1994). This results in a conformational change of the protein to stimulate the formation of cyclic guanosine 3', 5'-monophosphate (cGMP; Boehning and Snyder, 2003; Kim et al., 2003). This leads to the activation of a cGMP dependent protein kinase (PKG), which is believed to phosphorylate Ca^{2+} channels, inhibiting Ca^{2+} influx [figure 1.3 (Schwarz et al., 1998; Barnes et al., 2001)]. PKG could also function by activating a phosphatase, leading to the dephosphorylation of the channels (Keef et al., 2001).

Hemoglobin (Hb) is a tetramer composed of two α- and two β-globin polypeptide chains (Han et al., 2004) that functions in O_2 and CO transport (Crawford et al., 2003). Each chain contains a heme-moiety to which these small molecules bind (Stamler et al., 1997). It has been suggested that NO could also be transported by Hb by binding to the Cys^{93} on the β–globin chain (Wolzt et al., 1999). Hb is believed to actually conserve NO in the form of a nitrosothiol moiety (SNO-Hb; Joshi et al., 2002) and could serve in the delivery of NO.

Hb exists in two alternative structures, named R (for relaxed, high O_2 affinity) and T (for tense, low O_2 affinity; Stamler et al., 1997). A SNO complex with Hb is particularly appealing because the environment of β-cysteine 93 is sensitive to the R<->T

conformational equilibrium of Hb (Gladwin et al 2002). Similar to the binding of O_2, Hb has a high affinity for NO in the R conformation and low affinity in the T conformation (Stamler et al., 1997). Thus, Hb would deliver to oxygen-deficient vascular beds not only the oxygen required for sustained metabolism but also a vasodilator in NO (Joshi et al., 2002). A similar hypothesis exists for myoglobin (Mb), a single polypeptide chain that is similar to Hb (Flogel et al., 2000).

The role of Hb, through S-nitrosothiol formation, in providing a protected route for delivery of NO is a matter of debate (Flogel et al., 2000). NO is irreversibly inactivated by oxy Hb at an extremely high rate (rate constant, $k = 10^7\ M^{-1}s^{-1}$) forming nitrate and methemoglobin (Joshi et al., 2002; Han et al., 2002). Therefore, it is unclear whether SNO-Hb could reach sufficient levels to be physiologically significant.

Although NO produced by all NOS isoforms would be expected to influence catecholamine levels (see below), eNOS and/or nNOS are the likeliest candidates based on their Ca^{2+} dependency and localization. Because eNOS is located primarily within endothelial cells, it is likely to be in close proximity to the chromaffin cells associated with the posterior cardinal vein. nNOS, on the other hand, has received much attention because this isoform is believed to be under neuronal control. In mammals, nNOS has been localized in the 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, nNOS was localized in the head kidney (Jimenez et al., 2001) but is only rarely located in the 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 eNOS (Barnes et al., 2001; Rubio et al., 1994); there is no evidence for a role for iNOS.

The effect of NO on basal catecholamine levels as well as on stimulus-evoked catecholamine secretion in mammals remains a matter of debate. With respect to basal catecholamine secretion, NO has been shown to increase catecholamine levels (Oset-Gasque et al., 1994; 1998), decrease catecholamine levels (Ward et al., 1996; Barnes et al., 2001), or to have no effect (Marley et al., 1995). As for stimulus-evoked catecholamine secretion, early studies showed an increase in catecholamine levels in response to NO (Breslow et al., 1992; O'Sullivan and Burgoyne, 1990; Uchiyama et al., 1994), while more recent studies reported an inhibitory role for NO (Nagayama et al., 1998; Schwarz et al 1998; Barnes et 2001; Kolo et al 2004). Oset-Gasque et al. (1994) demonstrated that with low doses of nicotine, NO has a stimulatory role, while at high doses of nicotine, NO inhibits catecholamine secretion in a dose dependent manner. Interestingly, a recent study demonstrated that NO can directly decrease the biological activity of catecholamines by converting them to their 6-nitro derivatives (Kolo et al., 2004).

All studies that have examined the effects of NO on catecholamine secretion have been conducted on mammals using either cultured cells or isolated perfused adrenal glands. The majority of these studies have suggested an inhibitory role of NO on catecholamine secretion (Torres et al., 1994; Oset-Gasque et al., 1994; 1998; Rodriguez-Pascual et al., 1995; Schwarz et al., 1998; Nagayama et al., 1998; Vicente et al., 2002; Kolo et al., 2004). While all of these studies measured catecholamine levels, none of them measured NO levels.

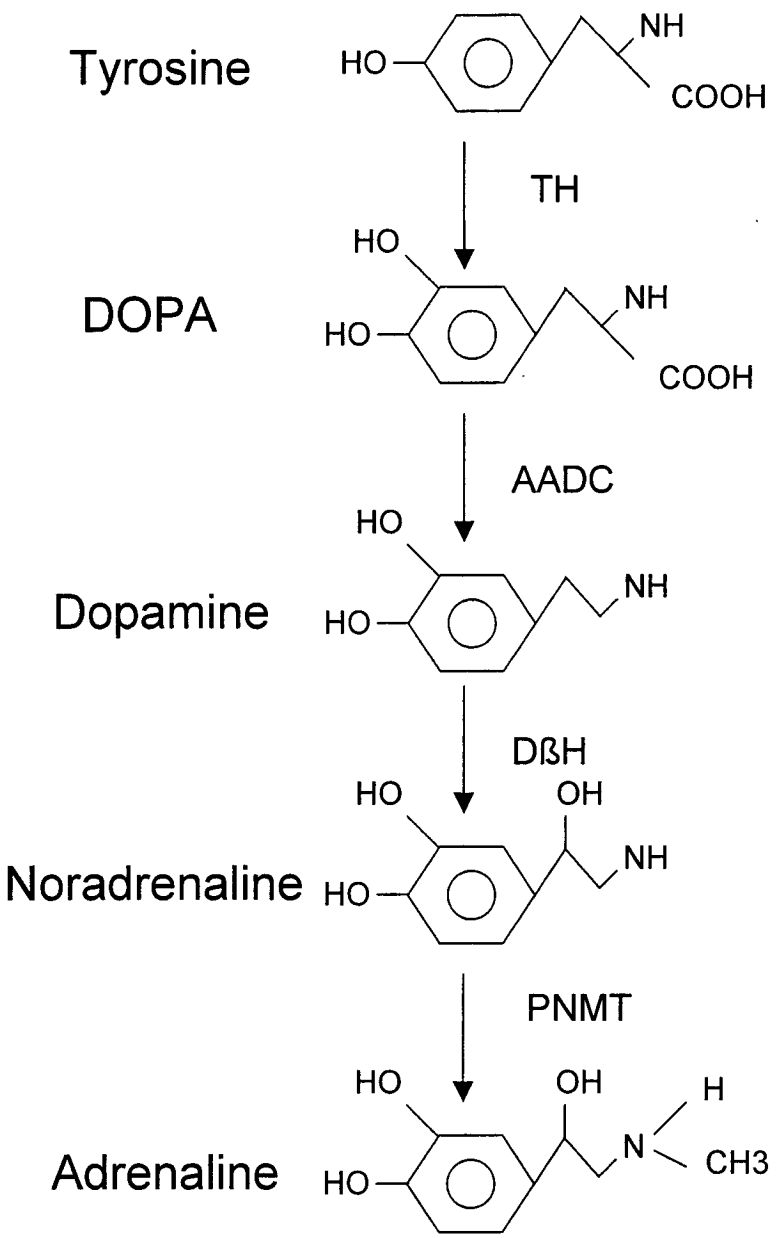
Goals of the Thesis

The goals of this thesis were to 1) investigate the effects of NO on basal and stimulus-evoked catecholamine secretion *in situ*, 2) determine which NOS isoform predominantly regulates catecholamine secretion and 3) investigate the effects of catecholamine secretion and NO production in fish repeatedly exposed to acute hypoxia *in vivo*.

Hypothesis

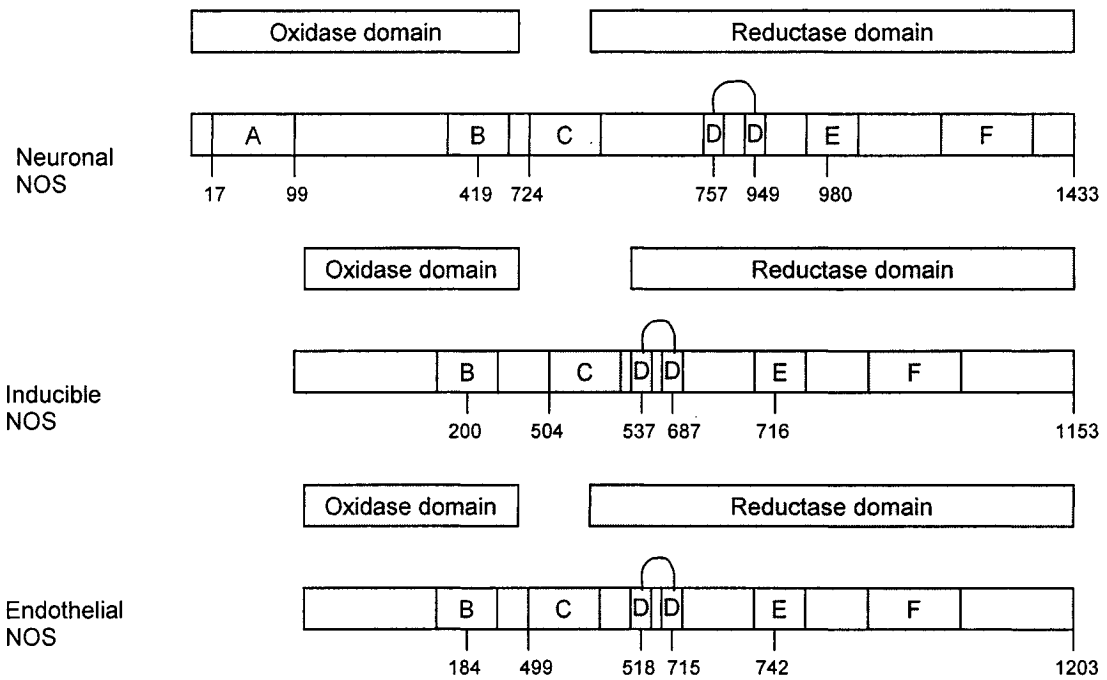
The principal hypothesis is that "NO, derived from the nNOS isoform, is an inhibitor of catecholamine secretion in trout".

Figure 1.1. The Blaschko pathway depicting the biosynthesis of catecholamines within the chromaffin cell. Enzyme abbreviations: TH, tyrosine hydroxylase; AADC, aromatic L-amino acid decarboxylase; DβH, dopamine beta hydroxylase; PNMT, phenylethanolamine-N-methyl transferase.



Adapted from Randall and Perry 1992

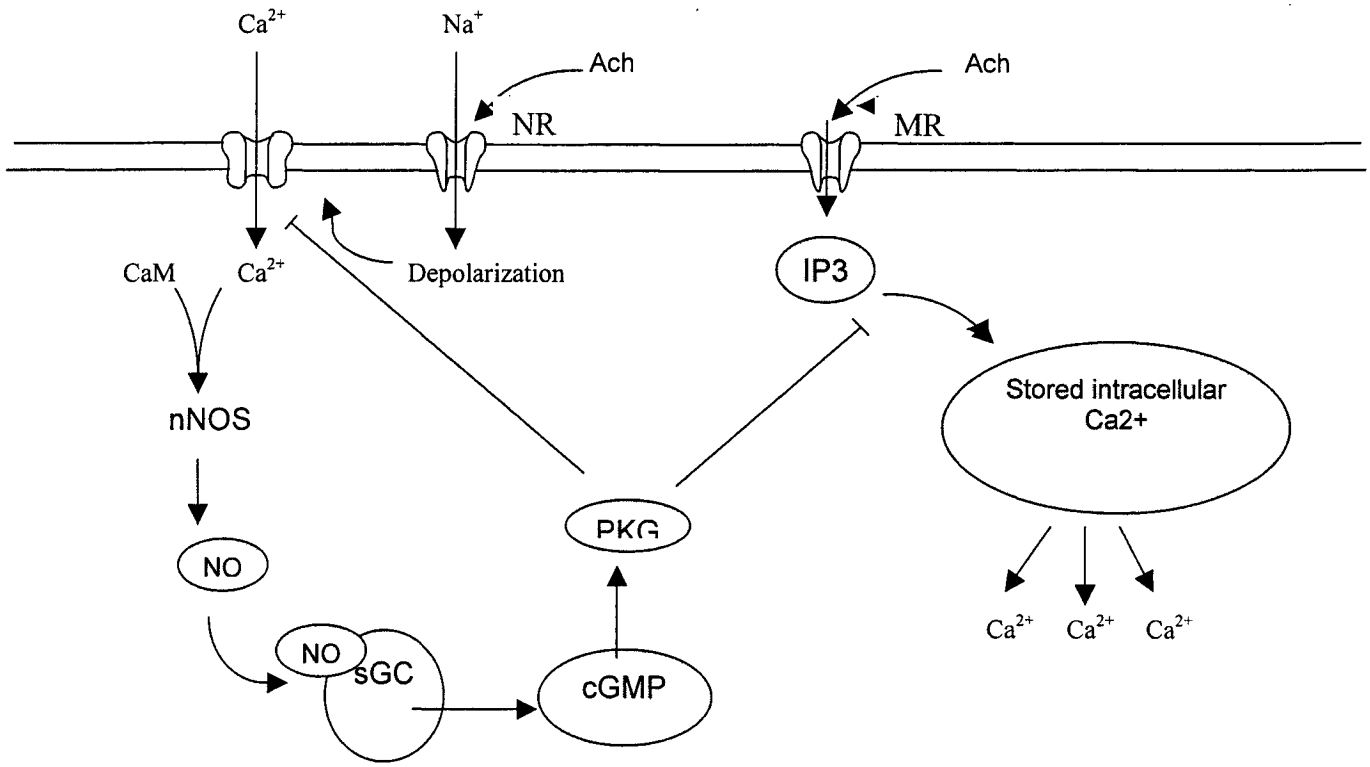
Figure 1.2. Domain structure of neuronal, inducible and endothelial NOS. Oxygenase, reductase and PDZ domains are denoted by solid boxes; the numbers refer to the amino acid residues at the start/end of each domain. Abbreviations: CaM, calmodulin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); PDZ , protein-interaction domain.



A ▶ PDZ domain
B ▶ Heme binding
C ▶ CaM binding
D ▶ FMN binding
E ▶ FAD binding
F ▶ NADPH binding

Adapted from Alderton et al.,2001

Figure 1.3. Control mechanism of catecholamine secretion by the endogenous NO/cyclic GMP pathway via a reduction in the magnitude of intracellular Ca^{2+} changes in response to acetylcholine (Ach). Abbreviations: sGC, soluble guanylyl cyclase; CaM, calmodulin; cGMP, cyclic guanosine monophosphate; MR, muscarinic receptor; NR, nicotinic receptor; PKG, protein kinase G; IP3, inositol 1,4,5-trisphosphate.



Edited from Schwarz et al., 1998

CHAPTER 2

EFFECT OF NITRIC OXIDE ON CATECHOLAMINE SECRETION *IN SITU*

Introduction

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 catecholamines.

Previous research using rainbow trout (*Oncorhynchus mykiss*) 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 current model for catecholamine release in fish contends that a number of cholinergic and non-cholinergic neurotransmitters interact either directly or indirectly with the chromaffin cells to influence secretion (Reid et al., 1998; McNeill et al., 2003). The primary stimulus that elicits catecholamine secretion is believed to be via activation

of the cholinergic nicotinic receptor (Nilsson et al., 1976; Guo and Wakade, 1994). It was recently demonstrated that different neurotransmitters are preferentially released from the pre-ganglionic fibers innervating the chromaffin cells at different action potential frequencies (Montpetit and Perry, 2000; McNeill et al., 2003).

In mammals, nNOS has been localized not only in chromaffin cells (Schwarz et al., 1998; Oset-Gasque et al., 1999), but also in the nerves innervating them (Bredt et al., 1990; Dun et al., 1992; Holgert et al., 1995). In rainbow trout, nNOS is present in nerve fibers in close proximity to the chromaffin cells (Jimenez et al., 2001; Gallo and Civinini, 2001), but only rarely within the chromaffin cells (Gallo and Civinini, 2001). The involvement of nNOS in regulating catecholamine secretion has not been investigated in fish. Thus, the goals of this study were to 1) clone rainbow trout nNOS and ascertain its tissue distribution, 2) determine the potential for NO to influence basal- and stimulus-evoked catecholamine secretion, and 3) ascertain whether NO is preferably produced at high or low action potential frequencies.

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 dechlorinated city of Ottawa tap water. The fish (mean mass of 231 ± 10.6 g; N = 69) were maintained at a temperature of 13°C on a 12h:12h 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.

In situ saline-perfused posterior cardinal vein preparation

The fish were killed by a sharp blow to the head, weighed and placed on ice. For electrical stimulation, electrodes were sutured to the skin on each side of the fish in the anterior region of the body, 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 posterior cardinal vein (PCV) 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; 125mM NaCl, 2.0 mM KCl, 2.0 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 7.5 mM glucose, 2.0 $CaCl_2$, and 1.25 mM 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 20 min 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 or was electrically stimulated using a previously validated field stimulation technique (Montpetit and Perry, 1999).

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 sec at 7500 g and the perfusate were quickly frozen in liquid N_2 and stored at -80°C until subsequent determination of catecholamine and NO levels.

Series 1: Cloning and tissue distribution of neuronal NOS

Tissue collection and RNA isolation

Fish were killed by a blow to the head, and tissues (brain, PCV, kidney, white muscle, spleen, heart and intestine) were collected and immediately frozen in liquid N_2 and stored at -80°C. Total RNA was isolated using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. RNA quantity and quality were verified by spectrophotometry (Eppendorf BioPhotometer) and gel electrophoresis. Aliquots of

RNA to be used in real-time PCR were treated with DNase1 DNA free kit (Ambion) before use.

Cloning and sequencing of nNOS

A 328 bp sequence was obtained by a 4th year honours student (Lihn-An Tuong). Primers were designed based on this sequence to be used for 3' and 5' RACE (Rapid Amplification of cDNA Ends). Other primers were designed based on the zebrafish (*Danio rerio*; NP_571735) and fugu (*Takifugu poecilonotus*; AAM46138) sequences acquired from GenBank (primers that generated sequence can be found in Table 2.1).

A typical PCR amplification consisted of cDNA or PCR product (0.5 µl) template, 0.1 µM of each primer (Table 1), 2 mM MgCl2, 0.2 mM of a dNTP mix, and 0.5 units of *Taq* DNA polymerase (Invitrogen) in PCR buffer supplied with the enzyme. PCR amplification was accomplished using a thermal cycler (Eppendorf Mastercycler) with the PCR cycle consisting of a 5 min 94°C denaturation step followed by 40 cycles of 94°C for 30 sec, 58°C for 30 seconds, 72°C for 1min/1kb, with a final extension for 10 min at 72°C. After gel electrophoresis (1.25% agarose gel containing ethidium bromide) PCR products were visualized using an ultraviolet light (BIORAD Chemi Doc attached to a camera) with digital images processed using commercial software (Quantity One, version 4.1.1)

PCR products of interest were cloned into pCR 2.1 (TOPO TA Cloning kit; Invitrogen). Plasmids from selected positive clones were purified (Wizard kit, Promega) and the DNA was commercially sequenced by either Canadian Molecular Research Services (CMRS Inc) or by the Core DNA Sequencing and Synthesizing Facility at the University of Ottawa.

Sequences were analyzed using the Basic Local Alignment Search Tool 2.0 (BLAST) algorithms at the National Center for Biotechnology Information (NCBI) using default settings. Sequence alignments were performed using DNAMAN (version 4.0, Lynnon Biosoft), the same program that was used for the construction of a homology tree.

Tissue distribution by real-time PCR

cDNA was synthesized using StrataScript reverse transcriptase (Strategene) and random hexamers using 5 μg of total RNA. mRNA levels were assessed by real time PCR on duplicate samples of cDNA (0.5 μl) using Brilliant® SYBR® Green QPCR (Stratagene) and a Stratagene MX-4000 multiplex QPCR system. The PCR conditions were as outlined by the manufacturer's instructions except that the reaction volume was scaled down from 50 to 25 μl. Gene-specific primers for rainbow trout nNOS and β-actin were designed using DNAMAN (version 4.0, Lynnon Biosoft) and DNA calculator (www.sigma-genosys.com) (Table 2.1). Relative expression of nNOS mRNA levels was determined (using actin as an endogenous standard) by the delta-delta Ct method (Pfaffl, 2001).

Series 2: Assessing the potential of 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; 5×10^{-3} M). Samples were collected for 6 min at which point the preparations were electrically stimulated at 60 Volts (V) at a frequency of 20 Hz.

Series 3: 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 NO production could be inhibited in the present study, a cocktail containing 7-nitroindazole (7-NI; 10^{-4} M) and N-nitro L-arginine methyl ester (L-NAME; 5 X 10^{-3} M) was used. 7-NI was used as a specific nNOS inhibitor (Bland-Ward and Moore, 1995; Barnes et al., 2001; Vicente et al., 2002, while L-NAME was used as a non-specific NOS inhibitor (Torres et al., 1994; Rodriguez-Pascual et al., 1995; Vicente et al., 2002). Preparations were either perfused with saline containing the combination of the inhibitors or with control saline. Following the collection of pre-samples, preparations received a bolus injection of 10 mM KCl (1 ml kg^{-1}).

Series 4: Stimulation at 30 V at varying frequencies

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 and either 1, 8 or 20 Hz.

Series 5: Effect of NO on catecholamine degradation

Noradrenaline and adrenaline (10^{-9} M) prepared in 0.1 M HCl were incubated separately in freshly prepared saline containing SNP (10^{-8} - 10^{-3} M). 0.5 ml of SNP was added to 0.5 ml of catecholamine and incubated for 5 min in a glass test tube. The reaction was stopped by the addition of 0.5 ml of 0.1 M perchloric acid and 0.1%

cysteine and the solutions were then placed on ice. Catecholamines were extracted and analyzed by HPLC, while a colorimetric assay was used to measure NO levels.

Series 6: Catecholamine secretion inhibition through NO activated sGC

Experimental preparations received saline containing the selective guanylyl cyclase inhibitor, 1H-(1, 2, 4) oxadiazole (4, 3-alpha) quinoxaline-1-one (ODQ; 10^{-5} M) for the entire experiment while controls received saline. Pre-samples were collected and the fish were stimulated at 30 V and 8 Hz.

Analytical procedures

Catecholamine determination

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

Nitric oxide assay

Quantification of NO is difficult 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. This method demonstrates high accuracy and reproducibility and adequately reflects actual NO production in an organism (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 mM. The stock solution was serially diluted in 0.14 M $KHPO_4$ to prepare standard curves. 50 µl of standard or sample along with (15 µl) of 0.8 mM NADPH (Sigma) were added to a 96 well plate. Next, 2.5 µl of FAD (100 µM; 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 for 45 min. 40 µl of Griess reagent I and II (Caymen chemicals) were then added and allowed to incubate for 5 min. Color development was assessed using a Spectra Max Plus 384 (Molecular Devices) micro-plate reader at a wavelength of 540 nM. This assay measures nitrate and nitrite concurrently and thus NO levels will be denoted as NO.

Statistical analysis

The data are presented as means ± 1 standard error of the mean (SEM). 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 were 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 simplicity, 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: Cloning and tissue distribution of neuronal NOS.

A 2166 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 722 amino acids of an expected 1431 amino acid sequence. An alignment of this sequence with selected vertebrate sequences is shown in Fig 2.1. A homology tree (Fig 2.2) illustrates that trout nNOS groups closely with nNOS of other vertebrates and is most similar to the nNOS of other fishes. Note that trout iNOS, like iNOS from other vertebrates is found within a separate grouping.

Figure 2.3 illustrates that the nNOS isoform is highly expressed in the spleen, intestine and brain, while lower expression is observed in the PCV; nNOS was undetectable in blood. As a validation, a standard curve was constructed using the primer combination for both nNOS and β-actin on a serial dilution of cDNA. The nNOS primer combination resulted in a slope of -3.215 with a correlation coefficient of 0.983, while β-actin had a slope of -3.200 with a correlation coefficient of 0.989, both within the accepted range.

Series 2: Assessing the potential effect of NO on 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 approximate 12-fold increase in noradrenaline, 7-fold increase in adrenaline and an 8-fold increase in total catecholamine secretion rates over the pre values (Fig 2.4A). The preparations receiving SNP were unresponsive to the electrical

stimulus. In control preparations, electrical stimulation evoked a 3-fold increase in perfusate NO levels (Fig 2.4B). In the presence of SNP, there was a 14-fold increase in NO in response to electrical stimulation.

Series 3: NO production during non-specific chromaffin cell depolarization

The addition of 10 mM KCl to the perfusate caused significant increases in catecholamine and NO secretion (Fig 2.5). However, in the presence of the NOS inhibitors, 7-NI and L-NAME, the increase in NO was prevented and there was a significant increase in catecholamine secretion (Fig 2.5A). The inhibitor combination reduced KCl-induced NO levels by approximately 48% (Fig 2.5B), while increasing catecholamine levels approximately 2.3-fold (Fig 2.5A).

Series 4: Stimulation at 30 V at varying frequencies

The highest NO production in response to electrical stimulation was observed at medium frequency (8 Hz) with an approximate 9-fold increase in NO levels when compared to the pre-value (Fig 2.6B). Low frequency (1 Hz) stimulation also evoked a significant response (5-fold increase, whereas high frequency (20 Hz) stimulation failed to elicit a NO response (Fig 2.6B). The frequency dependency of the catecholamine secretion response to 30 V electrical stimulation is depicted in Fig 2.6A. Catecholamine secretion was lowest at 1 Hz (~6-fold increase), intermediate at 8 Hz (~8-fold increase) and greatest at 20 Hz (~28-fold increase).

Series 5: Effect of NO on catecholamine degradation

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 2.7. Percentage degradation was calculated by comparing the catecholamine levels between the samples containing SNP and the saline control. The extent of degradation was maximal at 10^{-3} M SNP and therefore was set to 100%.

Series 6: Catecholamine secretion inhibition through NO activated sGC

ODQ treatment resulted in an approximate 8-, 5.6- and a 5.4-fold increase in noradrenaline, adrenaline and total catecholamine secretion rates, respectively, when compared to saline treated fish (Fig 2.8A). NO levels for ODQ treated preparations showed an approximate 1.5-fold increase over saline treated fish (Fig 2.8B).

Table 2.1. A list of nNOS primers used to generate sequence and for use in measuring relative gene expression levels by real time PCR. Shown are the primer sequences and the location based on the zebrafish sequence.

Primer	Sequence (5'-> 3')	Position
Forward		
F1	GAT GCA AAG GTA ATG TCA ATG	2370
F2	AGA GGA CCG AAA GAG TTA CAA G	2539
FN	TCA CCA CNC ACC TGG AGA C	326
FQ	TGG AGA GAA ATT CGG AGC TG	2476
Reverse		
R1	CAT CTC CAT CAA GGC AGC TC	2471
R2	GAA ACG CAC CTT GTA ACT CTT	2521
RQ	CGG GTG TCA GAA TAG GAG GA	2551

Figure 2.1. A multiple sequence alignment comparing trout nNOS and nNOS of selected vertebrates prepared using ClustalW (EMBL-EBI) software. An asterisk indicates 100% homology, a colon > 75 % homology and period > 50% homology. GenBank accession numbers; zebrafish (*D. rerio*), NP_571735; Fugu, (*T. poecilonotus*) AAM46138; Xenopus (*X. laevis*), AAD55136; rat (*R. norvegicus*), CAA42574.

```
Trout       LKEIEPVLQLIKNSKREINGEEQRTIQRRDAEVQVDRDLGMGNGMSPQLASDKNSVCEDL 60
Zebrafish   LKEIEPVLKLIKNSKKEINGEGQRVIDRKDAEVQVERDLGVGNGQNAQLASDNDRVFDDL 60
Fugu        MKEIEPVLRLVKNSKKEINGEGQRHVGRRDAEIQVTWGAGVGIDTSLQLDSCKN------ 54
Xenopus     LKEIEPIVSLLQNAGKELNGDEHRNVEQKDAEVQVE----SNSQTQPSMQKDQ---VNGI 53
Rat         LKEIEPVLSILNSGSKATNRGGPAKAEMKDTGIQVDRDLDGKSHKAPPLGGDNDRVFNDL 60
            :*****:: ::::.. :  *         :*: :**            :   :

Trout       WRKDNNMQTVLTNPYSEKDEPPAQGRISPNKALMNGSPSKCPRFMKIKNVETGVTYNDTL 120
Zebrafish   WRKDN-MPTVLNNPYSESDKPQSYERVSPTKAVQNGSPSKCPRFMKVKNWETGALYNDTL 119
Fugu        -KMPEKEPGVPQN--ADNDKPPAEARTSPTKSLQNGSPSKCPRFLKIKNWETGAIQNDTL 111
Xenopus     WKNNNKMPVVLNNPYLECEQVTLTGRHSPAKSQVNGSPSKCPRYLKIRNWDSNTTLNDTL 113
Rat         WGKDN-VPVVLNNPYSEKEQSPTSGKQSPTK---NGSPSRCPRFLKVKNWETDVVLTDTL 116
                :    *  *   : ::     : ** *   *****:***::*::* ::..  .***

Trout       HHSSS-NPLCTECLCFGSIMTPSQGSRKPDESRTKEELLPLATDFIDQYYTSIKRFGSKA 179
Zebrafish   HHSSSKVPICTEHVCIGSIMVPNQHAHEPDEDRRKEELLPLATDFIDQYYTSIKRCCSKA 179
Fugu        HNSSTKTPMCPENVCYGSLMMPNLHARKPEEVRSKEELLKLATDFIDQYYTSIKRYGSKA 171
Xenopus     HSKASMPTPCTQQICMGAVMTPPHYVRKPEDIRTKEELLPLAKEFIDQYFSSIKRFGSKA 173
Rat         HLKSTLETGCTEHICMGSIMLPSQHTRKPEDVRTKDQLFPLAKEFLDQYYSSIKRFGSKA 176
            * .::  . *.: :* *::* *    ::*:: * *::*: **.:*:***::****  ***

Trout       YVDRLEEVTKEIEASGTYQLKDTELIYGAKHAWRNAARCVGRIQWSKLQVFDARDCTTAH 239
Zebrafish   HVDRLEEVKKEIETSGTYQLKDTELIYGAKHAWRNAARCVGRIQWSKLQVFDARDCTTAH 239
Fugu        HTDRLEEVTKEIEATGTYQLKDTELIYGAKHAWRNAARCVGRIQWSKLQVFDARDCTTAH 231
Xenopus     HMDRLQEVTKEIEATDTYQLRDTELIYGAKHAWRNASRCVGRIQWSKLQLFDARDCTTAH 233
Rat         HMDRLEEVNKEIESTSTYQLKDTELIYGAKHAWRNASRCVGRIQWSKLQVFDARDCTTAH 236
            : ***:**.****::.****:***************:***********:**********

Trout       GMYNYICNHIKYATNKGNLRSAITIFPQRTDGKRDFRVWNTQLIRYAGYKQPDGQILGDP 299
Zebrafish   GMFNYICNHIKYATNKGNLRSAITIFPQKTDGKHDFRVWNSQLIRYAGYKQPDGSILGDP 299
Fugu        GMYNYICNHIKYATNKGNLRSAITIFPPRTDGKHDFRVWNSQLIRYAGYKQPDGQILGDP 291
Xenopus     GMFNYICNHIKYATNKGNLRSAITIFPQRTDMKHDFRIWNAQLIRYAGYKQPDGSVLGDP 293
Rat         GMFNYICNHVKYATNKGNLRSAITIFPQRTDGKHDFRVWNSQLIRYAGYKQPDGSTLGDP 296
            **:******:***************** :** *:***:**:*************. ****

Trout       ANVEFTEICIQQGWKAPKGRFDVLPLLLQSNGNDPELFEIPQDLILEVQITHPKYDWFKD 359
Zebrafish   ASVELTEICMQQGWKAPKGRFDVLPLLLQANGNDPELFEIPDDLVLEVPIIHPKFEWFKE 359
Fugu        ANVEFTEICMQLGWKAPKGRFDVLPLLLQANGNDPEQFEIPEDLVLEVPIIHPKYEWFKE 351
Xenopus     ANVELTEICIQQGWKALHGRFDVLPLMLQADGNDPELFEIPPELIMEVNIRHPRLEWFKD 353
Rat         ANVQFTEICIQQGWKAPRGRFDVLPLLLQANGNDPELFQIPPELVLEVPIRHPKFDWFKD 356
            *.*::****:* **** :********:**::***** *:** :*::** * **: :***:

Trout       LNLKWYSLPAVFSMLLKVGGLEFTACPFSGWVMGTEIGVRNFGDSSRYNFCEEVATMMGL 419
Zebrafish   LNLKWYGLPAVSNMLLEIGGLEFTACPFSGWYMGTEIGVRDFCDSSRYNILEEVATMMGL 419
Fugu        LALKWYALPAVSNMMLEIGGLEFTACPFSGWYMGTEIGVRDFCDTSRYNMLEEVANKMGL 411
Xenopus     LGLKWYGLPAVSNLLLEIGGLEFSACPFSGWYMGTEIGVRDYCDNSRYNILEEVALKMNL 413
Rat         LGLKWYGLPAVSNMLLEIGGLEFSACPFSGWYMGTEIGVRDYCDNSRYNILEEVAKKMDL 416
            * ****.****  .::*::*****:******* ********:: *.****: ****  *.*

Trout       DTRKTFLLWKDQALVEINIAVLYSFNFSKVTIVDHHSATESFMKHMENEIRVRGGCPGDW 479
Zebrafish   DTRKTSSLWKDQALVEINIAVLYSYQMSKVTIVDHHSATESFMKHMENEYRVRGGCPGDW 479
Fugu        DTRKTSSLWKDQALVEVNIAVLHSFQSCKVTIVDHHSATESFMKHMENEYRVRGGCPGDW 471
Xenopus     DTRKTSSLWKDQALVEINIAVLYSFQSDKVTIVDHHSATESFIKHMENEYRCRGGCPADW 473
Rat         DMRKTSSLWKDQALVEINIAVLYSFQSDKVTIVDHHSATESFIKHMENEYRCRGGCPADW 476
            * ***  *********:*****:*::  **************:****** * *****.**
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Trout          VWIVPPMSGSITPVFHQEMLNYRLTPSFEYQADPWNNHVRKVNNGTPTKKRAIGFKKLAK 539
Zebrafish      VWIVPPMSGSITPVFHQEMLNYRLTPSFEYQPDPWNTHVWKGVNGTPTKKRAIGFKKLAK 539
Fugu           VWIVPPMSGSITPVFHQEMLNYRLTPSYEYQLDPWHTHVWKGVNGTPTKKRAIGFKKLAK 531
Xenopus        VWIVPPMSGSITPVFHQEMLNYRLTPSFEYQPDPWNTHVWKGVNGTPTKKRAIGFKKLAK 533
Rat            VWIVPPMSGSITPVFHQEMLNYRLTPSFEYQPDPWNTHVWKGTNGTPTKRRAIGFKKLAE 536
               ****************************:*** ***:.** *  ******:*********:

Trout          AVKFSAKLMGQAMAKRVKATILFATETGKSQDYAKTLCEIFKHAFDAKVMSMDEYDMVDL 599
Zebrafish      AVKFSATLMGQAMAKRVKATILFATETGKSQDYAKTLCEIFKHAFDAKVMSMDEYDTVDL 599
Fugu           AVKFSAKLMGHAMAKRVKATILFATETGKSQDYAKTLCEIFKHAFDPKVMSMDDYDVVDL 591
Xenopus        AVKFSAKLMGQAMAKRVKATILYATETGKSQVYAKTLCEIFKHAFDAKVMSMDEYDVVHL 593
Rat            AVKFSAKLMGQAMAKRVKATILYATETGKSQAYAKTLCEIFKHAFDAKAMSMEEYDIVHL 596
               ******.***:***********:******** **************.*.***::** *.*

Trout          EHETLVLAVTSTFGNGDPPENGEKFGAALMEMRHPTSNTEDRK 642
Zebrafish      EHETLVIVVTSTFGNGDPPENGEKFGAALMEMRHPTTSVEDRK 642
Fugu           EHETLVLVVTSTFGNGDPPENGEKFGAALMEMREPTSNTEDRK 634
Xenopus        EHETLVLVVTSTFGNGDPPENGEKFGCALMEMRHPNSNLEERK 636
Rat            EHEALVLVVTSTFGNGDPPENGEKFGCALMEMRHPNSVQEERK 639
               ***:**:.******************.******.*.:  *:**
```

Figure 2.2. A homology tree using selected amino acid sequence from GenBank for both nNOS and iNOS prepared using DNAMAN (Lynnon Biosoft) software. GenBank accession numbers for nNOS; zebrafish (*D. rerio*), NP_571735; Fugu (*T. poecilonotus)*, AAM46138; Xenopus (*X. laevis*), AAD55136; rat (*R. norvegicus)*, CAA42574; human (*H. sapiens*) , AAR07069; mouse (*M. musculus*), NP_032738. GenBank accession numbers for iNOS; human (*H. Sapiens*), AAC19133; mouse (*M. musculus*), AAM11887; rat (*R. norvegicus)*, CAB46089; trout (*O. mykiss*), CAC82808.

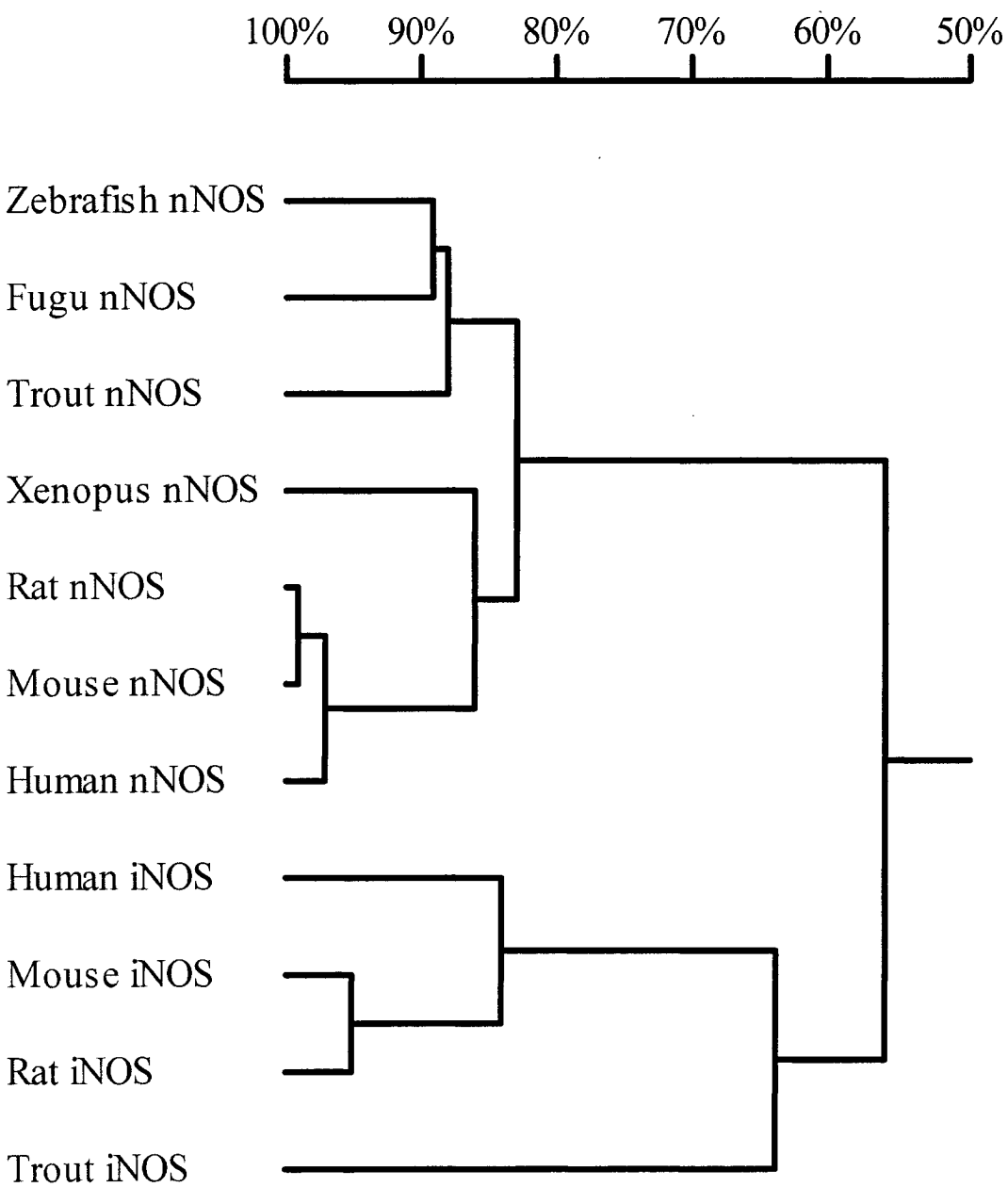

100%
90%
80%
70%
60%
50%
Zebrafish nNOS
Fugu nNOS
Trout nNOS
Xenopus nNOS
Rat nNOS
Mouse nNOS
Human nNOS
Human iNOS
Mouse iNOS
Rat iNOS
Trout iNOS

Figure 2.3 Real time PCR results showing the expression of rainbow trout (*O. mykiss;* N = 5) nNOS relative to brain (cross-hatched, gray bar). Relatively high levels occur in the spleen, intestine and brain, while lower levels are found in posterior kidney; nNOS mRNA was not detected in the blood.

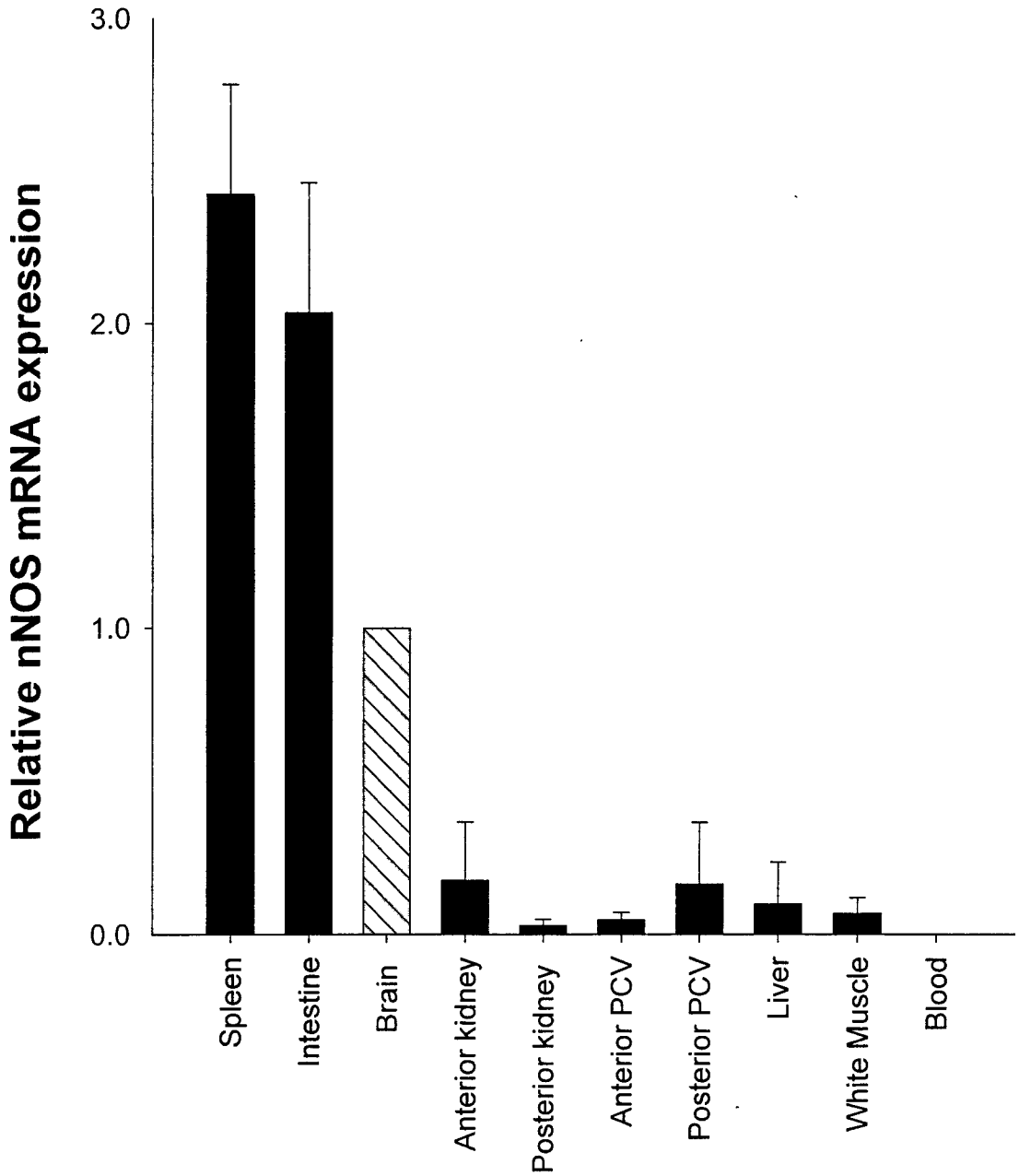

Relative nNOS mRNA expression
3.0
2.0
1.0
0.0
Spleen
Intestine
Brain
Anterior kidney
Posterior kidney
Anterior PCV
Posterior PCV
Liver
White Muscle
Blood

Figure 2.4. The effects of the NO donor SNP (5×10^{-3} M; cross-hatched bars; N = 6) on (A) noradrenaline (unfilled bars), adrenaline (filled bars), total catecholamine (sum of noradrenaline and adrenaline) and (B) perfusate NO levels in response to electrical stimulation of 60 V and 20 Hz. Values are shown as means ± 1 SEM. 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 control (N = 6) and the SNP treated group (cross-hatched).

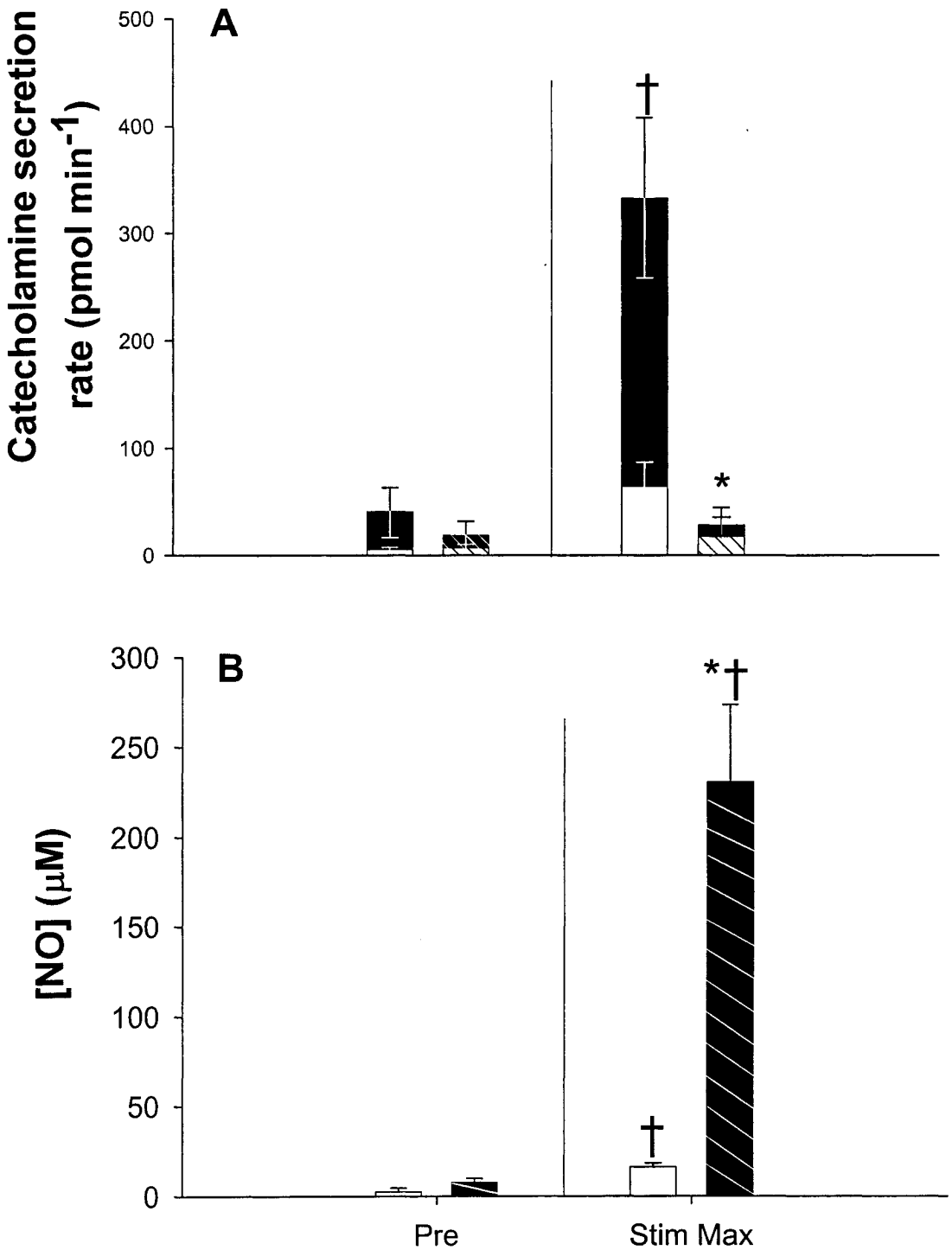

A
Catecholamine secretion rate (pmol min-1)
500
400
300
200
100
0
†
*
B
[NO] (μM)
300
250
200
150
100
50
0
*†
†
Pre
Stim Max

Figure 2.5. The effects of NO inhibitors on (A) noradrenaline (unfilled bars), adrenaline (filled bars), total catecholamine (sum of noradrenaline and adrenaline) and (B) perfusate NO production levels in response to a bolus injection of 10 mM KCl. Preparations were either pre-treated with saline (N = 6) or with saline containing the combination of 7-NI (10^{-4} M) and L-NAME (5 X 10^{-3} M; cross-hatched bars; N = 6). Values are shown as means ± 1 SEM. 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 control and the inhibitor treated group (cross-hatched).

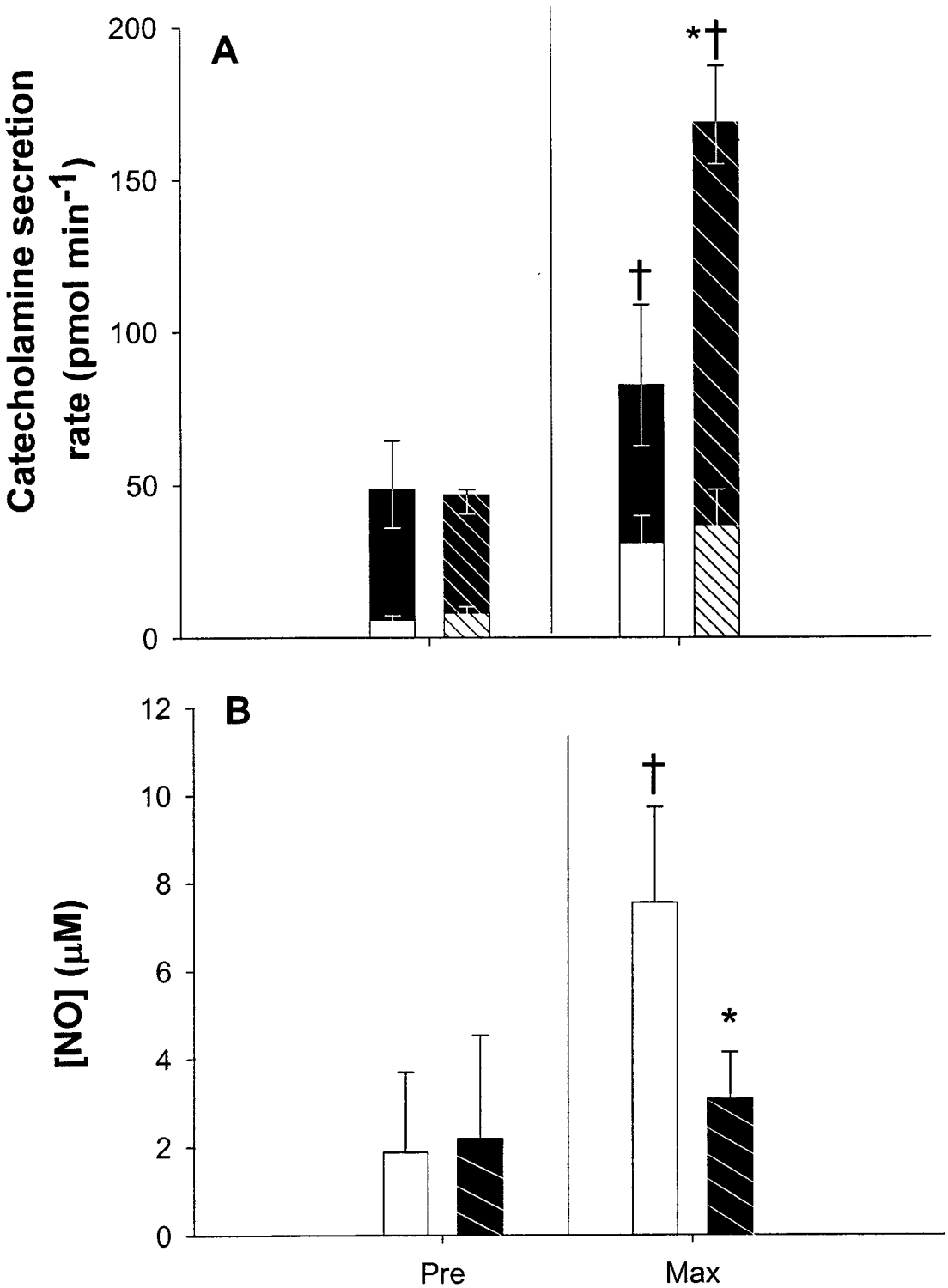

A
Catecholamine secretion rate (pmol min-1)
200
150
100
50
0
*†
†
B
[NO] (μM)
12
10
8
6
4
2
0
†
*
Pre
Max

Figure 2.6. The effects of field stimulation at 30 V and 1 Hz (N = 5), 8Hz (N = 14) or 20 Hz (N = 8) on (A) noradrenaline (unfilled bars), adrenaline (filled bars), total catecholamine (sum of noradrenaline and adrenaline) and (B) perfusate NO levels. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) and stimulated samples (cross-hatched bars).

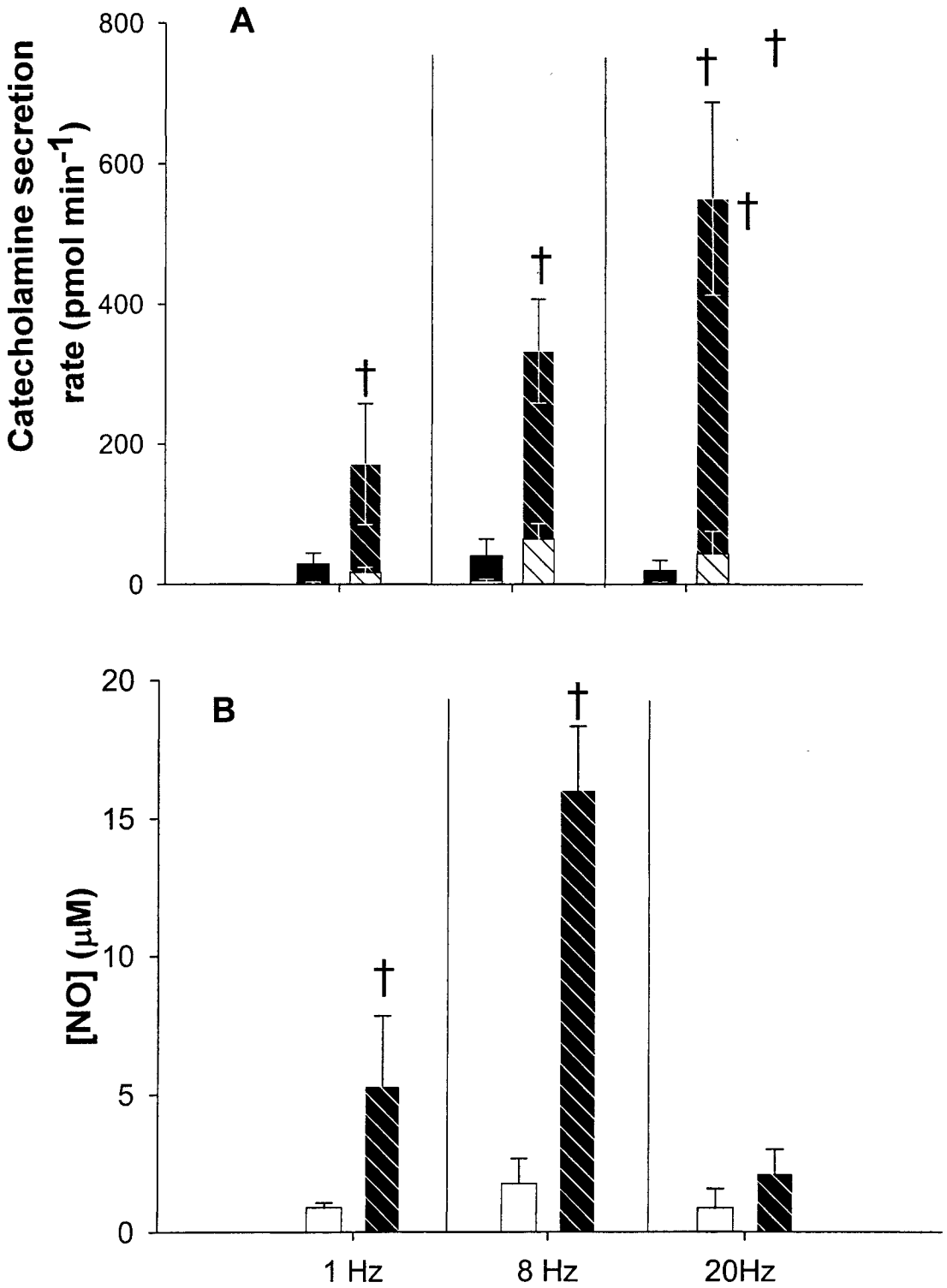

A
Catecholamine secretion rate (pmol min-1)
800
600
400
200
0
†
B
[NO] (μM)
20
15
10
5
0
1 Hz
8 Hz
20Hz

Figure 2.7. The dose-dependent effects of SNP on catecholamine degradation *in vitro* (A). The sigmoidal dose-response curve was calculated using curve-fitting software (Sigmaplot). The data constituting the dose-response curve in A was transformed to generate the Hill plot given in B. The following linear regression was calculated: $y = 0.4454x + 2.8884$; $r^2 = 0.97$. The EC_{50} for the Hill plot was calculated to be 2.63×10^{-7}.

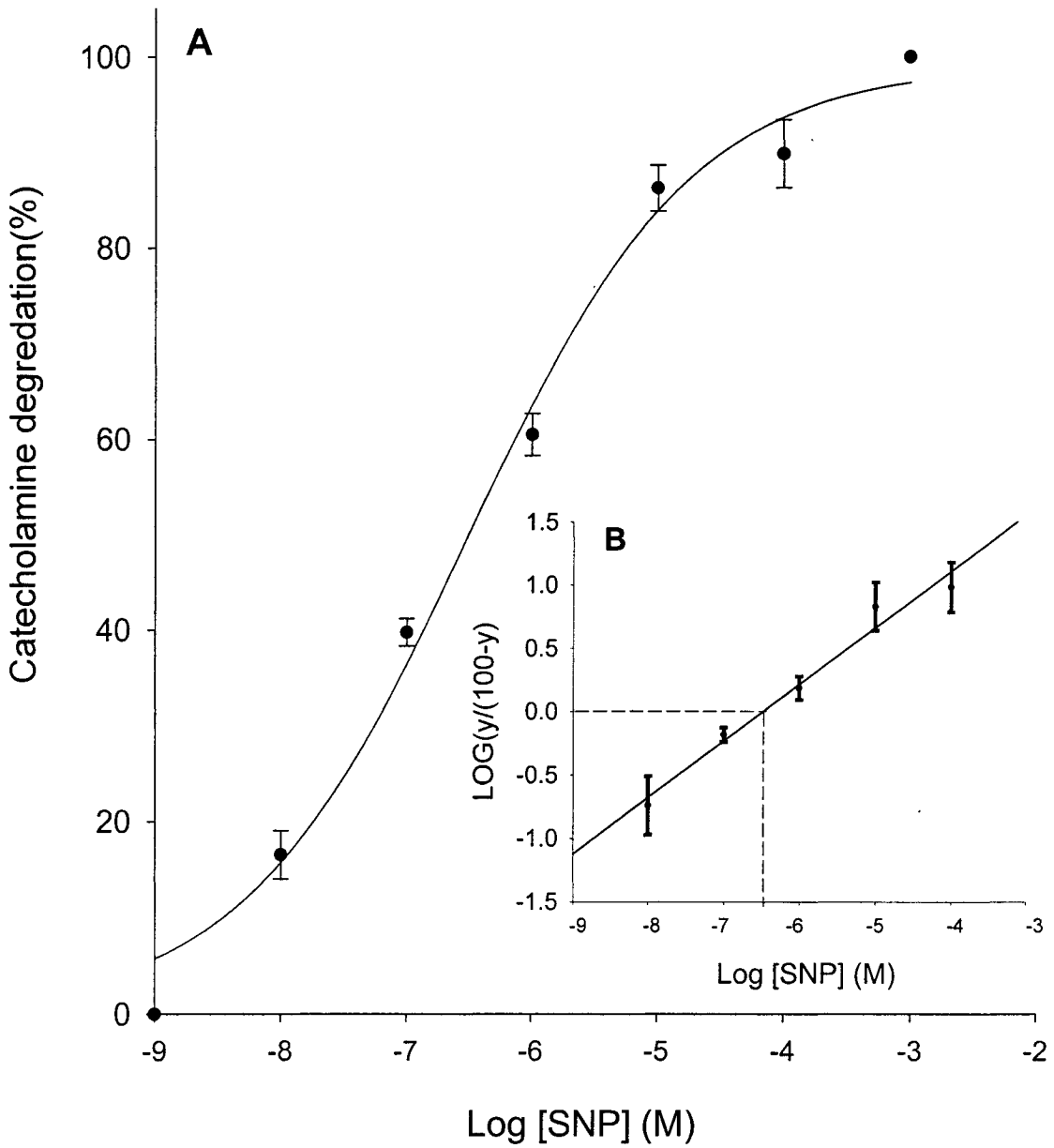
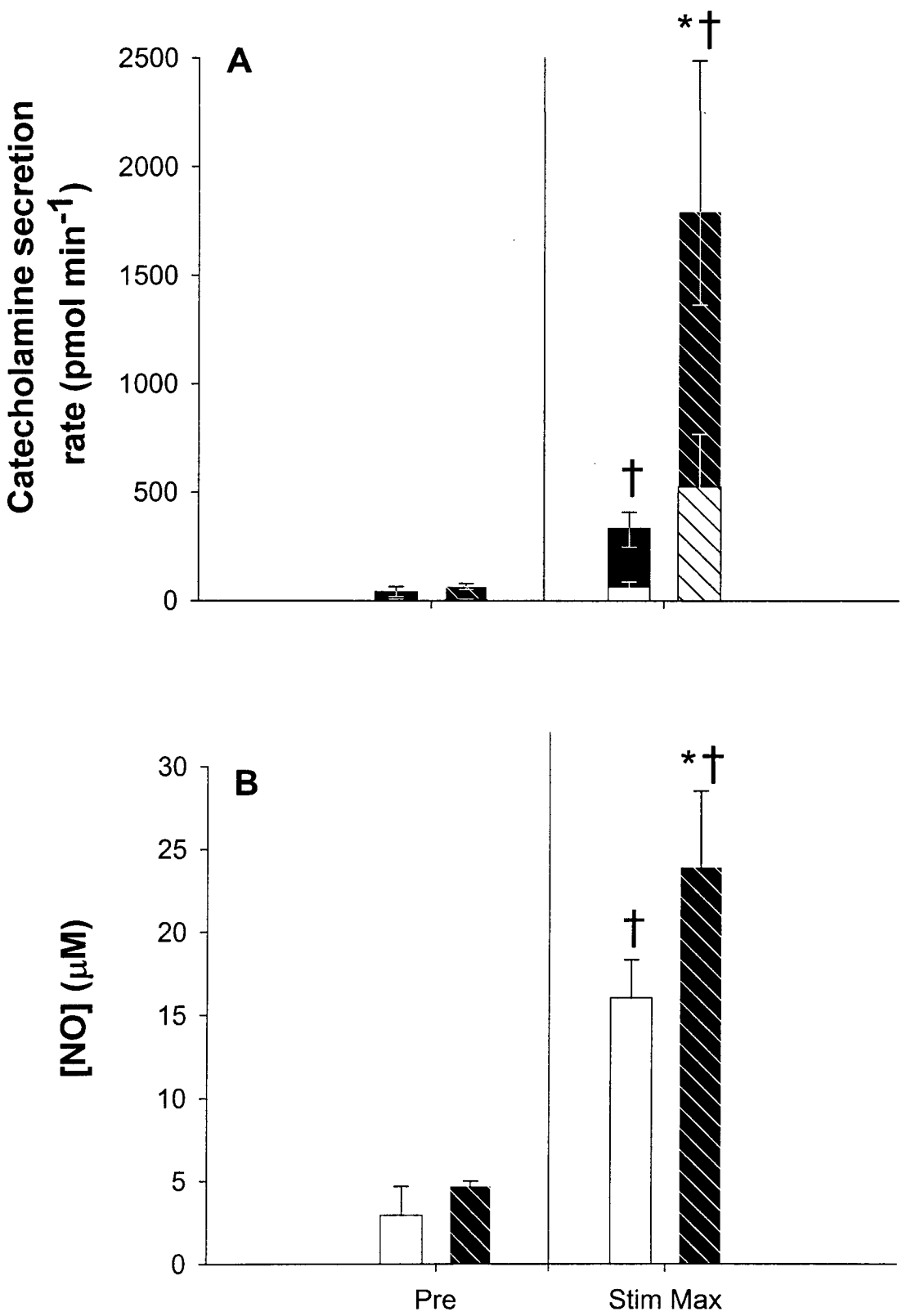

A
B
Catecholamine degredation(%)
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
-2
Log [SNP] (M)
LOG(y/(100-y)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-9
-8
-7
-6
-5
-4
-3
Log [SNP] (M)

Figure 2.8. The effects of the sGC inhibitor ODQ (10^{-4} M; cross-hatched bars; N = 6) on (A) noradrenaline (unfilled bars), adrenaline (filled bars), total catecholamines (sum of noradrenaline and adrenaline) and (B) perfusate NO levels in response to field stimulation of 30 V and 8 Hz. Values are shown as means ± 1 SEM. A dagger denotes a significant difference between pre-sample (Pre) and stimulation. An asterisk denotes a significant difference between the control (N = 6) and ODQ (cross-hatched bars) treated group.


A
Catecholamine secretion rate (pmol min-1)
2500
2000
1500
1000
500
0
*†
†
B
[NO] (μM)
30
25
20
15
10
5
0
*†
†
Pre
Stim Max

Discussion

The results of this chapter support the previous studies that used heterologous antibodies to localize nNOS in the head kidney region (Jimenez et al., 2001; Gallo and Civinini, 2001). By using a partial nNOS cDNA sequence, I was able investigate the distribution of nNOS in various tissues using real-time semi-quantitative PCR. Not only was nNOS localized in the anterior kidney, but also in the posterior cardinal vein, the primary site of chromaffin cells in the rainbow trout.

Role of NO

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. The NO donor, SNP, almost completely inhibited the stimulus-evoked catecholamine secretion. However, as in mammals (Rodriguez-Pascual et al., 1995; Marley et al., 1995) a rise in NO levels does not appear to have any effect on 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 might act 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 secretion in the presence of NO, namely an effect of NO on catecholamine stability and/or intracellular signaling events triggered by 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 binding to the heme iron group of sGC (Schwarz et al., 1998; Vicente et al., 2002) producing a conformational change that results in an increased conversion of guanisine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP; Ferrero et al., 2000). The rise in cGMP leads to the activation of protein kinase G (PKG), which in turn phosphorylates the Ca^{2+} channels (Schwarz et al., 1998; Hirooka et al., 2000), resulting in an attenuation of the inward Ca^{2+} flux in response to stimulation. In the present study, the sGC inhibitor, ODQ, was used to prevent PKG activation (and thus 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 from the perfused PCV preparation, the activation of sGC would appear to be the primary mechanism. In the presence of ODQ, both NO and catecholamine secretion levels were significantly elevated 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 binding of Ach to 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 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 (Figure 2.4B), it is possible that a similar situation may exist for NO production and release.

Results from the present study suggest that NO production/release is frequency dependent, as demonstrated in Fig 2.4. 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.

Conclusions

The results of this chapter suggests that NO inhibits stimulus-evoked catecholamine secretion through the activation of sGC and that NO production during electrical stimulation may be frequency dependent. In addition to its role in inhibiting secretion, NO can contribute to a lowering of plasma catecholamine levels by increasing catecholamine degradation. With the finding that NO can regulate catecholamine secretion and stability in trout, further research (Chapter 3) will address the relative involvement of the various NOS isoforms.

CHAPTER 3

ASSESSING THE ROLES OF THE DIFFERENT NITRIC OXIDE SYNTHASE ISOFORMS IN THE REGULATION OF STIMULUS EVOKED CATECHOLAMINE SECRETION

Introduction

In 1980, Furchgott and Zawadski discovered a substance released from the endothelium that was implicated in blood vessel relaxation. They named this substance the endothelial-dependent relaxing factor (EDRF). The EDRF was later identified in 1987 as NO (Khan and Furchgott, 1987; Palmer et al., 1987). Since then, more than 14000 papers have been published (Moncada et al., 1997) implicating NO in a wide range of physiological functions including memory (Xu et al., 2001), immune response (Chi et al., 2003) and neurotransmission in both the central (Pepicelli et al., 2004) and peripheral (Bland-Ward and Moore, 1995) nervous systems.

To date, three isoforms of nitric oxide synthase (NOS), the enzyme responsible for NO production, have been identified. The nomenclature used to describe the isoforms reflects the tissue from which the enzyme was initially characterized. The nomenclature can be misleading, however, because it is now known that the isoforms are broadly distributed in tissues. For example, although neuronal NOS (nNOS) was originally purified and cloned from neuronal tissue (Moncada et al., 1997), it is also found in the epithelium of the bronchi and trachea (Kobzik et al., 1993), as well as in skeletal muscle (Kobzik et al., 1994). Inducible NOS (iNOS), originally purified and cloned from a macrophage cell line, has since been identified in cells as diverse as cardiac myocytes, glial cells and vascular smooth muscle cells (Michel and Feron, 1997). Endothelial NOS (eNOS), originally purified and cloned from vascular endothelium, is now known to occur in cardiac myocytes, blood platelets and brain (Michel and Feron, 1997).

The NOS enzymes are homodimers whose subunits are comprised of a reductase domain containing FAD, FMN, and NADPH, an oxygenase domain containing 6(R)-tetrahydrobiopterin (H4B), iron protoporphyrin IX (heme), and a binding site for the L-arginine substrate (Santolini et al., 2001). For all three isoforms, NO synthesis depends upon the enzyme's binding of the ubiquitous Ca^{2+} regulatory protein CaM (Michel and Feron, 1997). The activities of eNOS and nNOS are dependent upon elevated intracellular Ca^{2+} concentrations for binding CaM (Boomershine et al., 1999). INOS, on the other hand, has a unique ability to irreversibly bind CaM independently of intracellular Ca^{2+} levels (Moncada and Higgs, 1993).

In rainbow trout, two of the three NOS isoforms, iNOS (Barroso et al., 2000) and nNOS (Gallo and Civinini, 2001; Jimenez et al., 2001), have been localized in the head kidney region which is where the main population of chromaffin cells lining the posterior cardinal vein are found (reviewed by Reid et al., 1998). The other form, eNOS, although not yet localized in rainbow trout, has been found in vascular smooth muscle of mammals (Cortes et al., 1999). Thus, in trout all isoforms of NOS could potentially be involved in the regulation of stimulus evoked catecholamine secretion that was demonstrated in Chapter 2. Therefore, the goal of this series of experiments was to evaluate the relative roles of the different NOS isoforms in the regulation of stimulus evoked catecholamine secretion.

Materials and methods

Experimental animals

Rainbow trout weighing between 180 and 260 g (mean mass 236.1 ± 12.4; N = 81) were maintained under the conditions outlined in Chapter 2.

Series 1: Tissue distribution of iNOS

Tissue collection and RNA extraction

Fish were killed by a sharp blow to the head and tissues were collected and frozen immediately in liquid N_2 and stored at –80°C. Tissues collected consisted of brain, posterior cardinal vein and kidney. Total RNA was extracted using Stratagene Absolute RNA RT-PCR miniprep kit (Stratagene) according to the instructions of the manufacturer. RNA concentrations and quality were verified using spectrophotometry (Eppendorf BioPhotometer).

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 (0.5 μg) 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 and β-actin for rainbow trout were designed using DNAMAN (version 4.0, Lynnon Biosoft) from the cDNA sequences obtained from GenBank. Relative expression of mRNA levels was determined (using actin as a standard) 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 same primers were used for nNOS as in Chapter 2.

Series 2: NO production during specific activation of iNOS.

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 mM) 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.

Series 3: The effect of NOS inhibitors on stimulus evoked NO production

Preparations were perfused for the entire experiment with a cocktail of the inhibitors, 7-nitroindazole (7-NI; 10^{-4} M; Sigma) and N-nitro L-arginine methyl ester (L-NAME; 5 X 10^{-3} M; Sigma). L-NAME is a commonly used non-specific NOS inhibitor (Rodriguez-Pascual et al., 1995; Dick and Lefebvre, 1997). 7-NI is a specific inhibitor thought to be selective for nNOS (Bland-Ward and Moore, 1995; Schwarz et al., 1998). While L-NAME could be added directly to saline, 7-NI was prepared in methanol prior to the addition to the saline. Following the pre-sample collections, preparations received an electrical stimulus of 30 V and 8 Hz.

Subsequent experiments were performed using L-NAME or 7-NI, alone. Perfused preparations either received saline containing 7-NI (10^{-4} M), prepared as described above, or L-NAME (5 X 10^{-3} M). Pre-samples were collected following the 20 min stabilization period, at which point all preparations were stimulated at 30 V and 8 Hz.

Series 4: Hypoxia-induced NO production from eNOS and the effects on catecholamine secretion.

Hypoxia is able to specifically induce eNOS to produce NO (Yamamoto et al., 2003). Thus, experiments were performed to evaluate whether hypoxia could directly affect NO production and if so, could the NO produced during hypoxia 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 PO_2 of the hypoxic saline solution was measured using a Foxy-AL300 fiber-optic probe and associated hardware and software (Ocean Optics). In all cases, the PO_2 of the saline was allowed to fall to 10 mm Hg 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.

Series 5: The effects of endothelium removal on stimulus evoked catecholamine secretion

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 sec, 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 ($PO_2 < 10$ mmHg, as described above), while the remaining

group continued receiving control saline. All preparations were perfused for an additional 10 min, with collections every 2 min. All preparations were then electrically stimulated at 30 V and 8 Hz.

Series 6: eNOS inhibition by 7-NI and L-NAME

This series of experiments were performed to determine if the combination of both inhibitors could inhibit hypoxia-induced NO production from eNOS. Preparations were perfused with either 7-NI (1×10^{-4} M) and L-NAME, (5×10^{-3} M) or regular saline. Following the collection of the pre-samples, fish were switched to hypoxic saline ($PO_2 < 10$ mm Hg). 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.

Analytical Procedures

Determination of NO and catecholamines levels in the perfusate were performed as outlined in Chapter 2.

Statistical analysis

The data are presented as means ± 1 standard error of the mean (SEM). All data sets were analyzed using two-way repeated measure 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).

Results

Series 1: Tissue distribution of iNOS.

Figure 3.1 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.

Series 2: NO production during specific activation of iNOS.

The control preparations responded to electrical stimulation with an approximate 11-fold increase in total catecholamine secretion and a 5-fold increase in NO production. Preparations perfused with Ca^{2+}-free saline did not exhibit an increase in catecholamine secretion (Fig 3.2A) or NO production (Fig 3.2B) in response to electrical stimulation.

Series 3: The effect of NOS inhibitors on stimulus evoked catecholamine secretion and NO production

In response to electrical stimulation, all preparations displayed an elevation in catecholamine secretion and NO production. However, the preparations treated with the combination of 7-NI and L-NAME showed an approximate 2-fold increase in adrenaline and total catecholamine secretion rates when compared to controls (Fig 3.3A). Noradrenaline levels were not different between the two groups (Fig 3.3A). Concurrently, the preparations treated with 7-NI and L-NAME showed an approximate 50% decrease in NO production (Fig 3.3B).

Preparations treated with 7-NI, alone, showed an approximate 1.8-fold increase in total catecholamine secretion rates when compared to saline perfused fish (Fig 3.4A). Catecholamine secretion rates of L-NAME treated fish were unaffected (Fig 3.4A). Stimulus evoked NO production was unaffected by either L-NAME or 7-NI, alone (Fig 3.4B).

Series 4: Hypoxia-induced NO production from eNOS and the effects on catecholamine secretion.

Preparations perfused with hypoxic saline showed no difference in basal catecholamine secretion (Fig 3.5A). However, hypoxia was associated with an approximate 3.5-fold increase in NO levels (Fig 3.5B). In response to electrical stimulation, there was an approximate 6-fold increase in noradrenaline secretion regardless of the perfusate O_2 status (Fig 3.5A). However, perfusion with hypoxic saline was associated with a 50% decrease in adrenaline secretion rates in response to electrical stimulation (Fig 3.5A). NO levels were increased 8-and 10-fold, respectively, in the normoxic and hypoxic treated preparations; there was no statistical difference between the two groups (Fig 3.5B).

Series 5: The effects of endothelium removal on stimulus evoked catecholamine secretion

In response to hypoxia, the control preparations showed an approximate 5-fold increase in NO levels whereas the saponin treated group was unaffected; levels of NO remained constant in the normoxic control group (Fig 3.6A). Catecholamine levels for

the saline injected preparations treated with hypoxia showed an approximate 41% decrease in adrenaline and a 40% decrease in total catecholamine secretion rates. Saponin treated preparations showed no statistical difference from the normoxia treated group for either catecholamine (Fig 3.6A) or NO (Fig 3.6B).

Series 6: eNOS inhibition by 7-NI and L-NAME

In response to hypoxic saline perfusions, preparations treated with the combination of 7-NI and L-NAME showed an approximate 1.8 -fold increase in adrenaline and total catecholamine secretion rates when compared to controls (Fig 3.7A). Noradrenaline levels were not different between the two groups (Fig 3.7A). The preparations treated with 7-NI and L-NAME showed an approximate 46% decrease in NO production (Fig 3.7B).

Figure 3.1. Real time PCR results showing the expression of rainbow trout iNOS relative to anterior kidney. The highest expression of this NOS isoform is seen in the anterior kidney and the lowest in the anterior posterior cardinal vein (PCV).

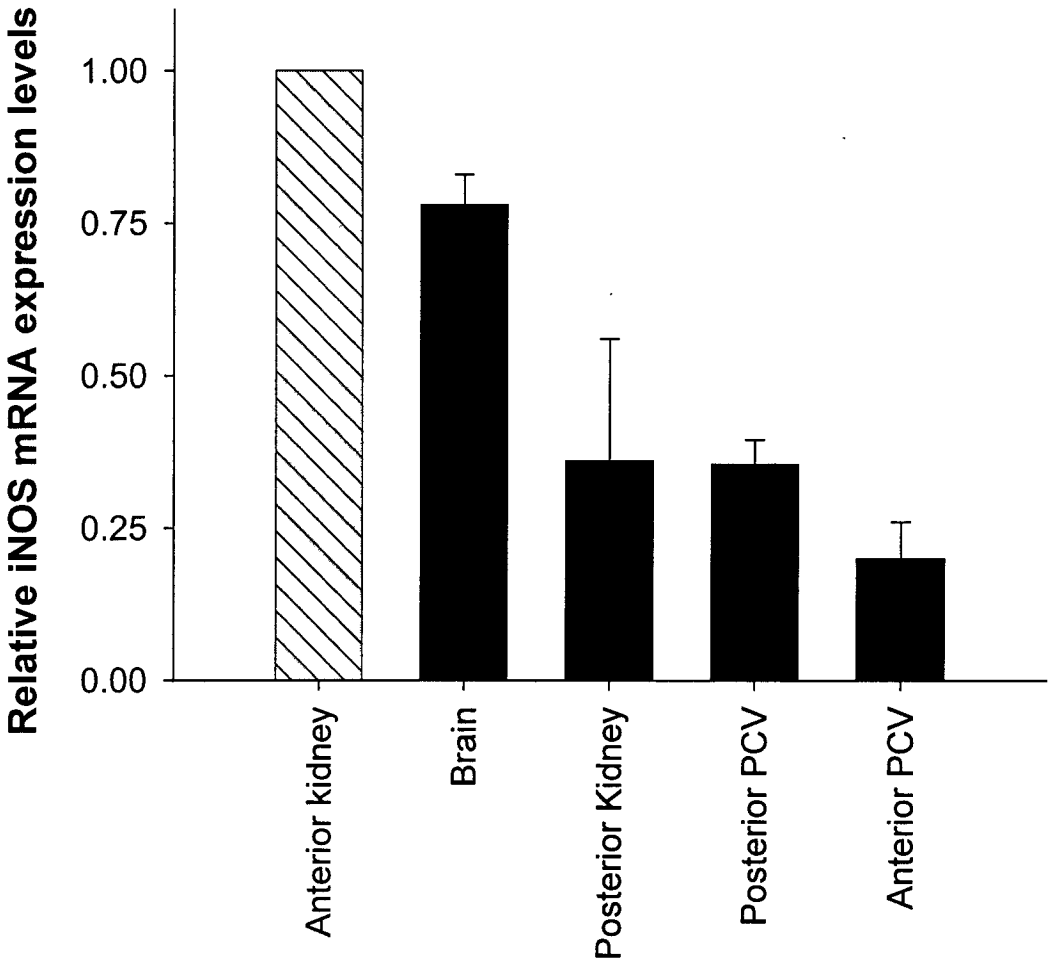

Relative iNOS mRNA expression levels
1.00
0.75
0.50
0.25
0.00
Anterior kidney
Brain
Posterior Kidney
Posterior PCV
Anterior PCV

Figure 3.2. The effects of calcium free saline (N = 6; cross-hatched bars) on *in situ* (A) noradrenaline (filled bar), adrenaline (unfilled bar), total catecholamines (sum of noradrenaline and adrenaline) and (B) perfusate NO production in response to electrical stimulation at 30 V and 8 Hz. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) 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).

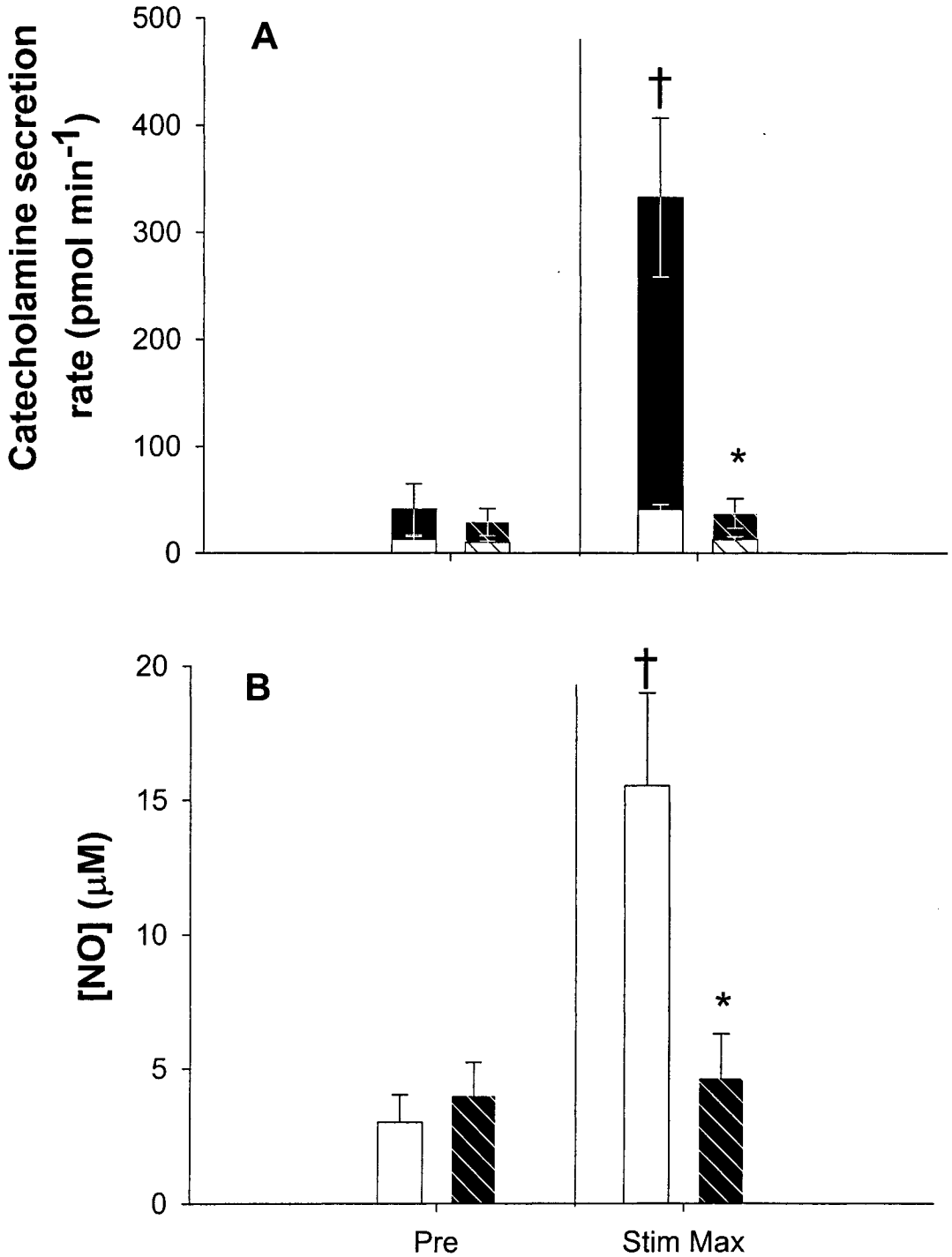

A
Catecholamine secretion rate (pmol min-1)
500
400
300
200
100
0
†
*
B
[NO] (μM)
20
15
10
5
0
†
*
Pre
Stim Max

Figure 3.3. The effects of NOS inhibition (using a cocktail of 7-NI and L-NAME; N = 9; cross-hatched bars) on (A) noradrenaline (unfilled bar), adrenaline (filled bar), total catecholamine secretion (sum of noradrenaline and adrenaline) and (B) perfusate NO levels of electrically stimulated preparations at 30 V and 8 Hz. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) and stimulated groups. An asterisk denotes a significant difference ($P < 0.5$) between the control ($N = 6$) and inhibitor treated group (cross-hatched).

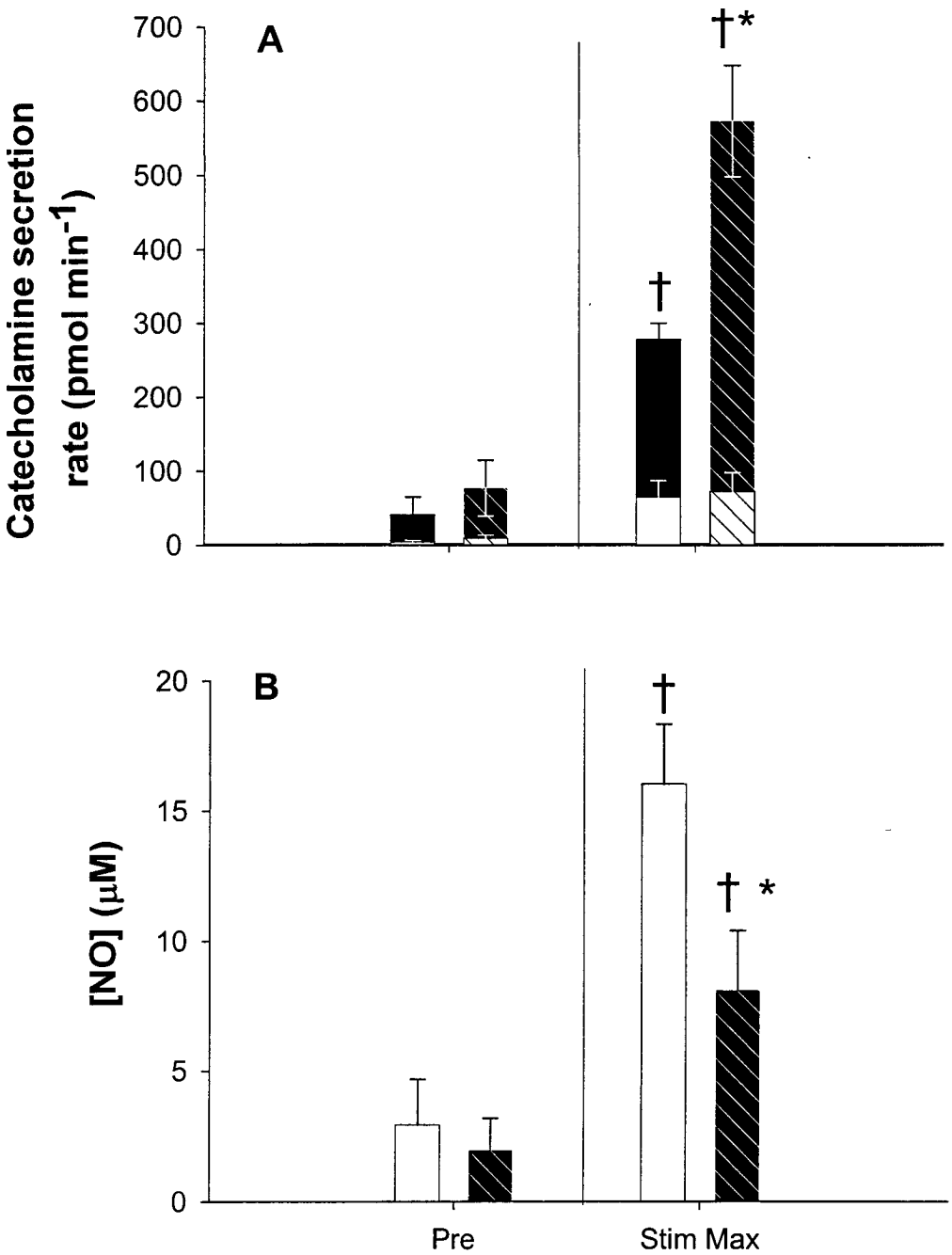
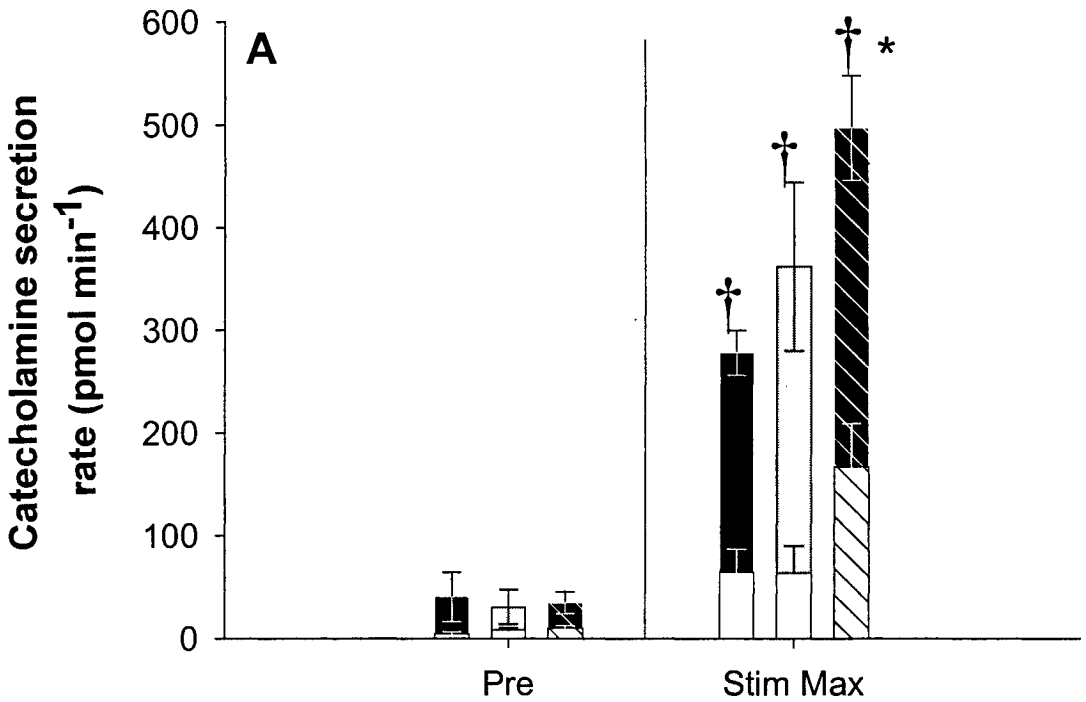

A
Catecholamine secretion rate (pmol min-1)
700
600
500
400
300
200
100
0
†
†*
B
[NO] (μM)
20
15
10
5
0
†
† *
Pre
Stim Max

Figure 3.4. The effects of 7-NI (10^{-4} M; N = 7; cross-hatched bars) or L-NAME (5 X 10^{-3} M; N = 9; gray bars) on (A) noradrenaline (white bars), adrenaline (black and gray bars), total catecholamine (sum of noradrenaline and adrenaline) and (B) perfusate NO levels of electrically stimulated preparations at 30 V and 8 Hz. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) and stimulated groups. An asterisk denotes a significant difference ($P < 0.5$) between the control and inhibitor treated group (cross-hatched).


A
Catecholamine secretion rate (pmol min-1)
600
500
400
300
200
100
0
Pre
Stim Max
*

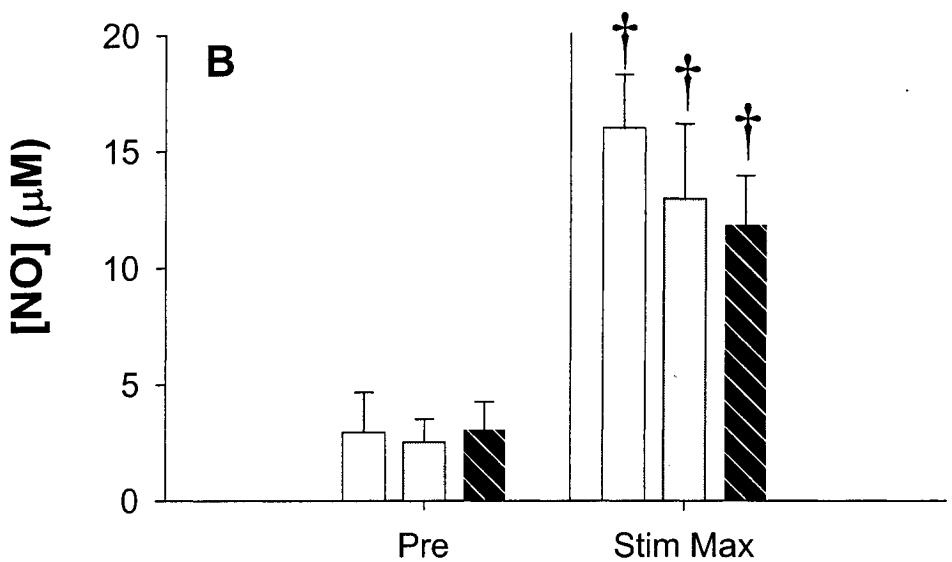

B
[NO] (μM)
20
15
10
5
0
Pre
Stim Max

Figure 3.5. The effects of hypoxic perfusate (N = 6; cross-hatched bars) on (A) noradrenaline (unfilled bars), adrenaline (filled bars), total catecholamine (sum of noradrenaline and adrenaline) and (B) perfusate NO levels in response to electrical stimulation of 30 V and 8 Hz. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) and stimulated groups. An asterisk denotes a significant difference ($P < 0.5$) between the normoxia (N = 6) and hypoxia treated group (cross-hatched).

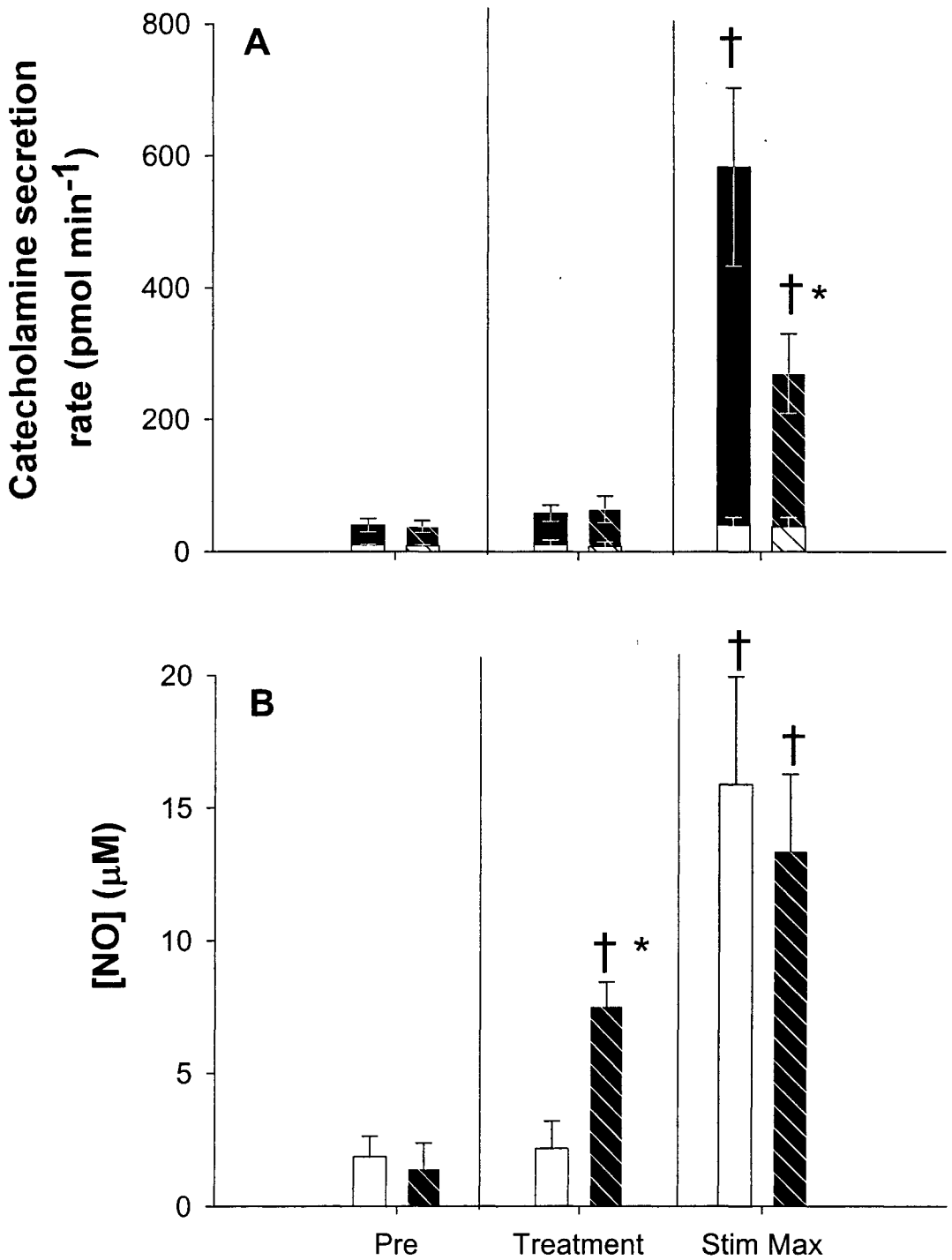

A
Catecholamine secretion rate (pmol min-1)
800
600
400
200
0
†
†*
B
[NO] (μM)
20
15
10
5
0
†*
†
†
Pre
Treatment
Stim Max

Figure 3.6. In response to hypoxia treatment, preparations injected with saponin (0.1%; cross-hatched; N = 6) showed no difference in basal (A) noradrenaline (unfilled bars), adrenaline (filled bars) and total catecholamine secretion (sum of noradrenaline and adrenaline) and (B) perfusate NO levels over untreated treated preparations (N = 6). Saponin treated fish showed a significant decrease in hypoxia induced NO perfusate levels (B), while stimulus-evoked catecholamine secretion was significantly increased (A). Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) and stimulated groups. An asterisk denotes a significant difference ($P < 0.5$) between the control and saponin treated group (cross-hatched).

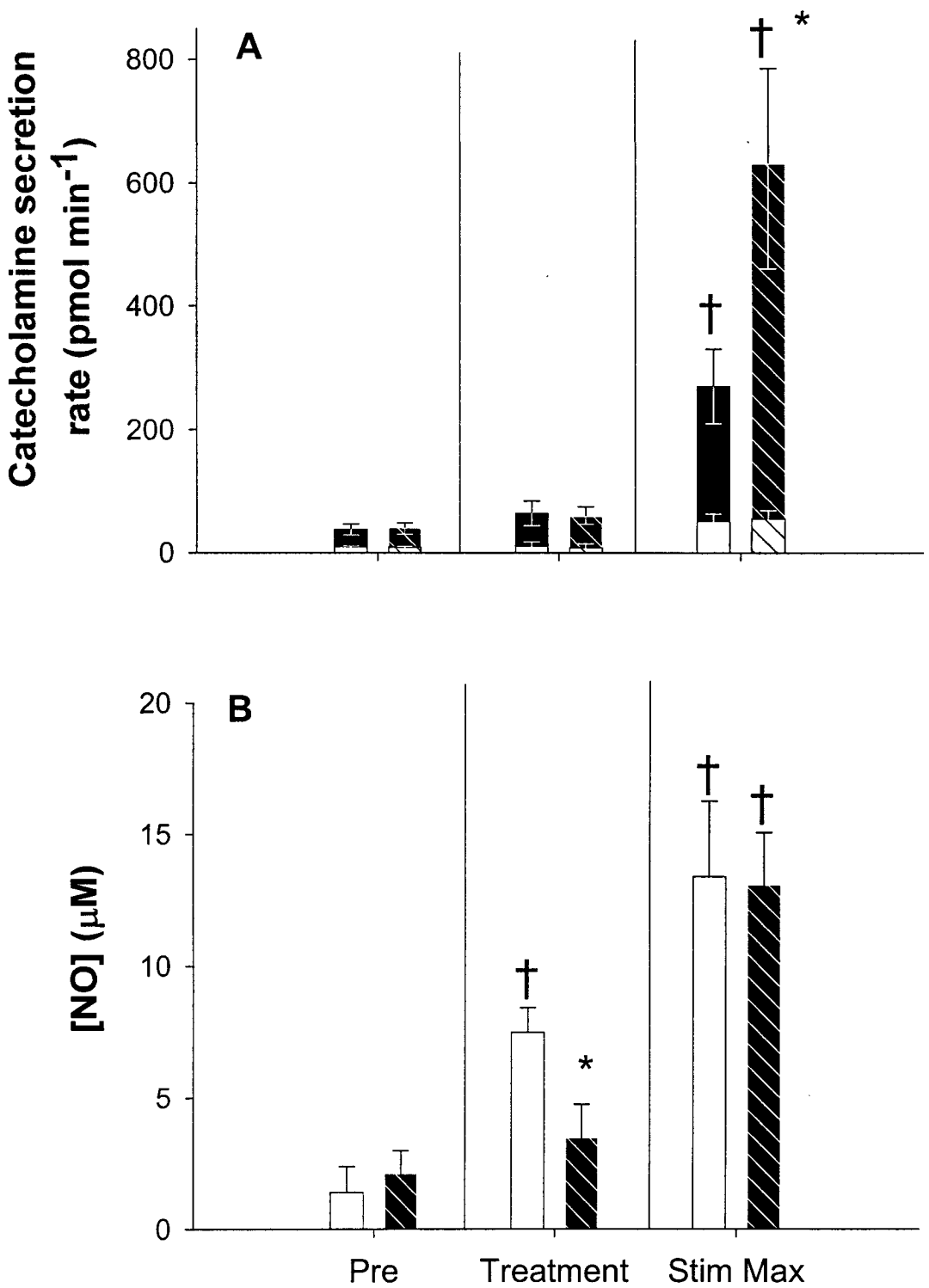
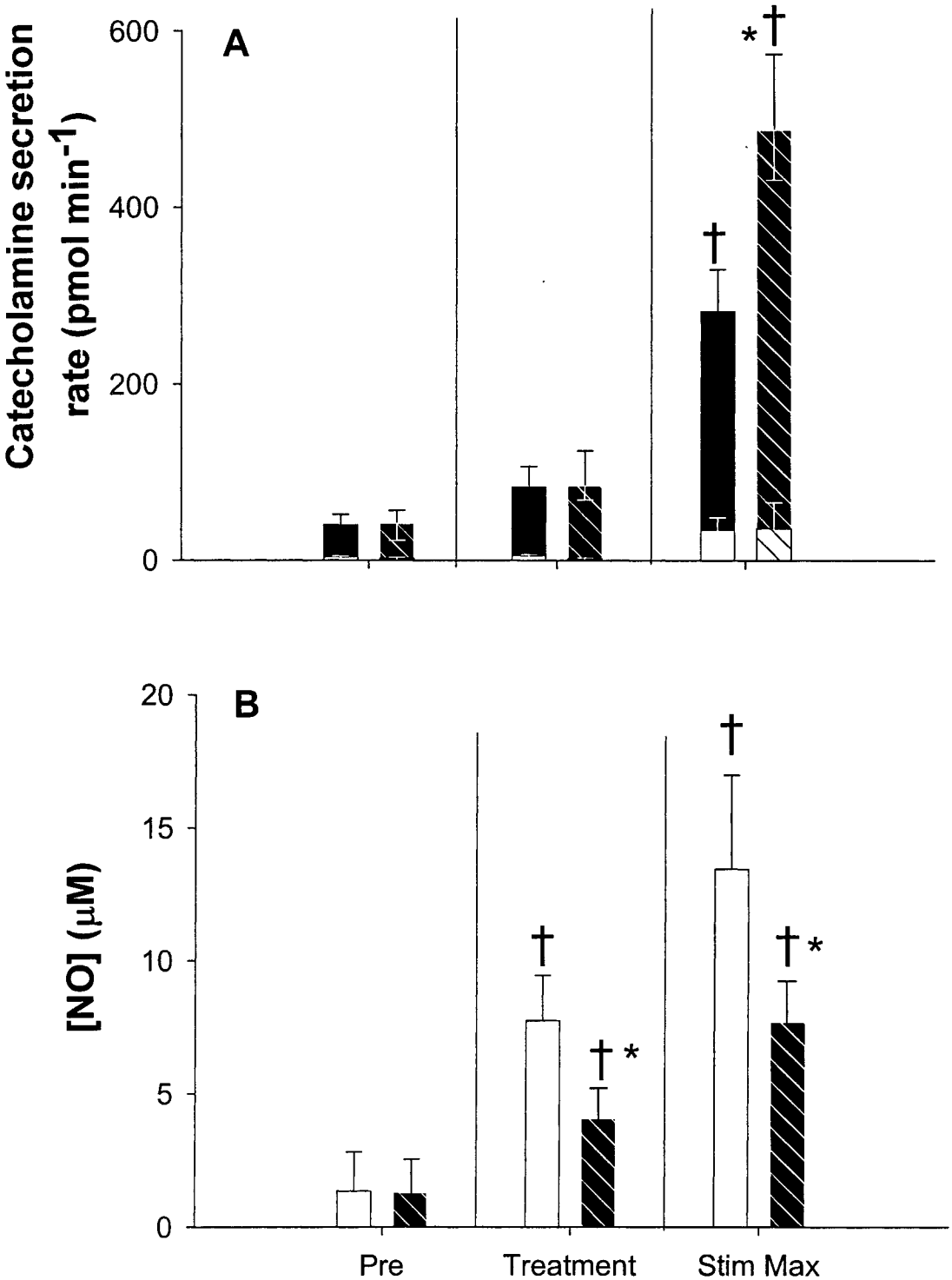

A
Catecholamine secretion rate (pmol min-1)
800
600
400
200
0
†
†
*
B
[NO] (μM)
20
15
10
5
0
†
*
†
†
Pre
Treatment
Stim Max

Figure 3.7. The effects of NOS inhibition (using a combination of L-NAME (5×10^{-3} M) and 7-NI (10^{-4} M; cross-hatched; N = 7) on hypoxia ($PO_2 < 10$ mm Hg)- or stimulus - evoked on (A) noradrenaline (unfilled bars), adrenaline (filled bars) and total catecholamine (sum of noradrenaline and adrenaline) secretion rates and (B) perfusate NO levels. Following the 10 min hypoxia treatment, preparations were electrically stimulated at 30 V and 8 Hz. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between pre-experimental (Pre) and stimulated groups. An asterisk denotes a significant difference ($P < 0.5$) between the control (N = 7) and inhibitor treated group (cross-hatched).


A
Catecholamine secretion rate (pmol min-1)
600
400
200
0
*†
†
†
B
[NO] (μM)
20
15
10
5
0
†
†*
†
†*
Pre
Treatment
Stim Max

Discussion

The results of this chapter show that iNOS is present in tissues known to contain high densities of chromaffin cells including the posterior cardinal vein and head kidney. Because of a high binding affinity for CaM, iNOS does not require an elevation of intracellular Ca^{2+} for its activation as required by both eNOS and nNOS (Oset-Gasque et al., 1994). Therefore, under Ca^{2+} free conditions it is expected that only iNOS would be activated by electrical stimulation. Assuming a similar situation in trout, the results of this study suggest that iNOS is not contributing 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) and confirmed that the perfusate was indeed devoid of Ca^{2+}. 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.

On the basis of previous studies on mammals, nNOS is believed to be the main isoform involved in the regulation of catecholamine secretion (Schwarz et al., 1998; Vicente et al., 2002). However, there is also evidence implicating eNOS (Torres et al., 1994; Barnes et al., 2001). In the present study, several inhibitors, alone or in combination, were used to inhibit NO production in response to stimulation and to distinguish the relative roles of eNOS and iNOS. Because there are no selective eNOS inhibitors, the non-selective NOS inhibitor L-NAME was chosen (Torres et al., 1994; Schwarz, et al., 1998; Vicente et al., 2002); 7-NI was used as a selective nNOS inhibitor (Mayer and Andrew, 1998; Vicente et al., 2002). The combination of both inhibitors was able to reduce stimulus evoked NO production by 50%, which could account for the

increase in catecholamine secretion observed in the inhibitor treated preparations. Although controversial, the inhibitory effects of NO on catecholamine secretion are believed to reflect the phosphorylation of Ca^{2+} channels, resulting in a reduced accumulation of intracellular Ca^{2+} in response to stimulus (Schwarz et al., 1998)

To determine the potential involvement of nNOS, the effects of 7-NI were assessed. In this series, catecholamine secretion rates following electrical stimulation were increased when compared to controls yet there was no difference in NO production. The reason for this apparent discrepancy is unclear; however one could speculate that other isoforms, specifically eNOS, could account for the high NO levels without affecting catecholamine secretion rates. The lack of an effect of NOS generated by eNOS activation might simply reflect the spatial separation between the site of NOS production and the chromaffin cells. In subsequent experiments, L-NAME, a non-specific NOS inhibitor, was used to try and block all NOS isoforms. It was observed that the effects of L-NAME treatment were similar to those of 7-NI, an increase in catecholamine secretion rates with no apparent difference in NO levels. Again, the reason for this is unclear, but it is possible that like 7-NI, L-NAME inhibited nNOS resulting in less NO production in the vicinity of chromaffin cells and thus an increase in catecholamine secretion. Conceivably, NO appearance in the perfusate would reflect incomplete inhibition of eNOS.

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 role of the different isoforms is proving to be difficult. In mammals, the NOS homodimers show high sequence similarity 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 this reason, 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). 7-NI, the inhibitor used in this study, as well as in several others (Barnes et al., 2001; Xu et al., 2001), as a selective nNOS inhibitor, has been 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. Thus, in an attempt to differentiate between nNOS and eNOS, other experiments were performed without further use of inhibitors. Yamamoto et al., (1999) demonstrated that hypoxia treatment is able to induce NO production from eNOS in blood vessels. The results obtained in the present study confirm that hypoxia treatment results in an increase in NO production, which was shown to decrease stimulus-evoked catecholamine secretion. To determine whether the increase in NO production during hypoxia was a consequence of eNOS activation, the results from another study were applied. Several previous studies have demonstrated that saponin treatment destroys the 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 is 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 then eNOS is the principal producer of NO during electrical stimulation of the chromaffin cells.

Conclusions

This chapter showed that iNOS, although present in the vicinity of the chromaffin cells, does not play a role in NO production in response to electrical stimulation. Although eNOS may play a role in regulating catecholamine secretion during hypoxia, the predominant source of NO (and hence catecholamine regulation) during electrical stimulation is via the nNOS isoform.

CHAPTER 4
THE INTERACTIVE EFFECTS OF REPETITIVE HYPOXIA *IN VIVO* AND NO ON CATECHOLAMINE SECRETION

Introduction

In response to severe acute stressors, the catecholamine hormones, noradrenaline and adrenaline, are released into the circulatory system (see review by 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 in the vicinity of the head kidney (Nandi, 1967). 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 (Thomas and Perry, 1992; Perry and Reid, 1994).

The primary mediator of catecholamine secretion from the chromaffin cells in rainbow trout is the activation of nicotinic cholinergic receptors by acetylcholine (Montpetit and Perry, 1998). This leads to an influx of extracellular Ca^{2+} into the chromaffin cell, which initiates a series of events culminating in catecholamine secretion via exocytosis (Burgoyne et al., 1993; Furimsky et al., 1996). Similarly, the increase in intracellular Ca^{2+} levels associated with stimulation of the chromaffin cell activates eNOS and nNOS through the binding of CaM (Schwarz et al., 1998; Vicente et al., 2002). The subsequent increase in NO levels leads to the activation of sGC, which, in turn, results in the phosphorylation of Ca^{2+} channels and a reduction in catecholamine secretion rates.

In rainbow trout, acute hypoxia is a potent stimulus for the secretion of catecholamines into the circulation (Ristori and Laurent, 1989). The abrupt release of catecholamines during hypoxia exposure appears to correspond with a reduction of arterial blood O_2 concentration of approximately 50% (Perry and Reid, 1994; Perry and Gilmour, 1996; Perry et al., 2004). Although the exact mechanisms causing catecholamine release during hypoxia are unknown, it is believed that both neuronal and non-neuronal mechanisms are involved (Perry et al., 1991; Reid and Perry, 1998). In mammals, hypoxia is known to increase NO production in various tissues including skeletal muscle (Javeshghani et al., 2000), brain (Prabhakar et al., 1996; Armstead, 1998) and lung (Vaughan et al., 2003). The NOS isoforms that are believed to be responsible are eNOS and nNOS, with their involvement varying between tissue and species (Vaughan et al., 2003). iNOS, which plays a significant role in the immune response (Chi et al., 2003), is not believed to participate in the hypoxia response (Javeshgani et al., 2000; Yamamoto et al., 2003). It has been suggested that a local elevation of NO during hypoxia would promote vascular dilation and increase regional blood flow (Prabhakar et al., 1996; Javeshghani et al., 2000; Yamamoto et al., 2003).

Previous chapters have clearly demonstrated the ability of NO to decrease stimulus-evoked catecholamine secretion. Moreover, it was demonstrated that the nNOS isoform is mainly responsible for the decrease in stimulus-evoked catecholamine secretion. These results confirm previous findings that indirectly suggested the involvement of nNOS is the regulation of catecholamine secretion in rainbow trout (Jimenez et al., 2001; Gallo and Civinini, 2001). It is unclear, however, to what extent nNOS is regulated *in vivo* to control catecholamine secretion. In the present study, fish

were subjected to repeated bouts of acute hypoxia over a 4 day period to induce repetitive episodes of catecholamine secretion. It was hypothesized that a consequence of repeated bouts of catecholamine secretion would be induction of nNOS and a resultant inhibition of catecholamine secretion during activation of chromaffin cells.

MATERIAL AND METHODS

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing between 160 and 300 g (mean mass = 231.6 ± 12.8 g; N = 25) were obtained from Linwood Acres Trout Farm and maintained under conditions outlined in Chapter 2.

In situ perfused posterior cardinal vein

An *in situ* perfused posterior cardinal vein preparation was prepared and utilized as outlined in Chapter 2.

Series 1: Effects of repeated hypoxia exposure *in vivo*

Animal Preparation

Rainbow trout were anaesthetized in an aerated solution of ethyl-P-amino-benzoate (benzocaine; Sigma; 2.4 X 10^{-4} M). 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) was implanted into the dorsal aorta via 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 and allowed to recover for 24 h prior to experimentation.

Experimental protocol

Trout were exposed twice daily for 30 min to acute hypoxia for a period of 4 days. Acute hypoxia was achieved by replacing the air supplying a water/gas

equilibration column with N_2. The PO_2 was dropped and kept at approximately 45 mm Hg, at which point the 30 min began. This degree of hypoxia was chosen on the basis of a previous study (Perry and Reid, 1992), showing significant catecholamine release at this PO_2. The water PO_2 was monitored using a Foxy- AL300 O_2 fiber optic probe (Ocean Optics). Blood samples were collected prior to, and following, each exposure to hypoxia and analyzed for catecholamine and NO levels.

Series 2: The effect of repeated hypoxia *in vivo* on *in situ* catecholamine secretion and NO production

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 PO_2 was decreased, as described above, to approximately 45 mm Hg. 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 posterior cardinal vein preparations were derived from these fish as outlined in Chapter 2. Control fish were placed in the Perspex boxes and received normoxic water for 4 days.

Preparations were perfused for 20 min at which point 2 pre-samples were collected. The preparations were then electrically stimulated at 30 V and 8 Hz. Following the collection of the final sample, tissues were collected and frozen in liquid N_2. The tissues included the brain, posterior cardinal vein, and kidney. The RNA was extracted as outlined in Chapter 3 and used for real-time PCR determination of nNOS mRNA levels using β–actin as the reference gene (see Chapter 3).

Analytical Procedures

Catecholamine and NO levels were determined as outlined in Chapter 2.

Statistical analysis

The data are presented as means ± 1 standard error of the mean (SEM). All data sets from *in situ* perfusions were analyzed using two-way repeated measure 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. Real-time PCR data were analyzed using Student's t-test. All statistical tests were performed using a commercial statistical software package (SigmaStat version 2.03).

Results

Series 1: Effects of repeated hypoxia exposure *in vivo*

Upon exposure of fish to acute hypoxia, a significant increase in circulating catecholamine levels was observed (Fig. 4.1A). Plasma NO levels were also elevated following each exposure to hypoxia (Fig 4.1B). Basal levels of NO were increased by approximately 2-fold prior to the final two exposures on the fourth day. Basal catecholamine levels, on the other hand, were unaffected while hypoxia-evoked catecholamine levels were decreased markedly during the final two exposures on day 4 (Fig. 4.1A).

Series 2: The effect of repeated hypoxia *in vivo* on *in situ* catecholamine secretion and NO production

This series was performed to determine if repeated hypoxia exposure was associated with any effects on NO enzyme activity or nNOS mRNA 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 approximate 50, 46 and 44% decrease in stimulus-evoked noradrenaline, adrenaline and total catecholamine secretion respectively (Fig 4.2A). There were no effects on basal catecholamine secretion. *In situ* preparations derived from fish exposed to repeated hypoxia displayed an approximate 2-fold increase in basal NO levels and a 1.8-fold increase in stimulus-evoked NO production (Fig 4.2B).

To investigate whether the increase in NO in fish repeatedly exposed to hypoxia was a result of increased levels of nNOS, , tissues were collected and processed by real-

time PCR to assess nNOS mRNA levels. An increase in mRNA, if translated to protein, would suggest an increase in nNOS quantity. On the other hand, if mRNA did not increase it would suggest that nNOS activity was increasing as a result of post translational modifications. In the fish repeatedly exposed to hypoxia, the mRNA levels in all tissues tended to be higher, however only brain and anterior posterior cardinal vein showed a statistically significant increase in nNOS mRNA (Fig 4.3).

Figure 4.1. The effects of 30 min exposure to acute hypoxia ($PO_2 < 45$ mm Hg) on plasma (A) total catecholamines (filled bars; N = 4) and (B) NO (filled bars; N = 3). Unfilled bars are basal levels prior to hypoxia exposure. Fish were exposed twice daily to acute hypoxia for a total of 4 days. Values are shown as means ± 1 SEM. A dagger denotes a significant difference ($P < 0.5$) between the pre-hypoxia values (unfilled bars) and 10 min into hypoxia exposure (filled bars). An asterisk denotes a significant difference ($P < 0.5$) in stimulatus-evoked catecholamine secretion from the peak value (cross-hatched). A double asterisk denotes a significant difference ($P < 0.5$) in basal and stimulus-evoked NO levels from the lowest values (cross-hatched)

I

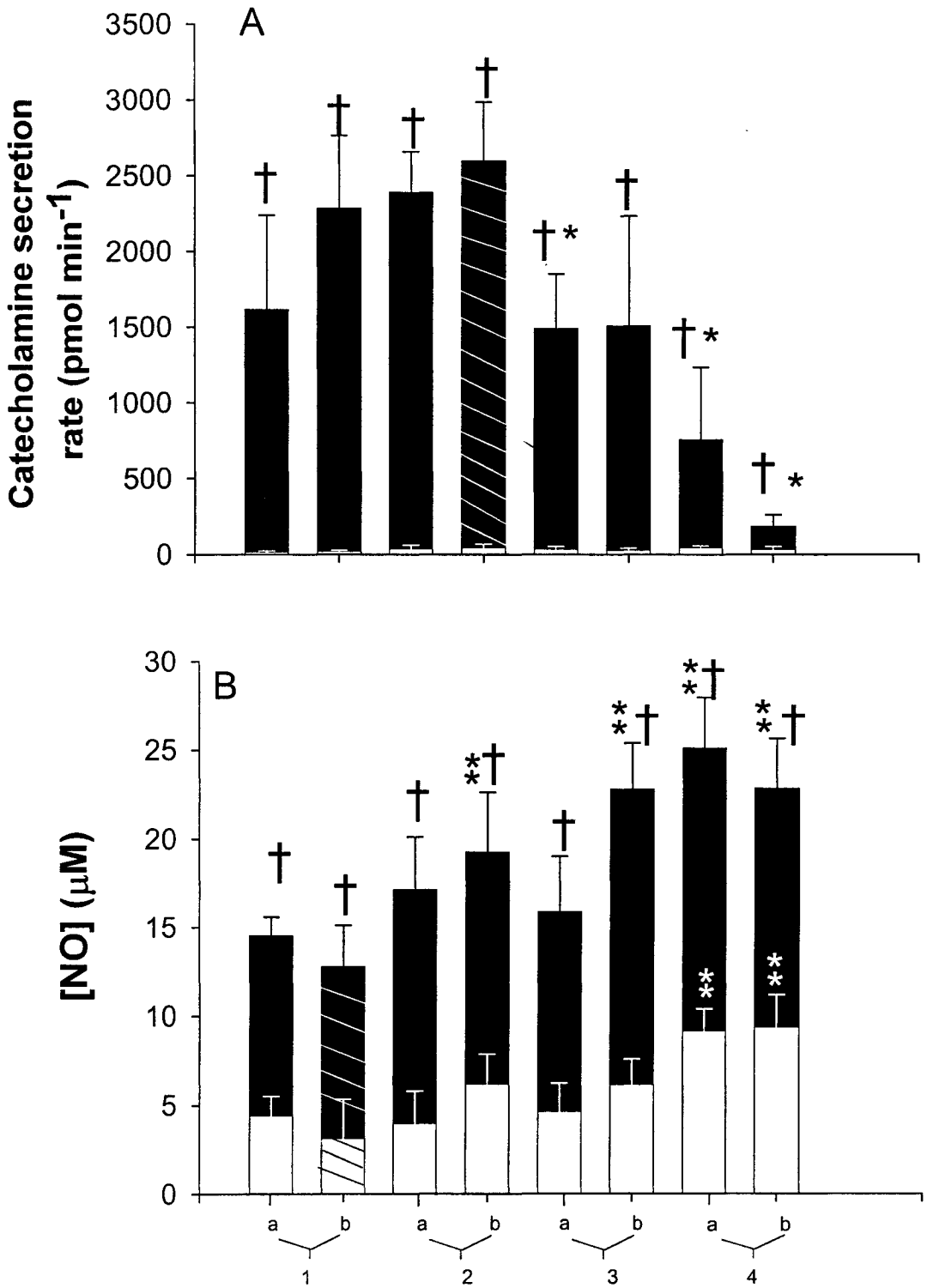

A
Catecholamine secretion rate (pmol min-1)
3500
3000
2500
2000
1500
1000
500
0
B
[NO] (μM)
30
25
20
15
10
5
0
a
b
1
2
3
4

Figure 4.2. The effects of *in situ* electrical stimulation on (A) noradrenaline (unfilled bars), adrenaline (filled bars) and total catecholamine secretion (sum of noradrenaline plus adrenaline) and (B) perfusate NO levels in preparations derived from fish repeatedly exposed to hypoxia (cross-hatched; N = 9) or control fish (N = 9). Following the stabilization period, the preparations were electrically stimulated at 30 V and 8 Hz. Values are shown as means ± 1 SEM. 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 control and hypoxic treated group (cross-hatched).

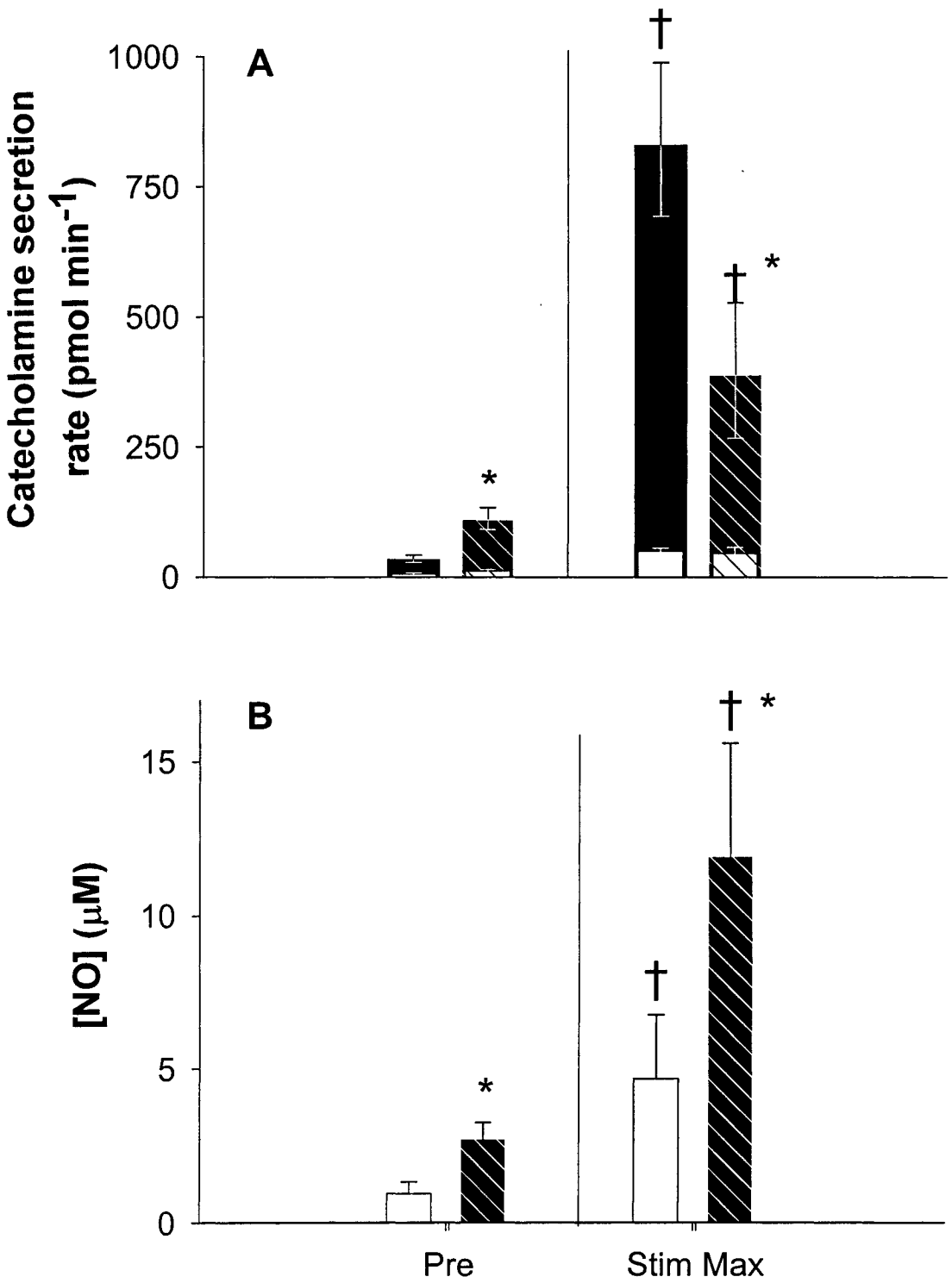
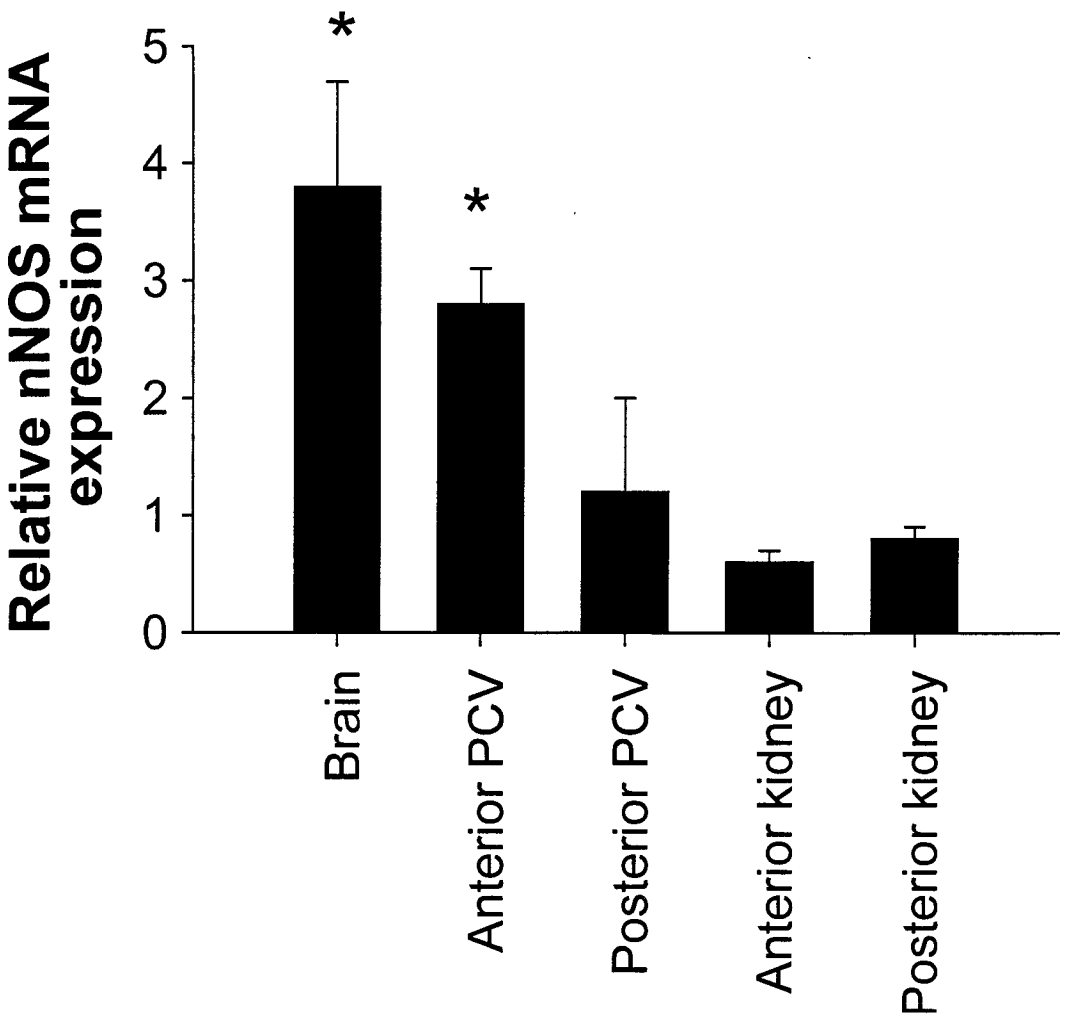

A
Catecholamine secretion rate (pmol min-1)
1000
750
500
250
0
*
†
‡
*
B
[NO] (μM)
15
10
5
0
*
†
† *
Pre
Stim Max

Figure 4.3. The effects of repeated hypoxia exposures on the relative nNOS mRNA levels in various tissues, using β-actin as the reference gene (N = 5) by real-time PCR. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference ($P < 0.5$) in the relative mRNA levels between control fish and hypoxia exposed fish in the respective tissue.


Relative nNOS mRNA expression
5
4
3
2
1
0
*
*
Brain
Anterior PCV
Posterior PCV
Anterior kidney
Posterior kidney

Discussion

The results of Chapter 3 demonstrated that NO produced in response to hypoxia *in situ* was probably the result of eNOS induction. The removal of the endothelium using saponin prevented the increase in NO during perfusion with hypoxic saline. Those data suggested that eNOS is the isoform responsible for NO production during hypoxia because it is known to be located predominantly in the endothelium (Torres et al., 1994). Furthermore, because removal of the endothelium (Chapter 3) did not prevent stimulus evoked NO production, it is apparent that the increase in NO levels associated with electrical stimulation is not via activation of eNOS. It was demonstrated in Chapter 3 that electrical stimulation does not induce iNOS. Therefore the production of NO during electrical stimulation in the absence/inhibition of eNOS must be from nNOS. This conclusion is consistent with previous demonstrations of nNOS localization in the nerve fibers within the head kidney (Jimenez et al., 2001, Gallo and Civinini, 2001).

In a previous study, Perry et al. (2000) investigated the direct effects of hypoxia on catecholamine secretion using a similar *in situ* setup. The results of that study also demonstrated that hypoxia was without effect on catecholamine secretion, but showed, as did this study, that hypoxia treated preparations exhibited an unexplained (at that time) blunted response to stimulation. The results obtained in this chapter suggest that the mechanism whereby hypoxia impairs stimulus evoked catecholamine secretion may involve NO. During inhibition of NOS (using the combination of L-NAME and 7-NI),

NO production in response to hypoxia was greatly reduced and in turn, stimulus-evoked catecholamine secretion was substantially increased.

In vivo experiments

Fish exposed to 30 min of hypoxia responded by elevating their circulating catecholamine levels. However, on day 4, after 6 prior hypoxic exposures, the fish showed a large reduction in the magnitude of the catecholamine response to the stress. A similar reduction in catecholamine secretion at the end of a repeated stress series was also shown by Hashiguchi and colleagues (1996) in rats. They suggested that the animals habituated themselves to the stress and no longer responded. With the results obtained from the present study, this effect could be, at least in part, a result of an increase in NO production, thereby decreasing catecholamine in response to stress. It was demonstrated that NO levels in plasma increased in response to hypoxia exposure, with the highest levels being found in the final two exposures. Basal NO levels also were elevated on the final day of the repeated hypoxia regimen. As observed elsewhere in this thesis, moderate increases in NO levels elicit an inhibitory effect on stimulus-evoked catecholamine secretion rates. Therefore, the elevated NO levels *in vivo* could theoretically elicit a similar effect on catecholamine secretion during exposure to acute hypoxia.

In situ perfused preparations derived from hypoxic 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. These results suggest

that NOS is up-regulated in the fish subjected to repeated hypoxia and that the net effect of the up-regulation is inhibition of catecholamine secretion both *in vivo* and *in situ*. Because nNOS appears to be the predominant isoform involved in regulating catecholamine secretion in trout (Chapter 3), the data suggest that nNOS is being up-regulated by repeated hypoxia. To assess this possibility, tissues were collected and analyzed by real-time PCR to determine the relative levels of nNOS mRNA. In the hypoxia exposed fish, there was a trend for higher nNOS mRNA levels in all tissues examined however the only statistically significant changes were in the brain and in the anterior posterior cardinal vein. Although these data do not indicate directly that enzyme levels (i.e. protein) have increased, the increase in mRNA levels certainly is suggestive of an increase in enzyme quantity.

Conclusion

Electrical stimulation results in an increase in NO levels that can be mainly attributed to the nNOS isoform. This increase in NO has been shown in the present study to have the ability to decrease stimulus-evoked catecholamine secretion. Therefore the *in situ* and real-time PCR results demonstrating an increase in basal NO and stimulus evoked NO perfusate levels, along with an increase in relative nNOS mRNA, suggests that nNOS could be involved in the physiological regulation of catecholamines.

CHAPTER 5
GENERAL DISCUSSION

Discussion

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 of this thesis demonstrate that NO, produced during stimulation of chromaffin cells, is able to inhibit catecholamine secretion. The technique used in this thesis to evaluate catecholamine secretion was an *in situ* perfused posterior cardinal vein preparation. Although measuring the levels of catecholamines in the outflowing perfusate provides a reliable index of secretion, ultimately the levels of catecholamines in the outflowing perfusate are set by the balance between secretion and degradation. Results presented in Chapter 2 demonstrated a profound effect of NO on accelerating the rate of catecholamine degradation *in vitro*. Nevertheless, it is clear from other data presented in Chapter 2 that the primary mechanism underlying the NO-induced decrease in stimulus-evoked catecholamine levels is through the activation of sGC. This conclusion concurs with the results of prior studies using mammalian models (Oset-Gasque et al., 1994; Schwarz et al., 1998; Vicente et al., 2002). These studies showed that the activation of sGC within chromaffin cells leads to increased cGMP levels and consequent activation of PKG. It is believed that PKG phosphorylates voltage-dependent Ca^{2+} channels, thereby reducing the stimulus-evoked rise in intracellular Ca^{2+} (Schwarz et al., 1998). It is likely that the activation of sGC in chromaffin cells of trout would activate a similar pathway leading to the eventual phosphorylation of Ca^{2+} channels, reduced Ca^{2+} entry and hence a decrease in catecholamine secretion.

Frequency dependency of NO production

The field stimulation technique employed throughout this thesis elicits catecholamine secretion by electrically stimulating the pre-ganglionic nerve fibers innervating the chromaffin cells (Montpetit and Perry, 1999). These fibers, when stimulated, release both cholinergic and non-cholinergic neurotransmitters, each able to initiate catecholamine secretion to varying degrees. Previous research using the field stimulation technique demonstrated that the relative proportion of cholinergic *versus* non-cholinergic neurotransmitters being released from these nerve fibers is dependent on the frequency of electrical stimulation (Montpetit and Perry, 1999, 2000; McNeill et al., 2003). The results of this thesis provide the first demonstration that NO production from the pre-ganglionic nerve fibers also displays a pronounced frequency dependency. 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 inhibition

To further demonstrate a role for NO in the regulation of catecholamine secretion, it was important to be able to block the NO response to stimulation. To do so, the combination of two inhibitors, 7-NI and L-NAME were used. L-NAME was chosen to inhibit non-specifically all isoforms of NOS (Dick and Lefebvre, 1997; Schwarz et al., 1998; Kolo et al., 2004). 7-NI, on the other hand, was selected as a specific nNOS inhibitor (Moncada et al., 1997; Xu et al., 2001). In mammals, nNOS is localized in the chromaffin cells (Oset-Gasque et al., 1994, 1998; Schwarz et al., 1998) and in the cholinergic nerve fibers (Afework et al., 1994; Holgert et al., 1995) and it is this isoform that is thought be most important in regulating catecholamine secretion (Oset-Gasque et al., 1994, 1998; Schwarz et al., 1998; Vicente et al., 2002). Thus, the rationale for using both inhibitors in this thesis was to ensure total NOS inhibition. The finding that NOS inhibition prevented NO production while increasing catecholamine secretion during electrical stimulation suggests that modulation of catecholamine secretion by NO may occur upon neuronal activation of chromaffin cells.

Involvement of different NOS isoforms

iNOS

The iNOS isoform, which has been mainly investigated for its involvement in the immune response (Boomershine et al., 1999; Chi et al., 2003), is not thought to be involved in catecholamine regulation in mammals. To investigate this in trout, perfusions were performed with Ca^{2+}-free saline containing the Ca^{2+} chelator EGTA. Because of the Ca^{2+} dependency of the eNOS and nNOS isoforms and the independence of iNOS on an

intracellular rise in Ca^{2+} levels, only the iNOS isoform would be expected to be activated under Ca^{2+}-free conditions. Since NO levels did not increase in response to electrical stimulation under Ca^{2+}-free conditions, it implies that either eNOS and/or nNOS is responsible for the increased NO production. Although the results of this thesis do not reveal an involvement of iNOS, the possibility for it's involvement cannot be ruled out under other situations because any non-specific NOS generated NO can activate sGC.

eNOS

In the investigation of eNOS, both saponin and hypoxia treatment were utilized. Saponin was previously shown to destroy the endothelium of blood vessels (Donoso et al., 1996; Cortes et al., 1999). Exposure of blood vessels to hypoxia is known to elicit NO production from eNOS (Yamamoto et al., 2003). Thus, to confirm the destruction of the endothelium by saponin, preparations were perfused with hypoxic saline. The increase in NO production normally associated with hypoxia was prevented by using saponin. These results demonstrate that i) hypoxia is able to induce eNOS to produce NO and ii) saponin is able to destroy the endothelium and thus eliminate eNOS. Because no decrease in either catecholamine secretion or NO production was observed in the saponin treated preparations, it implies that the destruction caused by saponin was limited to the endothelium and did not affect the chromaffin cells. Perfusion with hypoxic saline and increasing NO production prior to stimulation resulted in a decrease in catecholamine secretion. Therefore, it is possible that eNOS could regulate catecholamine levels, if induced. However, it does not seem that regulation of catecholamines by eNOS is under neuronal control because the removal of the endothelium had no effect on the maximum

NO produced during stimulation. Therefore, the principal source of NO during stimulation is likely via the nNOS isoform.

nNOS

As mentioned earlier, the NO produced during stimulation seems to be mainly derived from the nNOS isoform. The strongest evidence for this conclusion is from the endothelium removal by saponin. Under this endothelium free posterior cardinal vein *in situ* perfusion set-up, NO levels increased as did preparations with intact endothelium. With the lack of specific NOS inhibitors it remains difficult to provide more conclusive evidence for the involvement of nNOS.

Repeated exposure to acute hypoxia In vivo

The majority of the literature suggests that nNOS is a leading candidate for playing a major role in the regulation of catecholamines. In mammals, nNOS has been localized in the chromaffin cells (Oset-Gasque et al., 1994, 1998; Schwarz et al., 1998) and in the innervating nerves (Afework et al., 1994). In rainbow trout, nNOS has been localized in some nerve fibers in the head kidney (Gallo and Civinini, 2001). The latter study relied on the use of heterologous nNOS antibodies. A different approach was taken in this thesis that relied on cloning a partial cDNA sequence of trout nNOS and measuring mRNA levels in the posterior cardinal vein, an area densely populated with chromaffin cells (Nandi, 1961). The results suggest that, as in mammals, the nNOS is found in the vicinity of chromaffin cells and thus may be involved in the control of chromaffin cell activity. Because of this, the nNOS isoform was chosen to be

investigated in response to repeated exposures to hypoxia *in vivo*. The initial response of the fish to hypoxia was a large increase in circulating catecholamine levels. This response was observed for the following exposures, however following the 5th to 6th exposure, catecholamine levels were greatly reduced. In contrast, NO levels were slightly elevated during these final hypoxia exposures. Although, further investigation is needed, the results obtained throughout this thesis would suggest that NO could potentially be involved in the blunting of the catecholamine secretion response associated with repeated hypoxia. Data obtained from perfusion experiments demonstrated that fish repeatedly exposed to hypoxia showed an elevated ability to produce NO, both basally and in response to stimulation. These data suggest an increase in nNOS enzyme activity either by post-translational modifications or an increase in gene expression, or both. The results of real time PCR experiments demonstrated an increase in the relative nNOS mRNA levels in both the brain and anterior kidney. Therefore, the increase in basal and stimulus-evoked NO could be a result, at least in part, of increased gene expression.

Possible explanation for the controversy surrounding the regulation of catecholamines by NO in other studies

There are relatively few studies that have investigated the effects of NO on catecholamine secretion, yet there is much controversy. Studies have found that in the presence of NO, basal catecholamine levels either increase (Oset-Gasque et al., 1994; 1998), decrease (Ward et al., 1996; Barnes et al., 2001), or have no effect (Marley et al., 1995). The results of the present study concur with the results by Marley et al. (1995) where NO has no effect on basal catecholamine secretion. During stimulation-evoked

catecholamine secretion, studies have shown the presence of NO results in an increase (Breslow et al., 1992; O'Sullivan and Burgoyne, 1990; Uchiyama et al., 1994) or decrease in catecholamine levels (Torres et al., 1994; Oset-Gasque et al., 1994;1998; Nagayama et al., 1998; Schwarz et al., 1998; Barnes et 2001; Kolo et al., 2004).

A possible explanation could involve voltage-gated Ca^{2+} channels. NO is believed to inhibit catecholamines through the phosphorylation of voltage-gated Ca^{2+} channels (Schwarz et al., 1998; Vicente et al., 2002). Chromaffin cells possess several types of voltage-dependent Ca^{2+} channels such as L-,P-,N, and Q-type (Nagayama et al.,1999). The channels type and involvement of the channels in the influx of Ca^{2+} is species dependent. For example, cat chromaffin cells posses L and N-type voltage-dependent Ca^{2+} channels which each carry 50% of the Ca^{2+} current (Albillos et al 1994). Bovine chromaffin cells possess L-, N- (Artalojo et al., 1992), P-(Mintz et al., 1992; Gandia et al., 1994) and Q-type-voltage-dependent channels (Lopez et al., 1994). Both L- and Q- type Ca^{2+} dominate the exocytotic process (Lomax et al., 1997). Rat chromaffin cells possess L-,N-,P-, and Q-type channels with both L-and N-type Ca^{2+} channels showing to be recruited during exocytosis (Kim et al., 1995). This could be why so much controversy surrounds the effects of NO on catecholamine secretion. It is currently unknown which type of Ca^{2+} channels get phosphorylated by NO, and if NO preferentially phosphorylate specific Ca^{2+} channels. Therefore, depending on which channels are present and the roles they play, could result in conflicting results between species.

Conclusions

During exposure to an acute stress, catecholamines function by modulating the cardiovascular and respiratory system as well as mobilize energy stores (Perry and Gilmour, 1999) in an attempt to diminish the often harmful effects that accompany stress (Carrasco and Van der Kar, 2003). Therefore the understanding of catecholamine regulation is very important. To date, the majority of studies have concentrated on the neurotransmitters/neuromodulators that stimulate catecholamine secretion, such as Ach (Kim et al., 1999), angiotensin II (Lapner et al., 2000), and VIP/ PACAP (Montpetit and Perry, 2000). There are relatively few studies however, that investigate neurotransmitters that inhibit catecholamine secretion. The results provided the first evidence for an inhibitory effect of NO on catecholamine secretion in a non-mammalian vertebrate. Moreover, the results of this thesis provided the fist evidence of a role for nNOS in regulating catecholamine secretion *in vivo*. Further experiments should continue to focus on the role of NO on modulating catecholamine secretion during repeated stress and to specifically ascertain whether the up-regulation of nNOS observed during repetitive stress is a general phenomenon or restricted to hypoxic stress.

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