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Molecular characterization of α_1-adrenoreceptor gene family and its role in salt-induced high blood pressure in rainbow trout (*Oncorhynchus mykiss*)

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Molecular characterization of α_1-adrenoceptor gene family and its role in salt-induced high blood pressure in rainbow trout (*Oncorhynchus mykiss*)

Xi Chen

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
α-AR	α-Adrenoceptor
α_1-AR	α_1-Adrenoceptor
α_{1A}-AR	α_{1A}-Adrenoceptor
α_{1B}-AR	α_{1B}-Adrenoceptor
α_{1D}-AR	α_{1D}-Adrenoceptor
α_2-AR	α_2-Adrenoceptor
ABA	Afferent branchial artery
AC	Adrenylyl cyclase
AR	Adrenoceptor
ADR	(-)-Adrenaline
β-AR	β-Adrenoceptor
β_1-AR	β_1-Adrenoceptor
β_2-AR	β_2-Adrenoceptor
β_2-AA	β_2-Adrenergic agonist
β_3-AR	β_3-Adrenoceptor
BA	Bulbus arteriosus
BCA	Bicinchoninic acid
BMY7378	8-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride
BP	Blood pressure
BSA	Bovine serum albumin
BW	Body weight
CA	Catecholamine
cAMP	Cyclic adenosine monophosphate
CEC	Chlorethylclonidine
CMA	Celiacomesenteric arteries

CO	Cardiac output
Ct	Cycle threshold
DA	Dorsal aorta
DAG	1,2-diacylglycerol
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
EBA	Efferent branchial artery
f_H	Heart rate
FW	Fresh water
GFR	Glomerular filtration rate
GH	Growth hormone
GIT	Gastrointestinal tract
G-proein	Guanine nucleotide binding protein
G_i-protein	G-protein involved in the α_2-AR signal transduction pathway
G_q-protein	G-protein involved in the α_1-AR signal transduction pathway
G_s-protein	G-protein involved in the β-AR signal transduction pathway
GPCR	G-Protein coupled receptor
GRK	G protein-coupled receptor kinase
GSP	Gene specific primers
HR	Heart rate
IP_3	Inositol-1,4,5-trisphosphate
K_d	Affinity (Concentration at 50% of maximum number of binding sites)
K_i	Displacement affinity (Concentration displacing 50% of specific binding)
k_s	Rate of fractional protein synthesis
MAPK	Ras-mitogen-activated protein kinase
Min	Minute
mRNA	Messenger ribonucleic acid
NOR	(-)-Noradrenaline
PCR	Polymerase chain reaction
PCV	Posterior cardinal vein

PHT	Phentolamine
PIP2	PI-4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC β	Phospholipase C β
PVR	Peripheral vascular resistance
QPCR	Quantitive polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RBC	Red blood cell
RM	Red muscle
RPA	RNase protection assay
Rs	Systemic resistance
RS17053	(*N*-[2-(2-Cyclopropylmethoxyphenoxy)ethyl]-5-chloro-*a*,*a*-dimethyl-1*H*-indole-3-ethanamine) hydrochloride
RT	Reverse transcription
SHR	Spontaneously hypertensive rat
SVR	Systemic vascular resistance
TMD	Transmembrane domain
VA	Ventral aorta
V_b	Cardiac output
WM	White muscle

ABSTRACT

α_1-Adrenoceptors (α_1-ARs) are members of the G-protein coupled receptor superfamily of membrane proteins that mediate the actions of the endogenous catecholamines, adrenaline and noradrenaline. α_1-ARs are the prime mediators of vascular smooth muscle contraction and hypertrophic growth, and play a key role in the regulation of arterial blood pressure.

Mammals possess a complex α_1-AR system that is divided into three distinct subtypes, α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR based on their different pharmacological and molecular properties. Relatively few studies have focused on α_1-ARs from early branching vertebrates such as fish, particularly at the molecular level. The goal of this thesis is to characterize the α_1-AR gene family and to investigate its role in salt-induced high blood pressure in the rainbow trout, *Oncorhynchus mykiss*.

Five putative α_1-AR genes called α_{1A}-AR, $\alpha_{1A'}$-AR, α_{1B}-AR, $\alpha_{1B'}$-AR and α_{1D}-AR were cloned and sequenced and further identified as homologs to their mammalian counterparts by phylogenetic analysis. Real-time PCR analysis showed α_{1D}-AR to be widely expressed in trout tissues, with relatively high expression levels in the peripheral vascular system, e.g. bulbus arteriosus (BA), afferent branchial artery (ABA), ventral aorta (VA) and the spleen. The α_{1A}-AR was highly expressed in brain, white muscle and efferent branchial artery (EBA); α_{1A}-AR was not detected in the ABA. Dose-response curves of α_{1A}- and α_{1D}-AR selective antagonists indicated that both α_{1A}- and α_{1D}-ARs play a potential role in the regulation of trout blood pressure.

Four weeks of high-salt diet (11% NaCl) significantly increased blood pressure (37.1%). An average two-fold decrease in α_{1D}-AR mRNA expression was observed in the DA, ABA and EBA in high-salt diet fed trout. The chronically hypertensive fish exhibited a blunted vascular response to hypercapnia and exogenous catecholamines. These results suggest either a lowered vascular response capacity or that α_{1D}-AR down-regulation occurred in high-salt diet fed fish as a consequence of the elevated blood pressure. The α_{1D}-AR is, at least in part, involved in salt-induced high blood pressure in rainbow trout.

This study is the first to characterize the α_1-AR gene family at molecular level in any fish species, and provides the first evidence for a link between α_1-ARs and salt-induced high blood pressure.

Résumé

Les α_1-adrénocepteurs (α_1-ARs) sont des protéines membranaires, faisant partie de la superfamille de récepteurs couplés à une protéine G, qui agissent en médiateur de l'action des catécholamines endogènes, soit l'adrénaline et la noradrénaline. Les α_1-ARs sont les médiateurs primaires de la contraction des muscles lisses vasculaires et de la croissance hypertrophique musculaire.

Les mammifères possèdent un système complexe d'α_1-AR qui se divise en trois sous-types distincts basés selon leur pharmacologie ainsi que leurs propriétés moléculaires de la façon suivante : α_{1A}-AR, α_{1B}-AR, et α_{1D}-AR. Il existe peu d'études au niveau moléculaire décrivant l'évolution des gènes codants pour la famille α_1-AR chez les descendants d'ancêtres qui se sont divisés tôt le long de l'arbre phylogénétique des vertébrés, tels les poissons. Le but de cette thèse est de caractériser les gènes codants pour la famille α_1-AR et de déterminer leurs rôles dans l'hypertension causée par une diète élevée en sel chez la truite arc-en-ciel (*Oncorhynchus mykiss*).

Les cinq gènes α_1-AR putatifs appelés α_{1A}-AR, $\alpha_{1A'}$-AR, α_{1B}-AR, $\alpha_{1B'}$-AR et α_{1D}-AR ont été clonés et séquencés pour ensuite être identifiés par analyse phylogénétique comme homologues aux gènes correspondants chez les mammifères. Une amplification enzymatique quantitative avec transcriptase inverse (RT-qPCR) a démontré une expression abondante de α_{1D}-AR chez la truite, particulièrement dans le système vasculaire périphérique (ex. bulbus arteriosus (BA), artère branchiale afférente (ABA), l'aorte ventral (VA) et la rate). α_{1A}-AR était exprimé de façon abondante dans le cerveau, le muscle blanc et l'artère branchiale efférente (EBA) mais n'a pas été détecté dans l'ABA. Des courbes de relation dose-effet, à

l'aide d'antagonistes sélectifs α_{1A}- et α_{1D}-AR, indiquent que α_{1A}- et α_{1D}-AR jouent un rôle potentiel dans la régulation de pression sanguine chez la truite arc-en-ciel.

Quatre semaines d'administration orale d'une diète élevée en sel (11% NaCl) augmente la pression sanguine de façon significative (37.1%). En moyenne, une diminution par un facteur de deux de l'ARNm α_{1D}-AR fût observée dans l'AD, l'ABA et l'EBA de poissons nourrit avec la diète élevée en sel. Ces mêmes poissons, souffrant d'une hypertension chronique, démontrent une diminution marquée de la réponse vasculaire à l'hypercapnie et aux catécholamines. Ces résultats suggèrent soit une diminution de la capacité à une réponse vasculaire ou une régulation négative de α_{1D}-AR dans le groupe traité à une diète élevée en sel résultant de l'hypertension chronique. α_{1D}-AR est en partie impliqué dans l'hypertension induite par une diète élevée en sel chez la truite arc-en-ciel.

Cette étude est la première à caractériser, au niveau moléculaire, les gènes de la famille α_1-AR chez une espèce de poisson et aussi à déceler le premier indice liant α_1-AR et l'hypertension induite par une diète élevée en sel.

Chapter 1. General Introduction

Hypertension or high blood pressure is a leading disease worldwide. It imperils human health not only as a direct result of high blood pressure, per se, but also because high blood pressure is associated with other serious diseases. Better understanding of the mechanisms that cause hypertension may provide valuable information to assist in the development of appropriate clinical therapies. Numerous avenues of study have contributed to investigating this disease and those factors related to it, including α_1-adrenoceptors (α_1-ARs) that primarily mediate the cellular responses to endogenous catecholamines, including blood vessel contraction. However, the molecular characterization of α_1-ARs and their involvement in high blood pressure is rare, especially in the early-branching vertebrates. The main objectives of this thesis are to characterize the rainbow trout α_1-AR gene family and to determine its potential role in salt-induced high blood pressure. In order to achieve these objectives, rainbow trout α_1-ARs were cloned and sequenced and phylogenetic analysis was performed to determine their subtype identity and evolutionary relationship to other vertebrate species. Real-time PCR was used to assess changes in α_1-AR mRNA expression in chronically hypertensive fish. To evaluate receptor responsiveness, cardiovascular parameters were assessed in trout exposed to hypercapnia or injected with catecholamines *in vivo*.

1.1 Overview of the adrenergic system

1.1.1 Catecholamine (CA) hormones and their receptors

Vertebrates, including teleost fish, produce the catecholamine (CA) hormones noradrenaline (NOR) and adrenaline (ADR) that are used within the nervous system as

neurotransmitters or in the circulation as neurohormones. In mammals, NOR and ADR are secreted into the circulation from chromaffin cells within the adrenal medulla, whereas NOR is the predominant neurotransmitter released from postganglionic sympathetic nerve fibers (Minneman and Esbenshade, 1994). In teleost fish, CAs are released into the bloodstream from chromaffin cells lining the posterior cardinal vein within the anterior kidney, CAs are also released by adrenergic neurons (Randall and Perry, 1992; Epple, 1993; Reid *et al.*, 1998). CAs have been widely studied in both mammals and fish, where these hormones play key roles in the acute response to stress by regulating cardiorespiratory function and energy metabolism (Minneman and Esbenshade, 1994). The CAs, ADR and NOR, affect target cells by binding to specific membrane receptors called adrenergic receptors or adrenoceptors (ARs).

1.1.2 Adrenoceptor (AR) classification

Adrenoceptors belong to the heterotrimeric GTP-binding protein (G protein)-coupled receptor (GPCR) superfamily that exists widely in both eukaryotes and certain prokaryotes (Rana *et al,* 2001). For instance, the human genome is thought to contain at least 700 GPCRs (Rubin *et al.,* 2000). This superfamily of receptors has several hundred members and is generally divided into five groups that include muscarinic cholinergic receptors, serotonin receptors, amine receptors, and neurokinin receptors, as well as the photoreceptor rhodopsin (Gether and Kobilka, 1998; Smiley *et al.,* 1998). It is estimated that 40 to 50% of current therapeutic drugs act on GPCRs (Rana *et al.,* 2001). Generally, GPCRs share a common structural feature that is comprised of a single polypeptide chain that serpentines across the membrane forming seven transmembrane domains (TMD) with an extracellular N terminus and a intracellular C terminus (Strader *et al.*, 1995; Wess, 1997; Gether and Kobilka, 1998; Ji

et al., 1998). Initially two distinct AR subtypes were recognized and called α- and β-ARs based upon pharmacological criteria (Ahlquist, 1948); by the 1970s, three AR subtypes were identified and called α_1-AR, α_2-AR and β-AR (Berthelsen and Pettinger, 1977). Subsequently, as molecular cloning technology became available, these three classifications of ARs were confirmed at the mRNA level, and more subtypes/types were reported (Lefkowitz and Caron, 1988; Harrison *et al.*, 1991; Lomasney *et al.*, 1991; Bylund, 1992; Minneman and Esbenshade, 1994). Currently, the existence of the three main AR subtypes, α_1-AR, α_2-AR and β-AR, is widely accepted. Each subtype has distinctive pharmacological profiles and different signal transduction pathways. These subtypes are now further subdivided into nine types, three α_1 (α_{1a}, α_{1b}, α_{1d}), three α_2 (α_{2a}, α_{2b}, α_{2c}), and three β (β_1, β_2, β_3) according to the criteria of molecular biology (gene sequence), pharmacology (drug specificity) and physiology (signal transduction pathway) (Cavalli *et al.*, 1997) (see Fig. 1.1). Each AR type is encoded by an unique gene, with sequence homologies between subtypes of about 40% and between types within a subtype of 70-75% (Lefkowitz and Caron, 1988; Lomasney *et al.*, 1991; Minneman and Esbenshade, 1994).

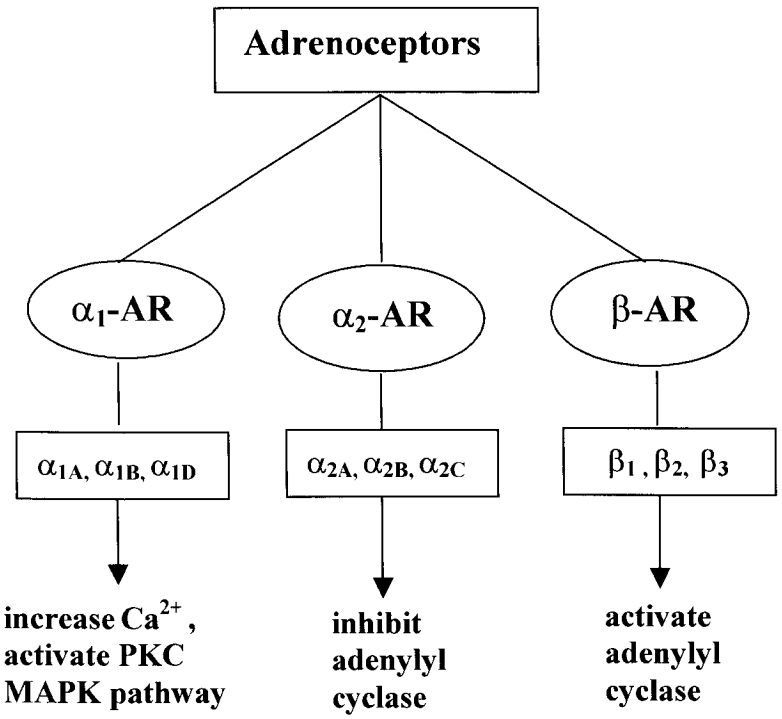

Figure 1.1. Adrenoceptors classification. The adrenoceptor family is divided into three subtypes and multiple types based on pharmacological properties, molecular characterization and signal transduction pathway. Modified from Zhong, (1999).

1.1.3 Adrenoceptor signal transduction pathways

Adrenoceptors belong to the amine receptor group of the GPCR superfamily and thus contain seven transmembrane domains that include 20-25 hydrophobic amino acid residues linked by three extracellular and three intracellular hydrophilic loops (Graham *et al*, 1996; Lomasney *et al*, 1996b; Varma and Deng, 2000). The amino acid sequence of the transmembrane spanning domains tends to be well conserved among most GPCRs including the ARs. All ARs bind ADR and NOR and activate a heterotrimeric G protein that transduces the hormone signal that eventually leads to changes in cellular functions. The ligand binding sites are located within the transmembrane domains (see Fig. 1.2) of the receptors and appear to be highly conserved in all ARs (Varma and Deng, 2000).

The signaling pathway of mammalian β-ARs was characterized earlier than that for the α_1- and α_2-ARs (Hein and Kobilka, 1995). The binding of ADR or NOR to the β-AR at the ligand binding site results in the G_s-protein associating with the β-AR at the 3rd intracellular loop and tail of the receptor. This ligand-βAR-G_s-protein complex causes the GDP located to the G_s-protein α subunit to be exchanged for GTP (intracellularly derived), thus activating the G_s-protein. The activated G_s-protein dissociates from the β-AR, separating into α and β/γ subunits (Valdenaire and Vernier, 1997); the activated G_s-α subunit activates adenylyl cyclase (ACase) at the inner surface of the cell membrane which catalyzes the conversion of ATP into cAMP (Millegan *et al.*, 1994; Lomasney *et al.*, 1995; Kompa *et al.*, 1999). Increasing the production of intracellular cAMP, the second messenger of the β-AR signaling pathway, activates the cAMP-dependent protein kinase or protein kinase A (PKA) that exerts numerous cellular responses by catalyzing specific serine-tyrosine phosphorylation of intracellular proteins (Exton, 1979; Bristow *et al.*, 1990; Mersmann,

1998). The binding of ligands (NOR or ADR) to the α_2-AR inhibits ACase through coupling to the inhibitory G-protein, G_i; this activation of G_i is responsible for inhibiting the opening of voltage-gated Ca^{2+} channels and activation of a Na^+/H^+ exchanger, resulting in changes to ion fluxes across the cell membrane (Cotecchia *et al.*, 1990; Surprenant *et al.*, 1992; Smiley *et al.*, 1998). The α_1-AR signal transduction pathway will be described in detail below.

1.1.4 Adrenoceptor functions

Adrenoceptors play key roles in the modulation of sympathetic nervous system activities as well as being the target sites for many therapeutic agents (Zhong *et al.*, 1999). α-ARs are involved in many important physiological functions including vascular contractility, systemic and pulmonary vascular tone, platelet aggregation, and insulin secretion. Activation of α-ARs provides enhanced inotropy and chronotropy of the heart, bronchodilation, vasconstriction, sedation, and analgesia, whereas inhibition of α-ARs results in vasodilation, decreased inotropy and chronotropy of the heart, and relaxation of prostate smooth muscle. Therefore, functional changes in α-ARs are associated with numerous diseases including congestive heart failure, hypertension, angina pectoris, cardiac arrhythmia, asthma, depression, glaucoma, pheochromocytoma, and benign prostatic hyperplasia (Ruffolo *et al.*, 1994; Smiley *et al.*, 1998). Other than the cardiovascular and neurological functions of α-ARs, they are also involved in metabolic regulation in liver (Nichols and Ruffolo, 1991; Fabbri *et al.*, 1999). β-ARs also have widespread functions within the cardiovascular system, on muscle growth and the respiratory system as well as metabolic regulation (Schauf *et al.*, 1990). Activation of β-ARs increases heart rate and force of contraction, induces vascular dilation, increases blood flow to the heart, increases glucose release (Randall and Perry,

1992; Fabbri and Moon, 1994; Reid *et al.,* 1998), and in certain fish species, stimulation a red blood cell Na^+/H^+ exchanger (Randall and Perry, 1992).

1.2 Overview of the α_1-AR system

1.2.1 α_1-AR classification

Over the past twenty years, numerous studies proposed that multiple α_1-ARs with homologous structures but unique characteristics existed in mammals (Guimaraes and Moura, 2001). The functional heterogeneity of α_1-ARs was first recognized from radioligand binding studies that demonstrated different affinities of agonists and antagonists (McGrath, 1982; Ruffolo, 1985; Morrow and Creese, 1986) and subsequently on the basis of sensitivity to chlorethylclonidine (CEC) (Han *et al.*, 1987). Additional subtypes (α_{1D}, α_{1L}, and α_{1N}) were proposed based on pharmacological studies; for instance, the putative subtype α_{1L}-AR was characterized by its low affinity for the antagonist prazosin in several lower urinary tract tissues (Muramatsu *et al.*, 1990; Ford *et al.*, 1994; Graham *et al.*, 1996). Thus, selective antagonists including prazosin are valuable tools for sub-classifying α_1-ARs (Robert and Ruffolo, 1985). From these early studies, efforts to classify the three α_1-AR subtypes through molecular analyses and pharmacological techniques took place. Cotecchia *et al* (1988) successfully cloned the first α_1-AR, the α_{1B}-AR in the hamster. Following this initial study, other α_1-AR types were cloned and characterized (Hieble *et al.*, 1995). In total, three α_1-AR types are now accepted (α_{1A}, α_{1B}, α_{1D}) and pharmacologically characterized (reviewed by Zhong *et al.*, 1999). Although it is often difficult to establish the relationship between pharmacologically defined and cloned α_1-ARs, the standardized nomenclature for α_1-ARs follows that recommended by the International Union of Pharmacology (IUPHAR) (Bylund

et al., 1994) which identifies three subtypes designated as α_{1A}-AR, α_{1B}-AR and α_{1D}-AR (Hieble *et al.*, 1995). In addition to these types, four α_{1A}-AR splice variants are reported in human prostate, although their physiological significance is currently unclear (Chang *et al.*, 1998).

1.2.2 α_1-AR signal transduction pathways and structure

Generally speaking, activation of the α_1-AR is initiated by an endogenous catecholamine disrupting an interhelical salt bridge formed by competition for an aspartic acid in TMD 3 and a lysine in TMD 7 (see Fig. 1.2 and Porter *et al.*, 1996; 1998). The coupling of the α_1-AR to its second messenger system has been examined in detail. α_1-AR agonist binding stimulates the G-protein, G_q, ultimately activating the phospholipase C-β isozyme, which hydrolyses membrane phophoinositides into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors located on the sarcoplasmic reticulum, resulting in increased free cytosolic calcium (Ca^{2+}) and in smooth muscle contraction (Smiley *et al.*, 1998). DAG remains in the membrane activating the Ca^{2+}-dependent protein kinase (PKC) that phosphorylates and changes the activity of many cellular proteins. There is generally a synergy between the IP_3 and DAG mechanisms (Exton, 1985). In addition to this classic pathway, α_1-ARs are also involved in activating the MAPK-Ras/Raf pathway that mediates the myocyte hypertrophic response (see Fig. 1.2 and Xin *et al.*, 1997). The nature of these mechanisms raise the possibility that α_1-ARs may interact with other cellular proteins that could play unanticipated roles in the α_1-ARs function (Hirasawa *et al.*, 2001). Currently, no close relationship can be established between specific subtypes and signaling mechanisms (Guimaraes and Moura, 2001).

As with other ARs, α_1-ARs have seven TMDs linked by three intracellular and three extracellular loops and an extracellular N-terminal and an intracellular C-terminal. The seven TMDs possess the ligand-binding pocket (see below), whereas the third intracellular loop and tail mediate receptor-G protein coupling (Khorana, 1992; Savarese and Fraser 1992). In addition, there are several important amino acids that are considered as potential phosphorylation sites in the third intracellular loop and the C-terminal end of the α_1-AR (Garcia-Sainz *et al.*, 2000). These phosphorylation sites are involved in α_1-AR down-regulation. Down-regulation of the AR is considered to result from prolonged catecholamine exposure (Liggett and Lefkowitz, 1993; Rokosh *et al.*, 1996). Down-regulation of AR may also result from a decrease in AR synthesis or an increase in degradation or both (Izzo *et al.*, 1994). The down-regulation of the β_2-AR by phosphorylation has been extensively characterized in response to agonists or other agents (Hausdorff *et al.*, 1990). The first α_1-AR that was reported to be subjected to phosphorylation was the hamster α_{1B}-AR (Diviani *et al.*, 1996; Garcia-Sainz *et al.*, 2004). The phosphorylation involved in α_{1B}-AR down-regulation may be activated by at least two protein kinases; PKC stimulated by phorbol esters and protein kinases belonging to the GPCR kinase or GRK family (Diviani *et al.*, 1996). Subsequently, two other α_1-AR subtypes were reported to be subjected to down-regulation; the α_{1A}-AR is phosphorylated in response to NOR and when PKC is activated by phorbol esters (Vazquez-Prado *et al.*, 2000). The α_{1D}-AR is also phosphorylated by NOR and PKC activation, but also by crosstalk with other receptors (Garcia-Sainz *et al.,* 2001).

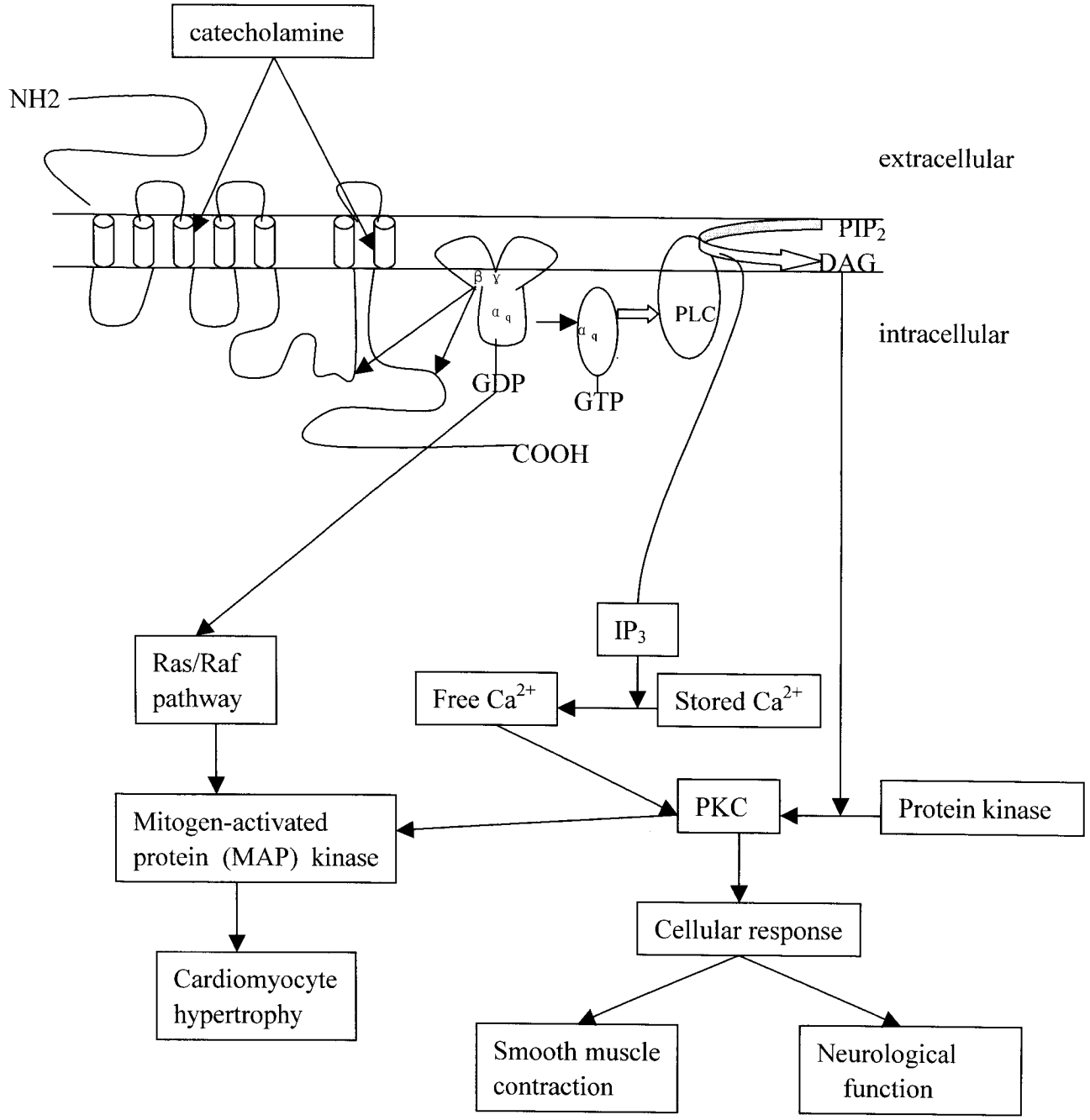

Figure 1.2. Signaling transduction pathway activated by α_1-ARs. Binding of endogenous catecholamine to membrane spanning α_1-AR is known to activate the heterotrimeric G proteins: $G_{q/11}$. The ligand binding site was considered to be on the extracellular surface within a pocket created by the transmembrane domains. G protein coupling possibly associates with the amino and carboxyl terminal ends of the third intracellular loop. The α and β/γ subunits of the G protein dissociate and stimulate phospholipase C (PLC)-β. This enzyme hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) into two products, inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on the endoplasmic reticulum to release stored intracellular Ca^{2+}. This secondary messenger acts synergistically with DAG to activate protein kinase C (PKC), which changes certain protein functions via phosphorylation. The G_q protein and PKC are also involved in activating mitogen-activated protein kinase (MAPK) through the Ras/Raf pathway, which causes cardiomyocyte hypertrophy. Modified from Smiley (1998), Zhong (1999), Varma (2000).

1.2.3 Major functions of α_1-AR

The sympathetic nervous system plays a key role in regulating peripheral vascular tone and hence in the control of blood pressure. The catecholamine neurotransmitters mediate cardiovascular responses by activating α_1-ARs. α_1-ARs are distributed in many tissues, where they mediate a diversity of effects. Activation of α_1-ARs is associated with important cardiovascular functions including smooth muscle contraction (Anyukhovsky *et al.*, 1992), growth of vascular smooth muscle cells (Chen *et al.*, 1995; Leech and Faber, 1996; Cavalli *et al.*, 1997; Hrometz *et al.*, 1999), enhanced cardiac contractility and in the hypertrophic response reported to occur in the myocardium (Milano *et al.*, 1994; Akhter *et al.*, 1997). Contraction of vascular smooth muscle is mediated predominantly through α_1-ARs, therefore these receptors are involved in regulation of peripheral vascular function. However, the expression of the three α_1-AR types varies from species to species and from vessel bed to vessel bed. The mediation of smooth muscle contraction and regulation of cardiovascular function has been extensively investigated. Results indicate that α_{1A}- and α_{1D}-ARs predominately mediate individual vessel contraction while α_{1B}-ARs play only a minor role (Daly *et al.*, 2002; Rokosh and Simpson, 2002; Tanoue *et al.*, 2002). Accumulating evidence indicates that it is difficult to establish the relationship between *in vivo* and *in vitro* studies with respect to α_1-AR function. Furthermore, recent studies report the involvement of α_1-ARs in the development and maintenance of hypertension based on hypersensitivity of vascular smooth muscle to α_1-AR stimulation in several hypertension models (Villalobos-Molina *et al.,* 1999; Villalobos-Molina and Ibarra, 1999; Varma and Deng, 2000; Guimaraes and Moura, 2001).

Apart from the cardiovascular system, many other tissues express α_1-ARs including liver and kidney. In these tissues, α_1-ARs regulate hepatic glucose metabolism (Nichols and Ruffolo, 1991; Fabbri *et al.*, 1999) and sodium and water re-absorption (Kopp and Dibona, 1996). α_1-ARs are also known to play an important role in neurological functions; analgesia (Kawabata *et al.* 1994), increasing motor tone (Ono and Fukuda, 1995), and enhancing sympathetic tone are good examples of the effects that are mediated by α_1-ARs, as well as regulating spinal cord signal transduction (Smith *et al.*, 1999). Interestingly, α_1-AR stimulation results in relaxation of most gastrointestinal smooth muscle (Bulbring *et al.*, 1981).

α_1-ARs are expressed in many tissues and most tissues possess more than one subtype, whereas very few tissues express only one subtype. Numerous studies investigated α_1-ARs tissue distribution at the mRNA level in humans. These studies demonstrate that α_{1A}-AR mRNA predominates in heart, cerebellum, cerebral cortex and prostate (Faure *et al.*, 1994; Price *et al.*, 1994); α_{1B}-AR mRNA is present in high concentrations in spleen and kidney (Price *et al.*, 1994); and, α_{1D}-AR mRNA predominates in aorta (Price *et al.*, 1994). However, it is difficult to establish the relationship between α_1-AR mRNA and α_1-AR protein expression in various tissues.

1.3 Overview of blood pressure regulation

1.3.1 The Mammalian system

1.3.1.1 Factors involved in regulating blood pressure

Blood pressure (BP) is the force exerted by the circulating blood volume on the walls of arteries, the veins, and the chambers of the heart. In a population, blood pressure estimates

are a good indicator of diseases including heart attack and stroke (Mulrow, 1995). A healthy body maintains BP within defined limits by a complex regulatory system whereby a change in any factor that may cause BP to rise will be compensated by a change in another factor (Guyton, 1979). The laws of hemodynamics describe the movement of blood in vessels. These laws express the relationship between pressure, flow and resistance such that

Blood pressure (BP) = cardiac output (CO) x systemic vascular resistance (SVR)

This relationship demonstrates that BP can change by changing CO and/or SVR. Cardiac output in fish is the volume of blood pumped out of the ventricle per unit time, or CO = HR (heart rate) x SV (stroke volume, or the volume of blood pumped with each beat). Blood pressure therefore can be controlled in several ways (Guyton, 1979; Schauf *et al.*, 1990) (see Figure 1.3). **1) Heart rate (HR)** - the pace of the heartbeat, counted in heartbeats per minute (bpm). Generally, when HR increases, BP rises; when HR decreases, BP drops. 2) **Stroke volume (SV)** - the volume of blood pumped out of the ventricle with each heartbeat. Under stressful conditions, the nervous system increases SV by positive inotropic effects. Stroke volume can also be affected by increases or decreases in the amount of blood in the circulation, called the blood volume. The mechanism by which blood volume affects BP is that changes in blood volume affect arterial pressure by changing CO. An increase in blood volume increases central venous pressure. This increases SV by the Frank-Starling mechanism. The 'Frank-Starling law of the heart' states that if the muscle walls of the heart are stretched prior to a stroke then they will squeeze harder on that stroke. To increase stretch, more blood must be in the heart. **3) Resistance** - is produced mainly in the small-diameter arteries called arterioles

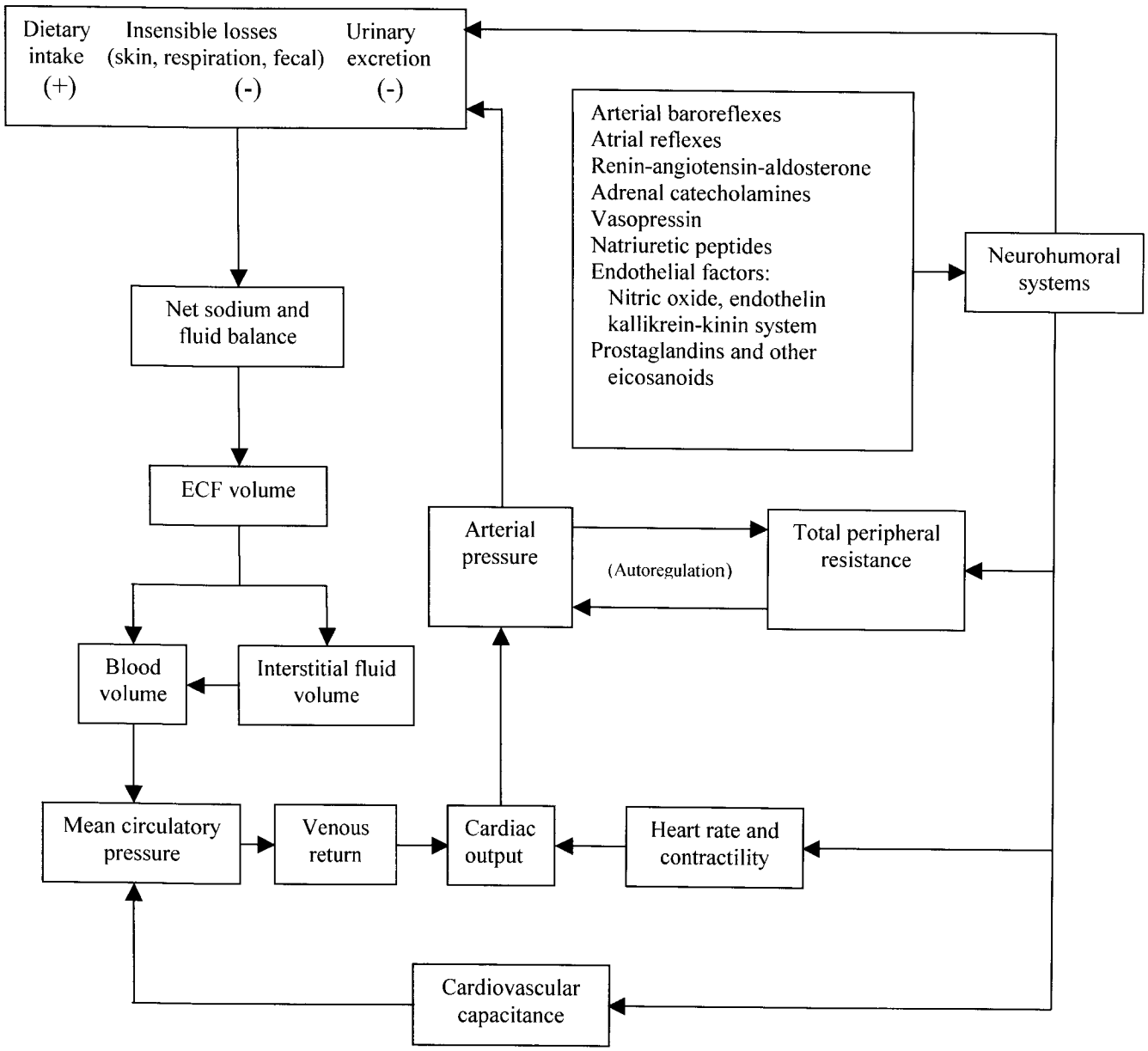

Figure 1.3. Generalized blood pressure regulation. The two major determinants of arterial pressure, cardiac output and total peripheral resistance, are regulated by a combination of short- and long-term mechanisms. In the short term, neural and humoral mechanisms listed regulate peripheral vascular resistance, cardiovascular capacitance, and cardiac performance. On a long-term basis, kidneys play the key role in regulating blood pressure by adjusting venous return that is related to blood volume and overall cardiovascular capacitance. Modified from Navar (1997).

and is known as the systemic vascular resistance (SVR) or the peripheral vascular resistance (PVR). The diameter of the vessels, fluid viscosity and the length of the vessels determine SVR. Therefore, BP can be regulated by adjusting the resistance of the arteries (arterioles) through changing the diameter of the blood vessels as appropriate, to prevent BP from swinging wildly. So, any factor(s) that directly or indirectly affect these three elements (HR, SV, SVR) may impact BP by a number of complex ways. For instance, the sympathetic nervous system (SNS), hormones, chemicals, body temperature, certain medications and various diseases may all affect BP. The SNS is key in animals to respond to environmental change (Mark, 1996) as well as to regulate BP in the short term, whereas in the long-term the kidneys play a vital role. The kidneys regulate BP by increasing or decreasing blood volume and also through the renin-angiotensin system. Hormones act on the kidneys to control the amount of sodium and water excreted. If too much sodium or water remains in the blood, blood volume increases. This increase in blood volume means that the heart will pump harder and thus BP increases. To maintain blood volume within a normal range, the kidneys regulate the amount of water and sodium lost into the urine. There are several mechanisms through which this regulation occurs. The renin-angiotensin-aldosterone system (RAAS) is a critical player in blood pressure control (Schauf *et al.*, 1990). These hormones regulate the amount of fluid in the blood, the diameter of the blood vessels, and the sodium and water balance by their action on the kidneys and blood vessels. Another important hormone regulating water balance is vasopressin (antidiuretic hormone; ADH). ADH stimulates water re-absorption in the collecting duct of the kidney, thereby decreasing water loss and increasing blood volume. In conclusion, BP is controlled by several physiological mechanisms acting in combination. The regulation of BP and blood volume is integrated and should be investigated as such. Both

ensure that the pressure is maintained within normal limits by adapting their responses in the short- and long-term to provide adequate perfusion to the body tissues (Cowley, 1992).

1.3.1.2 Hypertension and risk factors

Hypertension is a major threat to human health in all industrial nations (e.g., 65 million Americans were reported to have hypertension in 2004; Fields *et al.,* 2004) because it increases the risk for pathologies such as atherosclerosis, cerebral vasospasm, congestive heart failure, coronary heart disease, kidney failure, stroke, thrombolysis and aneurism (Gavras *et al.*, 1988; Cowley, 1992). Hypertension is caused by an increase in peripheral vascular resistance associated with structural changes in the walls of blood vessels and hypersensitivity of blood vessels to vasoconstrictor stimuli, although it can also be caused by prolonged periods of elevated cardiac output (McPhee *et al.*, 1997). Furthermore, the structural changes accompanying hypertension may lead the individual to other cardiovascular disease, renal failure, and stroke (Williams, 1994; Timiras, 1995). Undoubtedly hypertension is the number one public health problem worldwide and while it mainly affects mature and elderly individuals, recent evidence suggests that even the youth are suffering from this disease (e.g., 4.4 million people were reported to have hypertension in the 18-34 age group in 2004; Fields *et al.*, 2004). There are two types of hypertension: essential hypertension (high blood pressure with no known cause) and secondary hypertension (high blood pressure for which there is a cause) (Guyton *et al.*, 1986; Hall *et al.*, 1996). A number of pathologies are associated with secondary hypertension including renal disorder, endocrine disturbances, neurological conditions, drugs and chemicals and others. The development of hypertension has many causes and may, in fact, involve one or more of many risk factors including stress, obesity, smoking, a diet high in salt and the heavy use of

alcohol among many (Kannel, 1996). Given the subject of this thesis, only high-salt induced hypertension will be discussed.

1.3.1.3 High-salt induced hypertension

The fact that a diet high in salt is association with hypertension is not a new observation. As long ago as 2,000 B.C. the famous Chinese "Yellow Emperor" Huang Ti reported this relationship between salt and blood pressure (O'Shaughnessy and Karet, 2004). However, the question of how salt contributes to high blood pressure is still not completely resolved. Some explanation may help understand this phenomenon.

Increasing the volume of blood within the enclosed circulatory system is one mechanism to explain how salt increases BP. High-salt diets result in fluid retention since maintaining plasma sodium concentrations at a constant level in the face of increased plasma sodium requires (in the short-term) more fluid in the system. This increased volume of blood induces the heart to pump harder (see section 1.3.1.1). When this happens and/or the blood vessel walls are unable to expand adequately to accommodate this volume, BP increases. Another way salt may elevate BP is through actions at the arterioles. By contracting under the influence of sodium through endogenous endothelin (d'Uscio *et al.,* 1997) arterioles effectively increase the resistance to blood movement and lessen the volume of blood that is returned to the heart; this action also increases BP (Swales, 1992; Luff, 1998; Siani *et al.*, 2000).

Other mechanisms linking sodium with hypertension are less well understood. Some humans are genetically more susceptible to the effects of sodium than others, and sodium sensitivity appears to increase with age (Piccirillo *et al.,* 1996; Weinberger, 2002). The kidneys also play an important role in the development of hypertension. In certain renal

diseases, the pressure-natriuresis (sodium loss through the urine) relationship is altered so that the kidneys retain more sodium and water at a given pressure, thereby increasing blood volume and BP (Fujita and Ando, 2004; Suzuki and Saruta, 2004).

In the clinical field, salt moderation is often recommended to protect against an excessive increase in BP since a high-salt diet is implicated in the pathogenesis of hypertension, particularly in salt-sensitive individuals (MacGregor, 1985; Houston, 1986; Law *et al.*, 1991; Kuller, 1997).

1.3.2 Blood pressure control in fish

The circulatory systems of fish, amphibians, reptiles, birds and mammals demonstrate numerous differences based upon both evolutionary and functional considerations. In fish, the circulatory system has only one circuit, with the blood being pumped by the heart through the respiratory surface of the gills and on to the body tissues; this is known as a "single circulation" (Evans, 1998). The heart of the fish is a single pump consisting of three chambers in series. From an evolutionary perspective, it is generally accepted that the control of cardiovascular system function is similar in all vertebrates, with a few exceptions. The fish cardiovascular system comprises four elements: blood vessels, blood, heart and regulatory elements. As noted for mammals above, the relationship amongst blood pressure, blood flow and resistance can be described as

Blood pressure (BP) = cardiac output (CO) x systemic vascular resistance (SVR)

Resistance is affected by characteristics of the vessels, i.e. length and diameter, and the viscosity of the blood. A change in blood volume will affect CO if there are no compensatory changes in vascular compliance or tone (vascular compliance describes the passive changes in blood vessel volume associated with changes in pressure; this constitutes an important

function of large arteries and veins; Safar *et al.*, 2004). Four factors affect blood volume: red cell production and turnover, fluid exchange across the gill epithelium, excretion by the kidney and transcapillary exchange (Evans, 1998).

The piscine circulatory system has two basic differences from other vertebrates: 1) the branchial vasculature is connected in series with its systemic counterpart, and 2) BP and blood volumes are considerably lower (Olson and Duff, 1992) when contrasted with terrestrial vertebrates such as mammals (Olson, 1992). Fish live in a relatively gravity-free environment that has the same density as their body fluids thus they are neutrally buoyant in their environment. They face a constant threat of body fluid expansion in freshwater or depletion in seawater as a result of osmotic differences between the external (environment) and internal (blood) media. These unique challenges may have forced fish to evolve distinctive strategies in blood pressure-volume regulation. Evidence indicates that fish have a considerably greater capacity to handle intravascular volume loading than mammals (Duff and Olson, 1989).

Numerous *in vivo* and *in vitro* studies have identified the parameters involved in piscine cardiovascular homeostasis and compared them with the mammalian system. For instance, the Frank-Starling mechanism noted in section 1.3.1.1, whereby an increase in cardiac filling increases the force of contraction and thereby stroke volume, is well documented in fish (Farrell, 1991; 1993; Farrell and Jones, 1992; Minerick *et al.*, 2003). Overall, fish and mammals are quite similar with respect to signaling mechanisms and vascular responsiveness, including the sympathetic nervous system (Nilsson, 1984), the renin-angiotensin system (Nishimura, 2001; Russell *et al.*, 2001), kallikrein-kinin system (Conlon, 1999), vasotocin/vasopressin (Chan, 1977; Conklin and Olson, 1994), natriuretic

peptides (Loretz and Pollina, 2000; Takei, 2000), and endothelin (Wang *et al.*, 1999; Hoagland *et al.*, 2000). Perhaps the only difference between the fish and the mammalian signaling systems is the apparent lack of nitric oxide release from the vascular endothelium of fish (Olson and Villa, 1991; Minerick *et al.*, 2003).

1.4 Rainbow trout as a model organism

The rainbow trout, *Oncorhynchus mykiss*, is a widely used model species that has significant economic value for the aquaculture industry. The fish cardiovascular system is the evolutionary prototype for all vertebrates. Many fish physiological systems are exquisitely sensitive to the environment and can be manipulated experimentally. Fish are sensitive to acute and chronic environmental changes and demonstrate a classic stress response (Wendelaar Bonga, 1997; Fabbri *et al.*, 1998). The rainbow trout is a euryhaline teleost fish meaning that it can adapt and thrive in both freshwater (hypoosmotic) and saltwater (hyperosmotic) environments making it potentially useful model for integrative cardiovascular studies. Notably, less than 5% of the more than 25,000 teleost fish species are euryhaline (Nelson, 1994). Euryhalinity also means that the rainbow trout has evolved physiological mechanisms for dealing with these two contrasting environments. Thus, the rainbow trout provides a non-mammalian model of blood pressure and volume control that is both natural and versatile. The trout respiratory (gill) circulation is relatively non-compliant (Randall and Daxboeck, 1984) and because it is in series with the systemic circulation, there is little chance of transient fluid movement between these two circuits (Minerick *et al.*, 2003). Furthermore, given the key role played by the α_1-AR in the peripheral vasculature of

mammals, fish and possibly other non-mammalian vertebrates may be excellent models to study the evolution of ARs and their mechanisms of action.

The rainbow trout has been used extensively as a model fish species, and the α_1-AR system has been characterized at the physiological level. Studies reveal that α_1-ARs are involved in numerous physiological process including smooth muscle contraction and thus participation in BP regulation (Perry *et al.*, 1999), and glucose metabolism (Fabbri and Moon, 1994; Fabbri *et al.*, 1998; 1999). To better understand this physiology, I will attempt to characterize the α_1-AR gene family of the rainbow trout. I hope that this may provide valuable information to the current physiological data. One study goal is to establish a natural salt-induced hypertension model in fish; in contrast, there are no known natural models of human hypertension.

1.5 Hypothesis

Based upon the substantial evidence in mammals that α_1-ARs play a role in the regulation of blood pressure and the fact that the fish vasculature responds to α_1-AR agonists and antagonists, I hypothesize that α_1-ARs will be involved in salt-induced high blood pressure in the rainbow trout.

1.6 Objectives

To test this hypothesis, the following objectives will be studied:

Objective 1: molecular characterization of α_1-ARs and their role in the regulation of blood pressure in rainbow trout. This objective will be achieved by 1) cloning complete sequences of members of the α_1-AR gene family of rainbow trout, 2) using phylogenetic

analysis to determine the relationships amongst the trout and other vertebrate α_1-AR genes, 3) establishing the tissue distribution of α_1-AR family members in trout using real-time PCR, and 4) using *in vivo* injection of α_1-AR subtype specific antagonists to demonstrate the role of these AR-types in the regulation of blood pressure.

Objective 2: α_1-ARs are involved in salt-induced high blood pressure in rainbow trout. This objective will be achieved by 1) inducing high blood pressure by feeding rainbow trout a high-salt diet, 2) comparing α_1-AR mRNA expression levels between high-salt and control diet fed fish by real-time PCR, and 3) investigating the consequences of salt-induced hypertension on the responsiveness of α_1-ARs to hypercapnia and injected adrenergic agonists and antagonists.

Chapter 2. Molecular characterization of the α_1-AR gene family and its role in regulation of blood pressure in rainbow trout (*Oncorhynchus mykiss*)

2.1 Introduction

α_1-Adrenoceptors (ARs) are members of the superfamily of seven transmembrane domain (TMD) receptors coupled to G-proteins that mediate the cellular effects of the endogenous hormones adrenaline (ADR) and noradrenaline (NOR). The α_1-AR signal transduction pathway is well characterized in mammals. Binding of the catecholamine activates the heterotrimeric G_q protein (Wise *et al.*, 1995). The activated α-subunit of the G_q protein stimulates phospholipase C (PLC)-beta which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) into two products, inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on the endoplasmic reticulum to release stored intracellular Ca^{2+}. This second messenger acts synergistically with DAG to activate the membrane-bound protein kinase C (PKC) that phosphorylates specific cellular proteins and transduces the catecholamine signal (Zhong *et al.*, 1999). The G_q protein and PKC are also involved in activating mitogen-activated protein kinase (MAPK) through the Ras/Raf pathway that at least in cardiomyocytes, results in tissue hypertrophy (Michelotti and Price, 2000). Despite early controversy and confusion, it is accepted that α_1-ARs are divided into three subtypes, α_{1A}-, α_{1B}- and α_{1D}-AR based on their distinct pharmacology and molecular profiles (Zhong *et al.*, 1999). The discovery of α_1-AR heterogeneity raises the question whether the different α_1-AR-mediated responses in various organs could be assigned to distinct α_1-AR subtypes. To address this question, the tissue distribution of the mRNA encoding the three α_1-ARs was

investigated in species including human and rat by RT-PCR, Northern blot analysis and RNase protection assay. The mRNA of the different α_1-AR subtypes is found in several tissues, including brain, heart, liver, kidney, spleen, blood vessels and prostate (Ruffolo, 1985; Ruffolo and Hieble, 1993; Minneman and Esbenshade, 1994; Suzuki, *et al.*, 1997).

Although α_1-ARs have a metabolic role (Lithell *et al.*, 1988; Moriyama *et al.*, 1997; Shen *et al.*, 2000), α_1-ARs are prime mediators of smooth muscle contraction and of both normal and hypertrophic growth of vascular smooth muscle (Fedida *et al.*, 1993; Varma and Deng, 2000). In addition, Slavi (2001) reported that α_1-ARs play a vital role in mediating ischemic preconditioning (IPC) of the myocardium. α_1-AR-mediated smooth muscle contraction is important in determining the tonic and reflex changes in the diameter of arterial and venous vessels (Kobinger and Pichler, 1982; Langer and Schoemaker, 1989; Guimaraes and Moura, 2001), thus contributing to the maintenance of blood pressure and venous return to the heart (Ruffolo, 1985; Rudner *et al.*, 1999). α_1-ARs play a crucial role in mediating sympathetic control of arterial pressure, potent vasoconstrictor responses and changes in cardiovascular structure (Chen *et al.*, 1995; Vecchione *et al.*, 2002). The tonic sympathetic activation of vascular α_1-ARs maintains vascular resistance and is vital to the regulation of arterial pressure (Vargas and Gorman, 1995). α_{1A}-ARs are expressed in resistance vessels and are required to maintain arterial blood pressure (Rokosh *et al.*, 2002). α_{1D}-ARs are expressed in the aorta and directly regulate arterial blood pressure by vasoconstriction (Tanoue *et al.*, 2002). However, α_{1B}-AR plays only a minor role in the vascular contraction that is important in regulating blood pressure, implicating other subtypes as important controllers of these processes (Zuscik *et al.*, 2001; Daly *et al.*, 2002).

Our knowledge of α_1-ARs in non-mammalian systems is much less complete. The existence of hepatic α_1-ARs was demonstrated in a number of fish species including rainbow trout (Fabbri and Moon, 1994; Fabbri *et al.*, 1992; 1995), catfish (Brighenti *et al.*, 1987; Zhang *et al.*, 1992; Moon *et al.*, 1993; Fabbri *et al.*, 1994; 1999), brook trout (Goetz and Bradley, 1994), goldfish (Krumschnabel *et al.*, 2001), and American eel (Zhang *et al.*, 1992). Filadelfi *et al.* (2002) first showed the presence of an α_1-AR in avian melanocytes and the first evidence for the presence of α_1-ARs in the liver of an amphibian and a reptile was demonstrated by Fabbri *et al.* (1997); Yamanouye *et al.* (1992) demonstrated the presence of an α_1-AR in the aorta of the snake. However, these studies were based on pharmacological methods (i.e. binding of radioligands to cells and/or membranes) and pharmacology does not always give a clear picture of AR-type or –subtype due to the absence of highly selective α_1-AR agonists and antagonists. In fact some adrenergic agonists/antagonists developed for mammalian studies give totally contrary results when used in fish (Fabbri *et al.*, 1999). Xu and Olson (1993) however, did report the existence of multiple AR-types in the rainbow trout vasculature including α_1-ARs using physiological methods (i.e. vascular injection followed by blood pressure changes).

Few studies have reported on the molecular biology of non-mammalian α_1-ARs. Yasuoka *et al.* (1996) cloned and functionally expressed a putative α_{1A}-AR from the Japanese medaka *Orizias latipes*. Their results indicated fish may contain multiple AR systems for receiving adrenergic signals, similar to the systems reported in mammals. Despite this study, many fundamental questions remain regarding the α_1-ARs of fish including the number of α_1-AR genes in fish, tissue expression patterns and gene regulation. In addition, the combination of molecular and physiological data may add to our understanding of the piscine

α_1-AR systems in general and allow for the design of new selective and specific agonists/antagonists that could be useful tools for understanding α_1-AR-subtype function.

Therefore, the present study characterizes the α_1-AR gene family and their potential role in the regulation of blood pressure in the rainbow trout *Oncorhynchus mykiss*. Based upon physiological and pharmacological studies in other vertebrates, I hypothesize the existence of an α_1-AR gene family as complex as that reported in mammals and that the tissue distribution of these genes will identify blood pressure regulation as a potential function of this receptor family.

2.2 Materials and Methods

2.2.1 Animals

Rainbow trout (*Oncorhynchus mykiss*), weighing approximately 450-500 g, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were transported to the University of Ottawa Aquatic Care Facility and were maintained in fibreglass holding tanks (1275 L) supplied with well aerated, dechloraminated City of Ottawa tap water at 13.0 ± 1.0 ^{o}C. Fish were subjected to a constant 12L:12D photoperiod and fed five times a week with commercial trout pellets [Martin Mills 5 PT, 5 mm in size and composed of: 41.0% crude protein (minimum); 11.0% crude fat (minimum); 3.5% crude fibre (maximum); 1.0% calcium (actual); 0.85% phosphorus (actual); 0.45% sodium (actual); 6,800 IU kg^{-1} vitamin A (minimum); 2,100 IU kg^{-1} vitamin D (minimum); 80 IU kg^{-1} vitamin E (minimum); 200 IU kg^{-1} vitamin C (minimum)]. Receptor characterization experiments were conducted between August and October; blood pressure experiments were undertaken between August and

September. All procedures used were approved by the University of Ottawa protocol review committee and followed normal Canadian Council on Animal Care guidelines on the use of animals in research.

2.2.2 Isolation of tissue RNA

Total cellular RNA was isolated from fresh tissues [brain, efferent branchial artery (EBA), afferent branchial artery (ABA), celiacomesenteric artery (CMA), ventral aorta (VA), dorsal aorta (DA), spleen, liver, kidney, gill, white muscle, posterior cardinal vein (PCV), heart, bulbus arteriosus (BA), intestine] of the rainbow trout using Trizol reagent (GibcoBRL, Burlington, ON, Canada) according to the protocol provided by the manufacturer with the following exception. Four µl linear acrylamide (2 mg/µl) (Ambion, Austin, TX) was added prior to the addition of isopropanol; samples were then placed on dry ice for 30 min. This modification assisted in the precipitation of RNA from tissues of small size especially the EBA, ABA, CMA, VA, and DA, thus increasing the yield of RNA (Gaillard and Strauss, 1990). RNA concentrations and quality were verified using spectrophotometry (OD at 260 nm) and RNA gel electrophoresis, respectively.

2.2.3 Amplification of rainbow trout α_1-ARs cDNA

An initial set of rainbow trout α_1-ARs clones, spanning the fourth to the sixth transmembrane domains (TMD) (approximately 350 bp) were amplified using routine RT-PCR strategies cDNA primed by random primer was synthesized using the first Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biologicals, Laval, QC). A preliminary round of PCR amplification was performed using degenerate primers AF and AR (designed by CODEHOP program http://blocks.fhcrc.org/codehop.html) for α_{1A}-ARs and α_{1D}-ARs and degenerate primers α_{1B}FR for α_{1B}-ARs (see Table 2.1). These degenerate primers were

designed based on sequences from the fourth to sixth TMD of rat and human α_1-ARs (Genbank accession number for human α_{1A}-ARs, NP_150646; α_{1B}-ARs, P35368; α_{1D}-ARs, NP_000669; for rat α_{1A}-ARs, NP_058887; α_{1B}-ARs, AAA63478; α_{1D}-ARs, P23944). Subsequently, as the sequence of TMD7 was required to design real-time PCR primers (a proposed intron is located between TMD6 and TMD7; see below), another 3' degenerate primer was designed for α_{1A}-ARs and α_{1D}-ARs (see Table 2.1) based on the previous alignment. In addition, an α_{1D}-AR 5' degenerate primer designed based on the sequence alignment of TMD1 of human and rat α_{1D}-ARs amplified TMD1 to TMD6 of the trout α_{1D}-AR once paired with the α_{1D}-AR 3' degenerate primer. Trout clones were sequenced (Ottawa Genome Centre) and gene specific primers (GSPs) were designed (Primer 3 program http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_slow.cgi) from these sequences for 5' and 3' RACE.

2.2.4 RACE PCR

The 5' and 3' RACE system for Rapid Amplification of cDNA Ends, Version 2 (GibcoBRL) was used to amplify the 5' and 3' ends of the trout α_1-AR cDNA. α_{1A}-AR 5' end sequences were obtained through 5' RACE using trout α_{1A}-AR gene specific primer 1, GSP1 (Table 2.1) to prime cDNA synthesis. This cDNA was then used as the template in an initial round of PCR amplification using a second trout α_1-AR gene specific primer, GSP2 (Table 2.1) and the 5' amplification primer provided with the kit. A 5 µl aliquot of this initial PCR amplification step was then used as a template for a second round of nested PCR using a third trout α_1-AR gene specific primer 3, GSP3 (Table 2.1) and the abridged universal amplification primer (AUAP) provided with the kit.

Synthesis of cDNA for α_{1D}-AR 3' RACE was primed with the 3' amplification primer provided in the kit. A first round of PCR amplification was performed using trout α_{1D}-AR gene specific primer 4, GSP4 (Table 2.1) and AUAP. A second round of semi-nested PCR amplification using trout α_{1D}-AR gene specific primer 5, GSP5 (Table 2.1) and the AUAP was then carried out.

To confirm sequence accuracy, all three α_1-AR subtype genes were cloned three times using different PCR reactions and each clone was sequenced on both strands as noted above.

All PCR amplifications described above used the following regimen of denaturing, annealing and extension: 1x 2 min at 94 °C, 35x (30 sec at 94 °C, 30 sec at 55 °C, 1 min at 72 °C), and 1x 10 min at 72 °C. Annealing temperature is 55 °C (Table 2.1). All primers were ordered from Invitrogen.

2.2.5 Phylogenetic analysis and prediction of phosphorylation sites

The rainbow trout α_1-AR amino acid sequences generated were aligned with Genbank sequences of various α_1-ARs subtypes from selected vertebrates using the default setting in CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The accession numbers for sequences used in the phylogenetic analysis are presented in Table 2.2.

The segment of the sequences used for phylogenetic analysis spanned from the conserved amino end of TMD1 to the end of the conserved TMD7. Question marks filled the gaps where sequence was unavailable. The tree was the same as when keeping consistance all sequences with the shortest bits. Maximum likelihood phylogenetic analysis was performed using PUZZLE version 4.0.2 (Strimmer and von Haeseler, 1996).

The following program settings were used: quartet puzzling tree search, compute exact quartet likelihood, 1000 puzzling steps, use the fugu fish dopamine receptor sequence as out-

group, branch lengths are not clocklike, JTT model of substitution, amino acid frequencies were estimated from the data set, and the model of rate heterogeneity was 1 invariable + 8 gamma rates. The selection of the fugu fish, *Takifugu rubripes* dopamine receptor sequence as the out-group is based on the close relationship between α_1-ARs and dopamine receptors (Fryxell, 1995).

NETPHOS 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict the phosphorylation sites in the third intracellular loop and C-terminal region of the rainbow trout α_1-ARs.

Table 2.1. Primers designed to amplify various sequences of trout α_1-ARs. Gene specific primers (GSPs) were designed online using the program Primer 3, and degenerate primers were designed using the CODEHOP program. Tm is the annealing temperature used in the PCR amplification.

	5' primers	3'primers	Tm(°C)
AFR	GGITGGMRISARCCIGCICCIRAIGAYGA	CTYTTIGCIGCYTTYTTYTCICK	55
α_{1A}R		TGGGGTTGATGCAGGAGTTNARRTANCC	55
α_{1A}GSP1		CATAGGCAGGCTCCTCTGTG	55
α_{1A}GSP2		TAGTGTGTGACTGTTCGTAG	55
α_{1A}GSP3		ATTGTCCCAAGTATCAGTCC	55
α_{1B}FR	CAAAGAGTGCGGCATCACCSARRARCCNTT	CTYTTIGCIGCYTTYTTYTCICK	55
α_{1D}FR	TGTCCGCCCAGGGCRTNGGNGTNGG	TGGGGTTGATGCAGGAGTTNARRTANCC	55
α_{1D}GSP4	CCCTCTTCTCCTCGCTCTTT		55
α_{1D}GSP5	GCACTGAAGCCATCTGACAT		55

	α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
human	NP_150646	P35368	NP_000669
rat	NP_058887	AAA63478	P23944
mouse	P97718	P97717	P97714
rabbit	O02824	AAF80280	O02666
pig	CAB62570	CAE46112	CAB59347
dog	O77621	P11615	
bovine	P18130		
guinea pig	AAD22540	AAD22542	AAD22541
medaka	Q91175		
golden hamster		P18841	
puffer fish	http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html		
zebrafish	http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html		
fugu dopamine	CAA56457		

Table 2.2. GenBank accession numbers of sequences used in the phylogenetic analysis (see Fig. 2.4).

2.2.6 Real-time PCR analysis of α_{1A}- and α_{1D}-AR gene tissue distribution

Real-time PCR analysis was undertaken using a Stratagene MX-4000 real-time PCR machine and DNA amplification was followed using SYBR green (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. α_1-ARs (α_{1A} and α_{1D}) and β-actin (internal control) were assessed. To avoid genomic DNA amplification in real-time PCR, primers that span the hypothetical intron-exon boundary within TMD6 and TMD7 were designed (Arai *et al.*, 1999). The following primers were designed and synthesized (Invitrogen) to yield 170 bp amplicons.

α_{1A}-AR:

5'-CTGCGCCTCCTCAAGTTCTC-3' (forward)

5'-CCCCAGCCAGAAGGTGATCT-3' (reverse)

α_{1D}-AR:

5'-CTGTCCGTGCGCTTGATGAA-3' (forward)

5'-AACCCAGCCAGAAGATGACC-3' (reverse)

β-actin:

5'-CGTCCCAGGCATCAGGGAGT-3' (forward)

5'-TCTCCATGTCGTCCCAGTTG -3' (reverse)

Each primer set amplified a 170 bp fragment. The real-time PCR products were cloned to ensure that the amplification was identical to the original sequence. Forty cycles of a two-step PCR protocol were used: 1x 2 min at 94 °C, 35x (30 sec at 94 °C, 30 sec at 58°C, 1 min at 72 °C), and 1x 10 min at 72 °C. A no template control for each master mix and a no RT (reverse transcriptase) control were included in each analysis. A threshold value is set above baseline that reflects the average of emittance during the first PCR cycles. The

Ct value (cycle number) is the point at which the emittance passes above the baseline (threshold) and thus mirrors the accumulation of PCR product in a specific well of the PCR reaction. The relative mRNA expression level of the gene of interest was determined by comparing the different Ct values between the gene of interest and that of the internal control. Thus the lower the Ct value difference, the higher mRNA expression level of the gene of interest and vice versa.

The slopes of the standard curves for α_{1A}-AR, α_{1B}-AR and α_{1D}-AR were –3.083, -3.002 and –3.015, respectively, giving a corresponding primer efficiency of 111%, 115.3% and 114.6%, respectively.

Real-time PCR data were reported as 2^(-ΔCt) with β-actin as the reference gene. ΔCt is the difference between the Ct values of the α_1-AR and β-actin genes.

2.2.7 Cannulation procedures

To monitor blood pressure, trout were fitted with a dorsal aortic cannula according to the method of Soivio *et al.* (1975). Briefly, trout were anesthetized by immersion in a cold, oxygenated benzocaine solution (0.1 g/l) until voluntary activity ceased. The fish was transferred to an operating table where the same anaesthetic solution was passed continuously across the gills. A single indwelling cannula (Clay-Adams PE50 polyethylene tubing) was placed into the dorsal aorta of fish at the level of the first gill arch. The cannula was sutured to the roof of the mouth and, following recovery of the fish from surgery, the free end of the cannula was connected to a pressure transducer. Dorsal aorta pressure (P_{DA}) was monitored using a UFI model 1050BP (UFI, Morro Bay, CA, USA) pressure transducer calibrated using a water column. The P_{DA} signal was recorded with a data acquisition system (Biopac System Inc., Goleta, CA, USA) and

collected at 0.04 sec intervals using Acknowledge III (Biopac System Inc.) data acquisition software; sampling frequency was 10 Hz. Mean blood pressure = (systolic pressure + diastolic pressure)/2 and Rs (systemic resistance) was calculated as mean P_{DA}/V_b (cardiac output). Four separate experimental series were performed (see Section 2.3).

When drugs were applied, the caudal vein was also cannulated. A cannula (Clay-Adams PE50) was implanted into the vein in the anterior direction following exposure of the haemal arch by a lateral incision (~2 cm long) at the level of caudal peduncle (see Axelsson and Frirsche, 1994). To monitor cardiac output, a 3S ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the bulbus arteriosus. The bulbus was exposed by a small (~1.5 cm) ventral midline incision into the pericardial antrum. Lubricating jelly (K-Y Personal Lubricant; Johnson & Johnson) was used with the flow probe as an acoustic couplant. The caudal cannula and flow probe were anchored to the skin and the incisions closed using silk sutures. The tubing and cannulae were flushed with cold heparinized saline (100 i.u./ml sodium heparin) and the trout were revived by flushing the gills with aerated water until opercular movements became pronounced and regular.

Following surgery, fish were placed into individual opaque acrylic chambers supplied with aerated, flowing aquaria water (flow rate: > 2.5 l/min). The fish were allowed to recover for 24 h before the experiments were initiated. The chambers were small enough to prevent the fish from turning or rolling, but did not interfere with ventilation or tail movements. After the fish were placed into the boxes, the room was darkened until completion of the experiment.

2.2.8 α_{1A}- and α_{1D}-AR selective antagonists: *in vivo* injection and drugs

The selective α_{1A}-AR antagonist RS17053 and α_{1D}-AR antagonist BMY7378 were purchased from Tocris Cookson Inc. (Ellisville, MO). BMY7378 was dissolved in distilled water to a stock concentration of 100 nM, then dilute in 0.9% saline to the required working concentrations. RS17053 was prepared in DMSO to a stock concentration of 100 nM, then dilute in distilled water; final DMSO concentrations did not exceed 5%. Equivalent concentrations of DMSO were added as controls for the RS17053 experiments. To maintain normal osmolarity, mannitol was added to the working RS17053 solutions. Control injections included 0.9% saline for BMY7378, and DMSO plus mannitol for the RS17053 experiments. Fish were injected (0.6 ml kg^{-1}) via the caudal vein cannula over a 45 to 60 sec interval with the antagonists. Blank or control and six different concentrations (0.001, 0.01, 0.1, 0.5, 1, 2.5 nM) of the drugs were slowly injected into the fish from the lower to the higher concentrations, at which time all cardiovascular variables were stable.

2.3 Results

2.3.1 Sequence analysis

Two α_{1A}-ARs were cloned and called α_{1A}- and $\alpha_{1A'}$-AR (Fig. 2.1.A. and Fig. 2.1.B.). The partial α_{1A}-AR cDNA encodes a 330 amino acid protein that extends from the N-terminal end to TMD7 and exhibits the same putative seven TMDs as its mammalian AR counterparts. The partial $\alpha_{1A'}$-AR cDNA encodes a 120 amino acid protein that spans from TMD4 to the end of third intracellular loop. Two putative α_{1B}-

ARs cDNA were also cloned from trout and called α_{1B}- and $\alpha_{1B'}$-AR (Fig. 2.2). These two cDNAs encode 103 and 100 amino acid proteins, respectively. As with the $\alpha_{1A'}$-AR, these putative α_{1B}-ARs span from TMD4 to the end of third intracellular loop. Finally, a partial α_{1D}-AR cDNA was also cloned that encodes a 468 amino acid protein that spans TMD1 to the C-terminal tail and has the same characteristic seven TMD structure of its mammalian counterparts (Fig. 2.3). As with other fish ARs (Nickerson *et al.*, 2001), alignment of the three rainbow trout α_1-AR subtypes with α_1-AR sequences from various vertebrate species shows the greatest residue conservation within the TMDs, whereas the greatest diversity is found at the N- and C-terminal regions. Table 2.3 presents the percent identity between the alignments of the trout α_1-AR amino acid sequences and those for human and other fish α_1-AR sequences. Similarities range between 56 and 89% depending upon the species and receptor subtype.

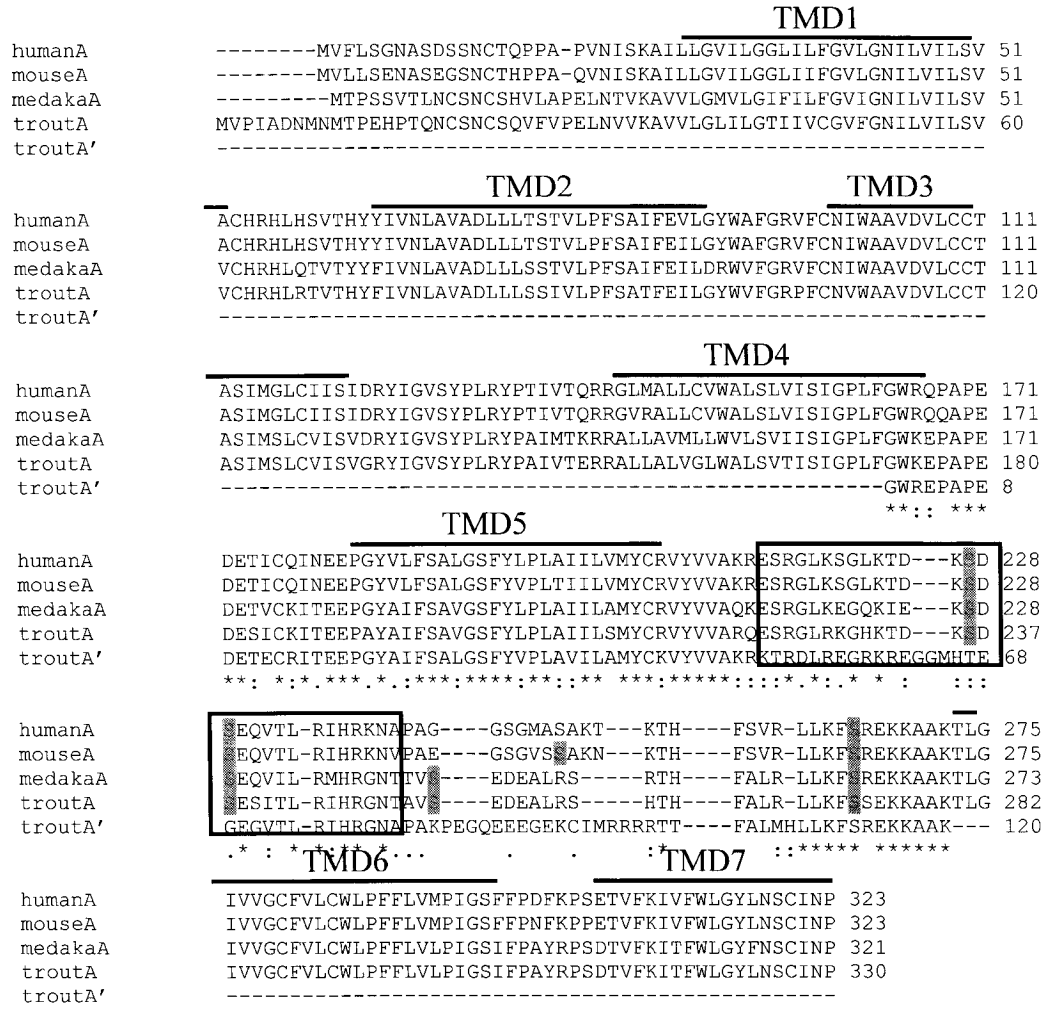

Figure 2.1.A. Amino acid alignments of two rainbow trout α_{1A}-ARs compared with mammalian (GenBank accession no. human NP_150646; mouse P97718) and medaka (GenBank accession no. Q91175) α_{1A}-ARs. (*) Indicates identical sequences, (:) indicates fully conserved 'strong groups', (.) indicates fully conserved 'weak groups'. All information is defined from CLUSTAL W (see Materials and Methods). Positions of the transmembrane domains (TMD) are shown as heavy lines and G-protein binding domains are indicated by a box. Shaded serine residues are potential protein kinase A, protein kinase C or G-protein coupled receptor kinase phosphorylation sites as defined by NETPHOS 2.0.

```
troutA          MVPIADNMNMTPEHPTQNCSNCSQVFVPELNVVKAVVLGLILGTIIVCGVFGNILVILSV 60
troutA'         ------------------------------------------------------------


troutA          VCHRHLRTVTHYFIVNLAVADLLLSSIVLPFSATFEILGYWVFGRPFCNVWAAVDVLCCT 120
troutA'         ------------------------------------------------------------


troutA          ASIMSLCVISVGRYIGVSYPLRYPAIVTERRALLALVGLWALSVTISIGPLFGWKEPAPE 180
troutA'         ----------------------------------------------------GWREPAPE 8
                                                                    **:*****

troutA          DESICKITEEPAYAIFSAVGSFYLPLAIILSMYCRVYVVARQESRGLRKGHKTD---KSD 237
troutA'         DETECRITEEPGYAIFSALGSFYVPLAVILAMYCKVYVVAKRKTRDLREGRKREGGMHTE 68
                **: *:*****.******:****:***:**:***:*****::::*.**:*:* :   :::

troutA          SESITL-RIHRGNTAVS----EDEALRS-----HTH-----FALR-LLKFSSEKKAAKTL 281
troutA'         GEGVTL-RIHRGNAPAKPEGQEEEGEKCIMRRRRTT-----FALMHLLKFSREKKAAK-- 120
                .*.:** ******:...    *:*. :.     :*      ***  ***** ******

troutA          GIVVGCFVLCWLPFFLVLPIGSIFPAYRPSDTVFKITFWLGYLNSCINP 330
troutA'         -------------------------------------------------
```

Figure 2.1.B. Amino acid alignments of two rainbow trout α_{1A}-ARs
See Fig. 2.1.A. for details.

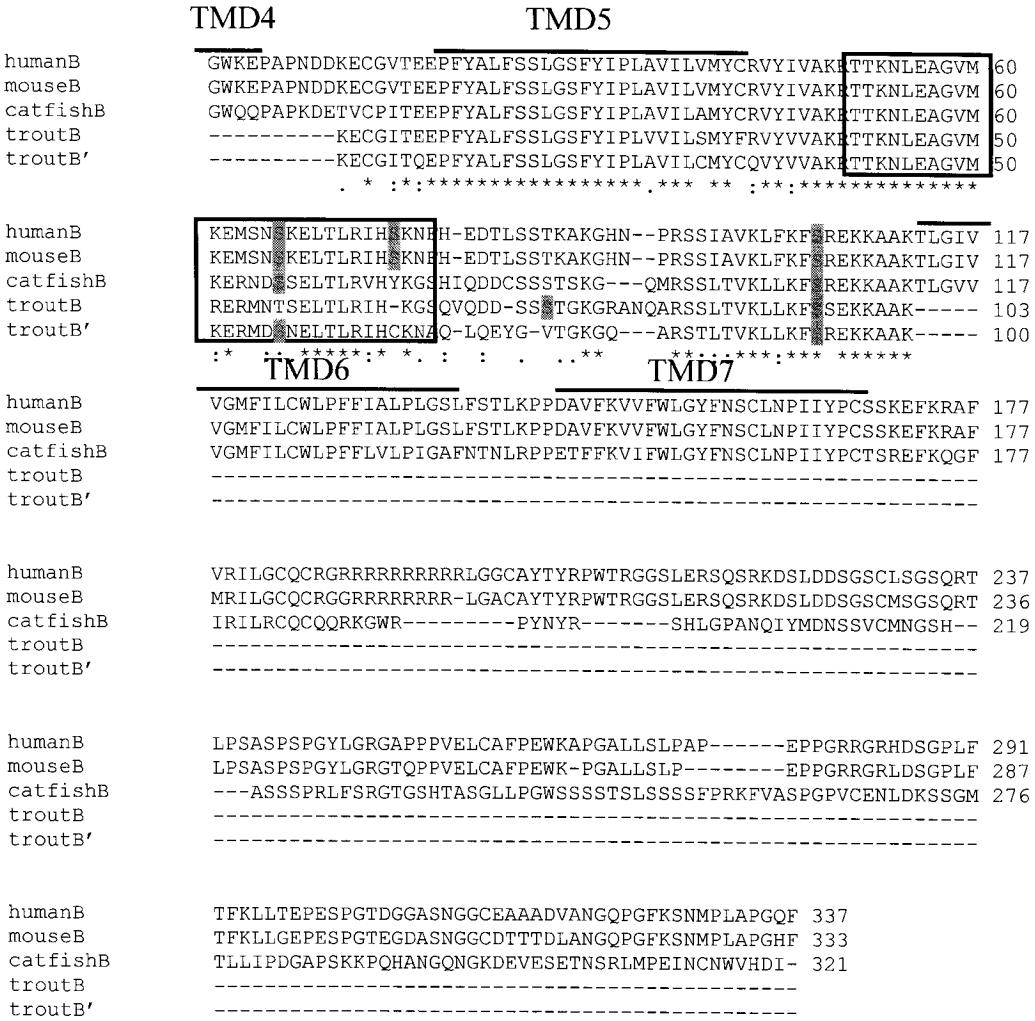

Figure 2.2. Amino acid alignments of two rainbow trout α_{1B}-ARs compared with mammalian (GenBank accession no. human P35368; mouse P97717) and catfish (see Appendix) α_{1B}-ARs. See Fig. 2.1.A. for details.

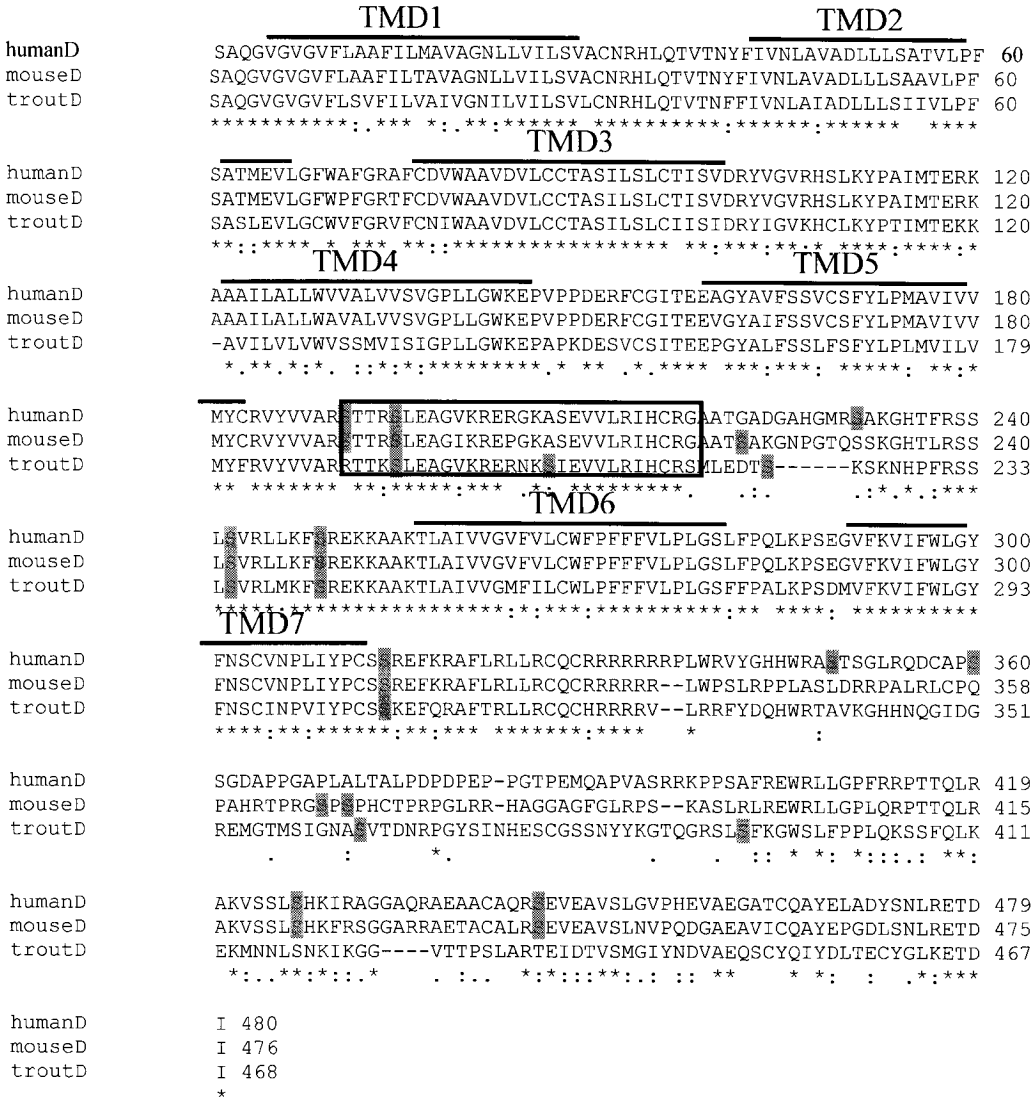

Figure 2.3. Amino acid alignments of rainbow trout α_{1D}-AR compared with the mammalian (GenBank accession no. human NP_000669; mouse P97714) α_{1D}-ARs. See Fig. 2.1.A. for details.

Table 2.3. Similarities between α_1-AR amino acid sequences of trout and human or trout and other fish α_1-ARs

	Human	Other Fish
α_{1A}-AR	74%	83% medaka (*Oryzias latipes*)
$\alpha_{1A'}$-AR	59%	62% medaka (*Oryzias latipes*)
α_{1B}-AR	71%	79% bullhead (*Ameiurus nebulosus*)
$\alpha_{1B'}$-AR	73%	76% bullhead (*Ameiurus nebulosus*)
α_{1D}-AR	56%	89% European eel (*Anguilla anguilla L*)

The similarity number is calculated from BLAST using GenBank accession numbers noted on Figures 2.1, 2.2 and 2.3 and amino acid multiple alignment (Clustal W). The bullhead and eel α_1-AR sequences are found in the Appendix.

2.3.2 Phylogenetic analysis

Phylogenetic analysis was used to trace the evolutionary relationship between all α_1-AR sequences mined from GenBank; Table 2.2 provides accession numbers for all sequences used to construct Fig. 2.4. Maximum likelihood analysis produced a tree comprising three major clades: α_{1D}-ARs, α_{1A}-ARs and α_{1B}-ARs. Each clade splits into two branches, one representing mammals and the other the homologous piscine receptors. The topology of the tree is consistent with the normal phylogeny of the organisms used, with the exception that the lungfish α_{1A}-AR was placed with the mammalian group. This may reflect the fact that the total number of α_1-ARs in GenBank is small, as support values at each major node varies from 53 to 75%, but more likely the recent data supporting the close phylogenetic relationship between the lungfish and tetrapods.

2.3.3 Potential phosphorylation sites

Several potential serine phosphorylation sites were found in each sequence. Trout α_{1A}-, α_{1B}- and α_{1D}-ARs share similar phosphorylation sites with mammals within the third intracellular loop and C-terminal tail; however the trout α_{1A}- and α_{1B}-ARs lack these phosphorylation sites at the same location (see Fig. 2.1.A., 2.2 and 2.3).

2.3.4 α_{1A}- and α_{1D}-AR mRNA tissue distribution

Real-time PCR was used to analyze α_{1D}- and α_{1A}-AR gene expression in 15 trout tissues. An unique probe of 20 bases (see 2.2.6.) was designed for each α_1-AR subtype corresponding to the region that varied the most between the two subtypes as determined by the multiple alignments of α_{1D}- and α_{1A}-AR gene sequences. Figures 2.5 and 2.6 demonstrate that both α_{1D}-AR and α_{1A}-AR are widely expressed in the trout tissues examined, including within the peripheral arteries, consistent with previous

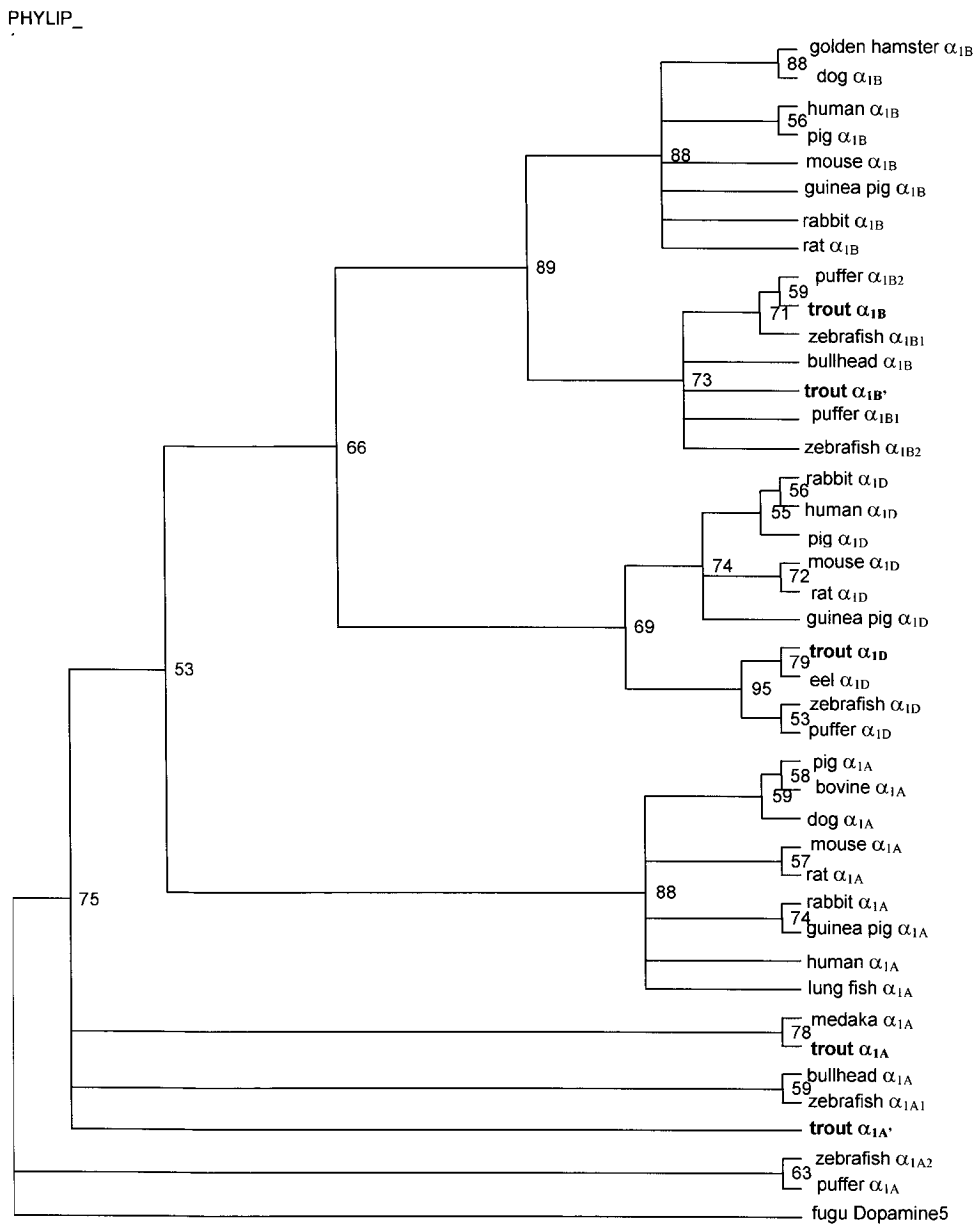

Figure 2.4. A maximum likelihood tree inferred from amino acid alignments (see Table 2.3) of vertebrate α_1-ARs, including five rainbow trout α_1-AR sequences identified in this study (in bold). Support values at the nodes are indicated as a percentage from 1000 puzzling steps. The lungfish α_{1A}-AR, the bullhead α_{1B}-AR and the eel α_{1D}-AR sequences are presented in the Appendix.

studies in the rat and human (Suzuki *et al.*, 1997). In general, the α_{1D}-AR mRNA is highly expressed in ABA, spleen, VA, brain, and EBA with highest expression in BA, and little in the heart (Fig. 2.5). α_{1A}-AR mRNA expression is relatively high in white muscle, EBA, DA, VA, gill and spleen with highest expression in brain but no detectable expression in the ABA and consistent with the α_{1D}-AR, expression of the α_{1A}-AR in heart is relatively low (Fig. 2.6). Comparing the expression patterns of these two receptors further indicates the predominance of the α_{1D}-AR subtype over that of the α_{1A}-AR in these tissues (Fig. 2.7). The relative expression ratio of α_{1D}-AR over α_{1A}-AR is especially high in BA, spleen and CMA.

2.3.5 α_{1A}- and α_{1D}-AR selective antagonists: *in vivo* injection

The involvement of α_1-ARs in the vascular response of the rainbow trout was confirmed with the use of α_1-ARs selective antagonists. Figure 2.8 demonstrates that blood pressure (BP) and systemic resistance (Rs) were inhibited in a concentration-dependent manner by the selective α_{1A}-AR antagonist RS17053 and α_{1D}-AR antagonist BMY7378, with complete inhibition achieved by an antagonist concentration of 2.5 nM. Within the dose range that the fish could tolerate, an EC50 between 0.5 nM and 1 nM was observed; an actual EC50 could not be calculated as the fish died at antagonist concentrations around 2.5 nM.

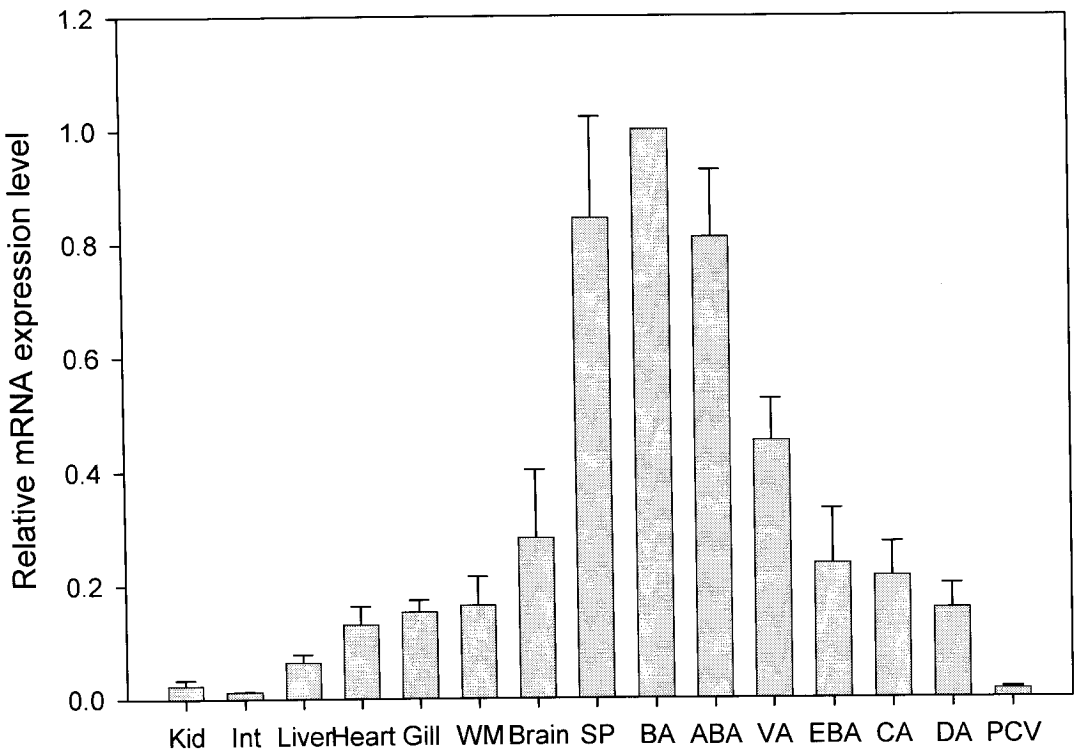

Figure 2.5. Real-time PCR analysis showing the tissue distribution of α_{1D}-AR mRNA in fifteen trout tissues. Equal amounts of total RNA were used in cDNA synthesis for real-time PCR. Data are reported as relative mRNA expression level by using β-actin as the internal control gene. All values are compared with the tissue with the highest expression (BA). Values are means ± SEM, n = 3. The fifteen tissues are kidney (Kid), intestine (Int), liver, heart, gill, white muscle (WM), brain, spleen (SP), bulbus arteriosus (BA), afferent branchial artery (ABA), ventral aorta (VA), efferent branchial artery (EBA), celiacomesenteric artery (CA), dorsal aorta (DA), and posterior cardinal vein (PCV).

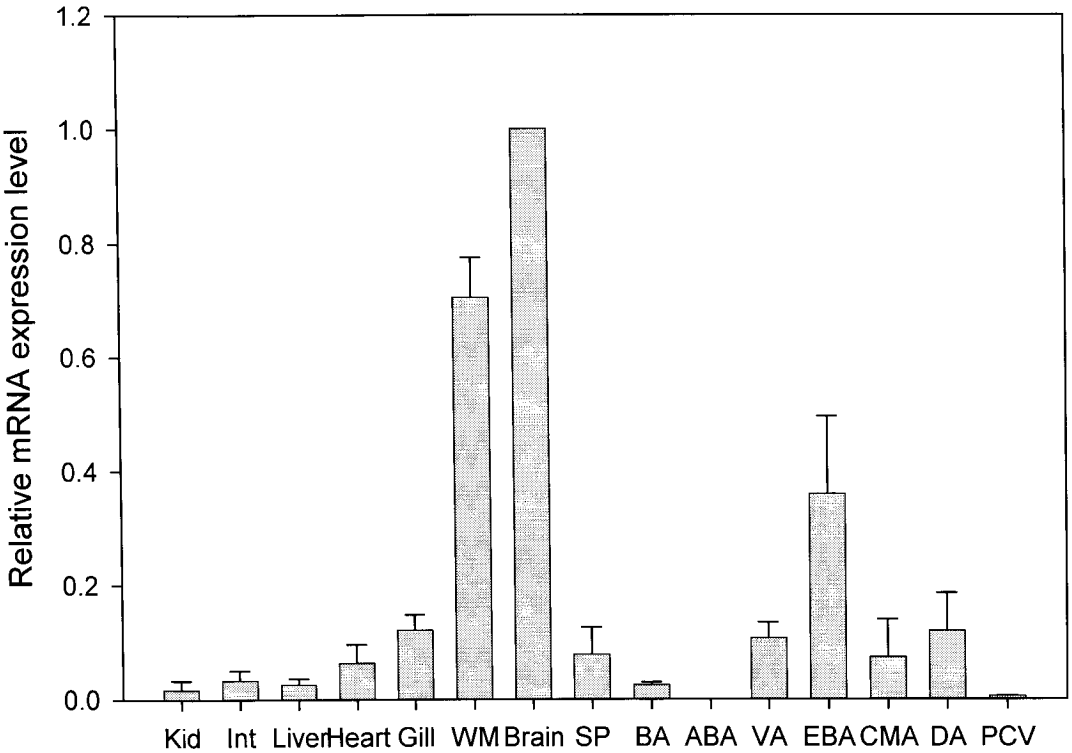

Figure 2.6. Real-time PCR analysis showing the tissue distribution of α_{1A}-AR mRNA in fifteen trout tissues. See Fig. 2.5 for a detailed description.

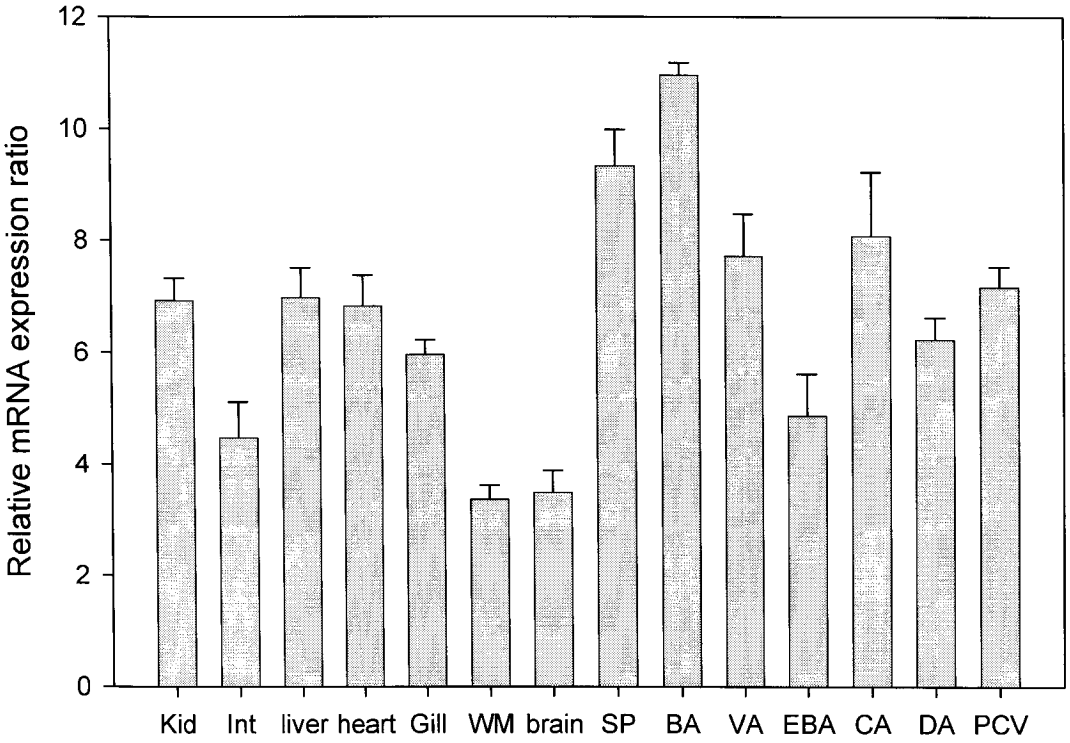

Figure 2.7. Relative mRNA expression ratio (α_{1D}-AR/α_{1A}-AR). The ratio was calculated based on individual α_{1D}-AR and α_{1A}-AR tissue distribution real-time PCR analysis in the 14 tissues (see Fig. 2.5 and 2.6). The ABA was not included as no α_{1A}-AR mRNA was detected in this tissue. Values are means ± SEM, n = 3. See Figure 2.4 for abbreviations.

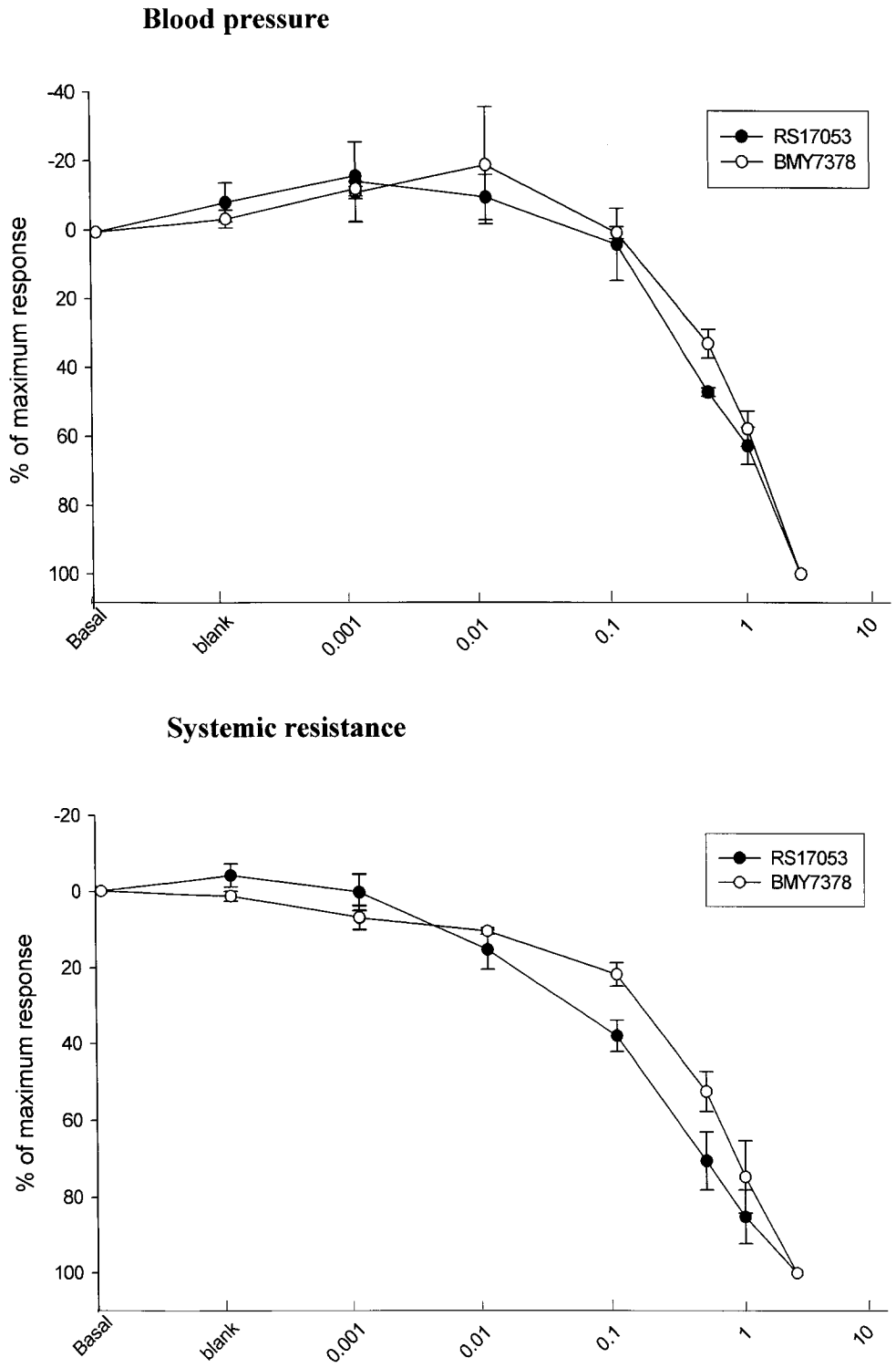

Figure 2.8. Dose-response curves for the α_{1A}-AR selective antagonist RS17053 and α_{1D}-AR selective antagonist BMY7378 on blood pressure and systemic resistance following intravenous drug injection. Data are % of maximum inhibition based upon the highest tolerated antagonist concentration of 2.5 nM. Basal (no injection), blank (saline or DMSO/mannitol; see Materials and Methods), and six drug concentrations. Values are means ± SEM., n = 4.

2.4 Discussion

The α_1-AR gene family has been well described in mammals at both the molecular and pharmacological levels. However information regarding α_1-ARs from fish is rare. Five rainbow trout α_1-ARs were identified that show highly conserved sequences with respect to mammalian α_1-ARs (Fig. 2.1.A., 2.2 and 2.3). The most highly conserved regions are found within the seven TMDs in contrast to the N- and C-terminal sequences that are variable; this is consistent with other ARs (Nickerson *et al.*, 2001). Not surprisingly, two α_{1A}-ARs and two α_{1B}-ARs were identified. Only a short α_{1B}-AR sequence was cloned, but the two α_{1A}-AR sequences do demonstrate differences in length and placement of phosphorylation sites within the third intracellular or G-protein binding domain (see Fig. 2.1.A; 2.1.B). Rainbow trout, like other salmonids, are tetraploid and a complete genome duplication occurred in the evolution of this group (Allendorf and Thorgaard, 1984). The precise role of this α_1-AR gene diversity in trout is unknown, but it is generally assumed to relate to the complex environmental challenges confronted by salmonid species.

Phylogenetic analysis revealed that the α_1-AR sequences from rainbow trout are closely related to their relevant mammalian counterparts. The position of the rainbow trout α_1-AR sequences in the tree relative to those of mammals is expected based upon the evolutionary relationships between fish and mammals (Ohno *et al.*, 1968). Moreover, the phylogenetic analysis supports the claim that the sequences are indeed rainbow trout α_1-ARs. The three well-defined (at the molecular, pharmacological and physiological levels) α_1-ARs subtypes in mammals all have homologs within teleost fishes. The multiple forms of the α_{1A}-

and α_{1B}-ARs identified in trout also fit within the relevant subtypes. This makes it likely that the teleost α-ARs system is more complex than that previously reported for mammals. Interestingly, the position of the lungfish α_{1A}-AR indicates that it is more closely related to the mammalian α_{1A}-ARs than the fish homolog. There are two possible explanations for this. First, the numbers of α_{1A}-AR sequences are not large, thus the certainly of the placement of this particular sequence may simply reflect the lack of available sequences. Second, and more likely, is that this placement reflects the evolutionary position of the lungfish relative to tetrapods. Molecular and paleontological evidence is accumulating to show that the lungfish rather than the coelacanth are the closest living relative of tetrapods (Brinkmann *et al.*, 2004). The placement of the lungfish α_{1A}-AR provides further support for this phylogeny.

Phosphorylation by protein kinases A or C and by specific G-protein coupled receptor kinases is an important mechanism involved in the regulation of receptor function, including that of the α_1-ARs (Alonso-Llamazares *et al.*, 1996; Diviani et al., 1997; Garcia-Sainz *et al.*, 2001). Phosphorylation is considered to be the initial step in the process of desensitization (Garcia-Sainz *et al.*, 2001). Previous studies determined that several serine and threonine residues located within the third intracellular loop and the C-terminal tail of the α_1-ARs are potential phosphorylation sites (Alonso-Llamazares *et al.*, 1996; Garcia-Sainz *et al.*, 2001). The third intracellular loop binds and activates the G protein (Franke *et al.*, 1990; Wong and Ross, 1994). The position of the G-protein binding domain for the α_{1B}-AR was proposed by several studies (Alonso-Llamazares *et al.*, 1996; Cotecchia *et al.*, 1990, 1998; Greasley *et al.*, 2001). Using this information and the multiple alignments of three subtypes of α_1-ARs, the proposed G-protein binding domains for the trout α_{1A}-, α_{1B}- and α_{1D}-ARs are noted in Figures 2.1.A., 2.2 and 2.3. This placement is consistent with that reported by Nickerson *et*

al. (2001) for the trout β_2-ARs. Multiple alignments of α_1-ARs show that the location of putative phosphorylation sites within the third intracellular loop and G-protein binding domain are conserved between mammalian and fish sequences, although not all trout α_1-AR sequences have putative phosphorylation sites within these regions. The trout α_{1A}-, α_{1B}- and α_{1D}-AR sequences all demonstrate this conserved pattern of phosphorylation sites (Fig. 2.1.A., 2.2 and 2.3). It has been stated that receptor phosphorylation is associated with signal turn-off/desensitization and receptor dephosphorylation with re-sensitization (Premont *et al.*, 1995; Ferguson *et al.*, 1997). The changes in the receptor molecules induced by phosphorylation likely affect their ability to interact with G proteins as well as other proteins (Garcia-Sainz *et al.*, 2000). The lack of such phosphorylation sites in the trout α_{1A}- and α_{1B}-AR sequences within the G-protein binding domain may affect signal transduction and the regulation of these receptor *in vivo*. One could postulate that the α_{1A}- and the α_{1B}-AR will be less sensitive to phosphorylation and desensitization during prolonged agonist exposure.

α_1-ARs are expressed at different levels in different tissues in mammals, but no study to date has reported α_1-ARs mRNA expression patterns in fish. Characterization of α_1-ARs is difficult due to the limited availability of specific agonists/antagonists for these receptors and the high degree of conservation of the α_1-AR subtypes. Molecular methods have quantified the tissue distribution of α_1-ARs in different animals, particularly in the peripheral vasculature (Perez *et al.*, 1994; Piascik *et al.*, 1994, 1995; Guarino *et al.*, 1996; Suzuki *et al.*, 1997). At the mRNA level, α_{1A}-AR mRNA predominates in heart, cerebellum, cerebral cortex and prostate (Faure *et al.*, 1994; Price *et al.*, 1994); α_{1B}-AR mRNA is present in high levels in spleen and kidney (Price *et al.*, 1994); and, α_{1D}-AR mRNA predominates in aorta (Price *et al.*, 1994). The expression of α_1-ARs varies with vessel bed and is modulated by

aging (chronic catecholamine exposure) (Rudner *et al.*, 1999). However, our study using real-time PCR analysis (Fig. 2.5 and 2.6) showed that the α_{1D}- and α_{1A}-AR genes are widely expressed in the tissues assessed and especially in the peripheral arteries including the ABA and the EBA. This result is consistent with some reports in mammals (Perez *et al.*, 1994; Piascik *et al.*, 1994, 1995; Guarino *et al.*, 1996). McGrath *et al.* (1989) and Muldowney and Faber (1991) also pointed out that α_1-ARs mediate constriction in large arteries, α_1-ARs and α_2-ARs in resistance vessels and α_2-ARs predominantly in capacitance vessels. This expression pattern demonstrates that α_1-ARs possess the capacity to regulate sympathetic vasoconstriction (Elhawary *et al.*, 1992; Piascik *et al.*, 1995; Bylund *et al.*, 1995; Zhou *et al.*, 1996; Piascik *et al.*, 1997) and thus modulate systemic vascular resistance (Jarajapu *et al.*, 2001), and implicates α_1-ARs in the control of blood pressure (Zuscik *et al.*, 2001). Compared with α_{1A}-AR mRNA expression, the α_{1D}-AR mRNA is expressed at greater levels in the 15 tissues examined in this thesis. Many tissues express more than one subtype, and in fact it is the rare tissue that expresses only one subtype (Guimaraes and Moura, 2001). In the trout, no α_{1A}-AR was detected in the ABA thus implicating the α_{1D}-AR subtype in the control of the branchial circulation that is reported to be controlled by α_1-ARs in fish (Axelsson *et al.*, 1994). However, as noted here, multiple forms of α_1-AR genes do exist in rainbow trout so it is likely that other AR-types may also be involved.

The relatively low level of α_{1D}-AR expression in the heart suggests that this tissue would be less sensitive to receptor blockade, likely a safety mechanism since receptor blockade could result in heart failure. In addition, previous studies in mammals showed α_{1D}-ARs are functionally expressed primarily in arteries including the aorta, carotid, mesenteric and femoral arteries (Piascik *et al.*, 1995; Gisbert *et al.*, 2000; Gomez-Zamudio *et al.*, 2002;

Arevalo-Leon *et al.*, 2003). It is important to remember that α_1-AR subtype expression is cell and species-dependent, so linking RNA expression in mammals to that in fish may be problematic. The biological significance of these receptors may be elucidated though the study of species-specific and tissue-specific expression patterns.

One further feature of the molecular characterization of α_1-ARs should be mentioned. Studies report that the mouse α_{1D}-AR gene possesses two exons and one large intron of at least 20Kb located between putative TMDs 6 and 7 (Arai *et al.*, 1999). The presence of this intron makes the α_1-ARs distinct from all other AR types that lack this intron, leading to a distinctive receptor subfamily (Ramarao *et al.*, 1992; Arai *et al.*, 1999). Analysis of α_1-AR genes was shown that they possess very similar gene structures supporting the hypothesis that all α_1-AR subtypes arose from one ancestral gene (Arai *et al.,* 1999). This distinctive characteristic allowed real-time PCR primers to be designed that spanned a putative intron-exon boundary in trout to avoid genomic DNA amplification.

The sympathetic nervous system plays a critical role in the response of animals to environmental change (Mark, 1996). The α_1-ARs primarily mediate vasoconstrictor responses of peripheral elastic and muscular arteries to adrenergic stimuli (Ruffolo *et al.*, 1991). Veelken and Schmieder (1996) reported that resistance and capacitance vessels are both dilated by α_1-AR antagonists. To examine the potential role of α_1-ARs in the regulation of blood pressure in trout, the alterations in cardiovascular parameters produced by α_1-AR selective antagonists in cannulated trout were monitored. The α_{1A}-AR selective antagonist RS17053 and the α_{1D}-AR selective antagonist BMY7378 were used in this experiment. Unlike agonists that may impact both affinity and total receptor binding, antagonists only affect receptor affinity (Ruffolo *et al.*, 1994). BMY7378 is a high affinity α_{1D}-AR antagonist

in mammals as established using cloned, functionally expressed receptors. Pharmacology studies using these expression systems reported that BMY7378 had approximately a 100-fold higher selectivity for the α_{1D}-AR over the other two subtypes [K_i values are 2, 800 and 600 nM for cloned rat α_{1D}, rat α_{1A} and hamster α_{1B} receptors, respectively (Goetz *et al.*, 1995; Piascik *et al.*, 1995;Yang and Endoh, 1997)]. It has been proposed that the binding site of the α_{1A}-AR is more flexible and topographically different when compare with the other two subtypes (Benedetti *et al.*, 1997). RS17053, an α_{1A}-ARs antagonist, has a very high affinity for the α_{1A}-AR [pK_i (compound binding affinity) and pA_2 (antagonist affinity values) estimates of 9.1-9.9; K_i and A_2 estimates of 0.126-0.79 nM] and a 30 to 100-fold selectivity over the α_{1B}- and α_{1D}-AR subtypes (pK_i and pA_2 estimates 7.7-7.8; K_i and A_2 estimates 16-20 nM) (Ford *et al.,* 1996; Marshall *et al.*, 1996). Figure 2.7 shows that both BMY7378 and RS17053 inhibit trout cardiovascular responses (BP, Rs) in a dose-dependent manner. This evidence supports the functional existence of both the α_{1D}- and the α_{1A}-ARs in trout blood vessels and their potential involvement in the regulation of blood pressure. In addition, as there was no difference found between the responses to BMY7378 and RS17053, both α_{1D}- and α_{1A}-ARs take part in the regulating blood pressure *in vivo.* To verify that BMY7378 and RS17053 exhibit similar binding patterns in trout as in mammals, antagonist binding sites can be compared. Previous studies showed that several single amino acids are involved in binding specific antagonists (Hein and Kobilka, 1995). Given the fact that there is a lack of structural homology between various classes of α_1-AR specific antagonists, antagonist binding to the α_1-AR is more complex and our understanding is limited. However, Wangh *et al.* (2001) proposed that the plane of the antagonist binding pocket on the AR lies above the agonist binding pocket. They also demonstrated that the α_{1A}-AR selective antagonists phentolamine

and WB4101 interacted with three consecutive residues (Gln177-He178-Asn179) in the second extracellualar loop of the AR (Guarino *et al.*, 1996; Zhao *et al.*, 1996; Waugh *et al.*, 2000). In addition, two conserved phenylalanine residues close to the extracellular surface of TMD7 are involved in non-selective binding for almost all α_1-ARs antagonists (Waugh *et al.*, 2001). The sequence of both the second extracellular loop and TMD7 are well conserved based upon the multiple alignments of the trout α_{1A}- and α_{1D}-ARs with the mammal homologs, suggesting that BMY7378 and RS17053 have similar binding sites and it can be speculated that BMY7378 and RS17053 have activities in trout similar to those in mammals. Thus, BMY7378 does inhibit α_{1D}-AR and RS17053 does inhibit α_{1A}-AR in trout. These data support the use of the subtype-selective antagonists BMY7378 and RS17053 in fish, and in particular in rainbow trout to characterize α_1-ARs.

2.5 Conclusions

The isolation and cloning of rainbow trout α_1-AR cDNAs were the initial steps in achiving a better understanding the entire α_1-adrenergic system in fish and other vertebrates. The present study provides a basic molecular characterization of the trout α_1-AR gene family, and its tissue distribution, and provides preliminary data for its potential role in regulating BP in trout. It is apparent that the number of α_1-AR genes is larger in the trout than in mammals, and as shown by differences in potential phosphorylation sites in the α_{1A}- vs $\alpha_{1A'}$-ARs, such multiple forms may have important physiological consequences. And finally, it is apparent that functional differences exist between the α_1-AR homologs of fish and mammals, as the

broad tissue distribution of trout α_1-ARs implicates these receptors as functioning in a variety of physiological processes.

Chapter 3. The effects of salt-induced high blood pressure on α_1-AR expression and cardiovascular physiology in rainbow trout (*Oncorhynchus mykiss*)

3.1 Introduction

Like all members of the GPCR superfamily, α_1-ARs possess seven transmembrane domains as well as three intracellular and three extracellular loops. Together with β- and α_2-ARs, the α_1-ARs mediate the endogenous actions of noradrenaline and adrenaline secreted either as neurohormones into the circulation or as neurotransmitters into synaptic clefts. The binding of catecholamines to α_1-ARs activates the G_q protein within the membrane resulting in the hydrolysis of PIP_2 to IP_3 and DAG by activation of PLC-β; ultimately, PKC is activated to stimulate and/or inhibit a suite of cellular functions. After several decades of discovery and debate, it is now accepted that α_1-ARs are comprised of three subtypes, α_{1A}, α_{1B} and α_{1D} based on the accumulated evidence of pharmacological and molecular studies.

Vascular adrenoceptors may be affected in numerous disease states, either contributing to the genesis of disease or being secondarily altered as a consequence of disease (Guimaraes and Moura, 2001). Stimulation of α_1-ARs elicits vasoconstriction contributing to basal vascular tone as well as to the acute regulation of systemic vascular resistance in the event of a sudden decrease in systemic blood pressure (Ruffolo *et al.*, 1985). Consequently, α_1-AR antagonists such as prazosin are used as efficacious clinical anti-hypertensive drugs (Vargas and Gorman, 1995). Despite the enormous progress made in identification and characterization of α_1-AR subtypes, several important questions remain to be answered. For

example, to what extent are each of the subtypes involved in the regulation of blood pressure? Recently, knockout techniques were used to investigate the function of three α_1-ARs subtypes in the vascular system of mice (Tanoue *et al.*, 2003). Based on the results of this, and other studies, it would appear that all three subtypes are involved in regulating arterial pressure. α_{1A}-ARs are expressed in resistance vessels and are required to maintain arterial blood pressure (Rokosh *et al.* 2002). α_{1D}-ARs are expressed in aorta and directly regulate arterial blood pressure by vasoconstriction (Tanoue *et al.* 2002). α_{1B}-ARs play a minor role in blood pressure regulation (Daly *et al.* 2002). Zhang *et al.* (1998) pointed out that the regulation of peripheral resistance and venous capacitance is similar between the rainbow trout and mammals and, in part, reflects the sympathetic control of α_1-ARs. So, studying the vascular function of α_1-ARs in fish could potentially provide important information relevant to blood pressure regulation in mammals.

Hypertension, characterized by an increase in peripheral vascular resistance (PVR), is the leading disease worldwide (Jackson and Insel, 1993; Marin, 1993). Essential hypertension is thought to be a familial disease resulting from environmental factors that accumulate over time in genetically susceptible individuals (Vecchione *et al.*, 2002). Several risk factors are related to the development of essential hypertension including stress, obesity, smoking and a diet high in salt. Among these factors, the high salt diet has been extensively investigated. The observation that salt intake is associated with hypertension is hardly new; it was noted in a Chinese medicine book dating from the first millennium BCE (O'Shaughnessy and Karet, 2004). Although the mechanisms underlying salt-induced hypertension are not completely clear, extracellular fluid or plasma volume expansion is considered one of the key mechanisms, in addition to changes in vascular smooth muscle and the sympathetic nervous

system (Janssen and Smits, 1989; Tobian, 1996). According to some authors, α_1-ARs may be involved in the pathogenesis/maintenance of some types of hypertension (Guimaraes and Moura, 2001). For example, a high density of α_{1D}-ARs was reported in the resistance vessels of hypertensive rats (Villalobos-Molina and Ibarra, 1999; Villalobos-Molina and Ibarra *et al.*, 1999). Furthermore, pharmacological studies of (spontaneously hypertensive rats) SHR and WKY rats showed α_{1D}-ARs may be involved in vascular supersensitivity leading to the hypertension state (Guimaraes and Moura, 2001). In addition, it has been shown that α_1-ARs regulate CNS sympathetic outflow. When subjected to subtotal nephrectomy followed by dietary salt loading, the increase in blood pressure was much higher in wild type mice than in α_{1D}-KO mice. These results demonstrate a functional role of α_{1D}-ARs in the development of dietary salt-induced hypertension in mice. Subsequently, Chu *et al.* (2004) showed that α_{1D}-ARs are involved in central salt-induced hypertension and water intake. Additionally, other authors reported that α_{1D}-ARs could be involved in the pathogenesis and maintenance of hypertension (Villalobos-Molina *et al.*, 1999; Villalobos-Molina and Ibarra, 1999; Ibarra *et al.*, 2000). Frank (1999) demonstrated that during developing hypertension, α_1-ARs increase sodium uptake and calcium in distal tubule (DT) cells of SHR rates, and expression of α_{1B}-ARs is selectively up regulated during developing hypertension. Increasing PKC activity, phosphoinositide metabolism and calcium mobilization (factors known to augment contractile responses to agonists) may also contribute to hypertension (Villalobos-Molina and Ibarra, 1996).

Vascular smooth muscle tissue contains a mixture of α_1-ARs, thus more than one subtype are potentially involved in the response to salt loading (Zhong and Minneman, 1999).

So, it has not yet been determined whether during hypertension there are changes exclusively to a specific α_1-AR subtype and/or alteration in the proportion of several receptor subtypes.

In teleosts, systemic vascular resistance is largely controlled by the interaction of circulating catecholamines and sympathetic neurotransmitters with α_1- and β-ARs (Perry *et al.*, 1999). Generally, the α_1-AR mediated vasoconstriction is the predominant response and thus an increase in sympathetic nerve activity would be expected to elevate systemic resistance (Perry *et al.*, 1999). To my knowledge there are no studies that have examined the role of α_1-ARs in the development of salt-induced hypertension in rainbow trout. Given the unique advantages associated with using trout to examine vascular physiology (see Chapter 1), it will be informative to determine whether the mechanisms involved in salt-induced hypertension in an early branching vertebrate, such as the rainbow trout, are similar to the mechanisms that operate in mammals. It will be important to examine which subtypes are specifically involved in the development of salt-induced hypertension.

Based upon the substantial evidence in mammals that α_1-ARs play a role in the regulation of blood pressure and the fact that the fish vasculature responds to α_1-AR agonists and antagonists, I hypothesize that α_1-ARs will be involved in salt-induced high blood pressure in the rainbow trout.

However, because the ligand extreme pharmacological similarities and the lack of clearly discriminating ligands, it has not been possible to differentiate which subtype plays the main role without molecular characterazition of these three subtypes. To shed light on this question, rainbow trout was used as a model organism, and gene expression of the two cloned α_1-ARs subtypes as well as their physiological roles was examined during the development of salt-induced hypertension in our study. Studying cases where high salt could induce high

blood pressure can yield molecular clues to the underlying biology and offer insights into treatment that may even apply to high blood pressure related diseases.

3.2 Materials and Methods

3.2.1 Animals

Rainbow trout (*Oncorhynchus mykiss*), weighing approximately 450-500 g, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were transported to the University of Ottawa Aquatic Care Facility and were maintained in fibreglass holding tanks (1275 L) containing well aerated, dechloraminated City of Ottawa tap water at 13°C. Fish were subjected to a constant 12L: 12D photoperiod and fed five times a week with commercial trout pellets [Martin Mills 5 PT composed of: 41.0% crude protein (min); 11.0% crude fat (min); 3.5% crude fibre (max); 1.0% calcium (actual); 0.85% phosphorus (actual); 0.45% sodium (actual); 6,800 IU kg^{-1} vitamin A (min); 2,100 IU kg^{-1} vitamin D (min); 80 IU kg^{-1} vitamin E (min); 200 IU kg^{-1} vitamin C (min)]. Receptor characterization experiments were conducted between August and October.

3.2.2 Normal and high salt diets

Fish were separated into four groups and each group was placed into individual tanks (1275 L). Two control groups were fed a commercial diet (see above) while the other two were fed a reconstituted diet containing 11% (w/w) NaCl (Salman and Eddy, 1987). The salt diet was prepared as follows: the normal diet pellet was ground; add 11% (w/w) salt and adequate water to moisten the mix; squeeze the mix through a pellet maker (Bizerba, Elpack Ltd); dry the new pellets. The size of the pellets was similar to that of the original ones. The

amount of diet containing salt was gradually increased to 11% over an 8-day period. The fish were then fed (1% body mass per day) a normal or full (11%) salt diet for 4-6 weeks.

3.2.3 Surgical procedures

Trout were anesthetized by immersion in an oxygenated benzocaine solution (0.1 g l^{-1}) until voluntary activity ceased. Fish were then transferred to an operating table that enabled continuous irrigation of the gills with the same anesthetic solution. An indwelling cannula (Clay-Adams PE 50 polyethylene tubing) was implanted into the dorsal aorta (Soivio *et al.*, 1975). The free end of the cannula was connected to a pressure transducer as noted in Chapter 2. To permit drug injections, the caudal vein was cannulated (Clay-Adams PE 50) in the anterior direction using standard surgical procedures (Axelsson and Fritsche, 1994). To measure cardiac output, a 3S ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the bulbus arteriosus. The bulbus was exposed by a small (~1.5 cm) ventral midline incision and removal of the pericardium. Lubricating jelly (K-Y Personal Lubricant; Johnson and Johnson) was used with the flow probe as an acoustic couplant. The caudal cannula and flow probe were anchored to the skin and all incisions were closed using silk sutures. The tubing and cannulae were flushed with cold heparinized saline (100 i.u. ml^{-1} sodium heparin). Fish were revived by flushing the gills with aerated water until opercular movements became pronounced and regular. The fish were placed in individual opaque acrylic chambers supplied with aerated, flowing water (flow rate > 2.5 l min^{-1}) where they were allowed to recover for 24 h prior to beginning an experiment.

Dorsal aortic blood pressure (P_{DA}) was measured using a UFI model 1050BP (UFI, Morro Bay, CA, USA) pressure transducer calibrated against a column of water. Cardiac output (V_b) was determined by connecting the ultrasonic flow probe to a small animal blood

flow meter (T106, Transonic Systems, Ithaca, NY). The analog signals (P_{DA} and V_b) were converted to digital data and stored by interfacing with a data acquisition system (Biopac Systems Inc.) using AcKnowledge™ data acquisition software (sampling rate set at 30 Hz) and a Pentium™ PC. Cardiac frequency (f_H) was calculated from the pulsatile V_b recordings using an automatic rate function feature of the data acquisition software. Systemic resistance (R_s) was calculated as mean P_{DA} (mm Hg)/mean V_b (ml min^{-1} kg^{-1}). Blood was sampled the day following surgery. Blood samples for sodium and chloride analysis were taken at the beginning of an experiment, before any treatment

3.2.4 Effects of external hypercapnia

Trout respond to external hypercapnia with a marked increase in R_s owing to α-AR mediated vasoconstriction (Perry *et al* 1989). To assess the effects of a high salt diet on this response, trout were exposed to acute hypercapnia. External hypercapnia was achieved by pumping mixtures of CO_2 in air (3 - 10%) through a gas equilibration column to achieve a water PCO_2 ($PwCO_2$) of 6-7 mm Hg. The mixtures were supplied by a Wosthoff gas-mixing pump (model M301 A/F). Once the $PwCO_2$ had stabilised (within 20 min), cardiovascular parameters were recorded. The CO_2 electrode (Cameron Instrument Company, model E201) was calibrated using mixtures of 0.5 % and 1.0% CO_2 in water from a gas-mixing flowmeter (Cameron Instrument Company). The CO_2 electrode was calibrated prior to each experiment. The CO_2 concentration was measured using a gas meter (BGM 200).

3.2.5 Catecholamine injection

In a separate experimental series, fish were injected (0.6 ml kg^{-1}) via the caudal vein cannula over a 30 sec interval with a catecholamine cocktail containing equimolar concentrations (2.5 x 10^{-5} mol l^{-1} in 150 mmol l^{-1} NaCl) (Bernier *et al.*, 1999) of NOR and

ADR; 30 min prior to injecting the catecholamines, 150 mmol NaCl l^{-1} (0.6 ml kg^{-1}) was injected as a vehicle control. Preliminary experiments were designed to ensure that the catecholamine dose was appropriate (i.e. able to distinguish potentially subtle effects of salt feeding on α_1-AR responsiveness). Several preliminary dose response curves were constructed that demonstrated that the selected dose was eliciting an approximate 50% vasoconstrictor response. Additional preliminary experiments were performed to ensure that the catecholamine injection was not causing down regulation or desensitization of α_1-ARs (which would confound the interpretation of the prazosin data). Serial injections (every 15 min) of the catecholamine cocktail into fish showed that the increases in P_{DA} and R_S were unaffected until the 9th injection. The results of these experiments ruled out the possibility that a single catecholamine injection could down-regulate α_1-ARs and thereby affect the prazosin injection results.

3.2.6 Prazosin injection

The α-AR antagonist prazosin (1 mg ml^{-1} dissolved in 1 part 150 mmol l^{-1} NaCl and 1 part methanol) was injected at 1 ml kg^{-1} fish via the caudal vein cannula over a 10 min period. The prazosin injection was performed 30 min after the catecholamine injection, at which time all cardiovascular variables had returned to baseline levels.

3.2.7 Plasma sodium and chloride assays

Plasma sodium concentration was measured by flame emission spectrophotometry (Varian, model SpectraAA 250 Plus). Plasma chloride concentration was determined by a mercuric thiocyanate spectrophotometric assay method (Zall *et al.*, 1956).

3.2.8 Isolation of RNA

Total RNA was isolated from fresh tissues [including efferent branchial artery (EBA), afferent branchial artery (ABA), coeliacomesenteric artery (CMA), ventral aorta (VA), dorsal aorta (DA) and spleen] using Trizol reagent (GibcoBRL, Burlington, ON, Canada) according to the protocol provided by the manufacturer. RNA concentrations and quality were verified using spectrophotometry (OD at 260 nm) and RNA gel electrophoresis, respectively. To aid the precipitation of RNA from the small tissue samples, 4 μl of linear acrylamide (2 mg μl^{-1}) (Ambion) was added before the addition of isopropanol; the samples were then placed on dry ice for 30 min. The following steps were as recommended by the manufacturer. This modification is for helping precipitate RNA from small tissues, i.e., EBA, ABA, CMA, VA, DA, thus increasing the yield of RNA.

Equal amounts of RNA were used to make cDNA. cDNA which is primed by random primer was synthesized using the first Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biologicals, Laval, QC, Canada).

3.2.9 Real-time PCR analysis of α_{1A}-AR and α_{1D}-AR mRNA expression

Real-time PCR analysis was performed using a Stratagene MX-4000 real-time PCR machine and DNA amplification was followed using SYBR green (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. α_1-ARs (α_{1A}-AR and α_{1D}-AR) and β-actin (internal control) were assayed. To avoid genomic DNA amplification, primers were designed to span a hypothetical intron. Based on previous studies of α_1-ARs genomic structure, there is a conserved intron region between transmembrane domain (TMD) 6 and TMD 7 (Arai *et al.*, 1999). The following primers (Invitrogen) were designed and synthesized to yield 170 bp amplicons.

α_{1A}-AR:

5'-CTGCGCCTCCTCAAGTTCTC-3' (forward)

5'-CCCCAGCCAGAAGGTGATCT-3' (reverse)

α_{1D}-AR:

5'-CTGTCCGTGCGCTTGATGAA-3' (forward)

5'-AACCCAGCCAGAAGATGACC-3' (reverse)

β-actin:

5'-CGTCCCAGGCATCAGGGAGT-3' (forward)

5'-TCTCCATGTCGTCCCAGTTG -3' (reverse)

Initially, the PCR products were cloned to ensure the specificity of the primers. Forty cycles of a two-step PCR protocol were used: 1 x 2 min at 94 °C, 35 x (30 sec at 94 °C, 30 sec at 58 °C, 1 min at 72 °C), and 1 x 10 min at 72 °C. A no template control (cDNA was replaced by water) for each master mix and a no reverse transcriptase (RT) control were included in all real-time PCR runs. Standard curves for each pair of primers were established by amplifying real-time PCR product in their specific plasmid. Dissociation curve analysis showed one specific peak, indicating a single PCR product was obtained. This product was further verified by gel electrophoresis that again showed a single band. The slopes of the standard curves for α_{1A}-AR, α_{1B}-AR and α_{1D}-AR were –3.083, -3.002 and –3.015, respectively, giving a corresponding primer efficiency of 111%, 115.3% and 114.6%, respectively.

Real-time PCR data were reported as 2^(-ΔCt) with β-actin as the reference gene. ΔCt is the difference between the Ct values of the α_1-AR and β-actin genes.

3.2.10 Measurement of plasma catecholamine levels

Blood samples (0.6 ml) were withdrawn from the caudal vein cannula prior to injecting catecholamines, and 2 and 15 min after injection. Samples were centrifuged immediately (10,000 X g for 30 sec) and the plasma was recovered and flash frozen in liquid N_2. Samples were placed at -80 °C prior to determination of catecholamine levels. Plasma catecholamine levels were measured on alumina-extracted samples by HPLC (Varian) with electrochemical detection (E.G. & G. Parc, model 400EC detector) according to the basic method of Woodward (1982). 3,4-Dihydroxybenzylamine was used as an internal reference standard for all analyses.

3.2.11 Statistics

Data are presented as mean ± 1 S.E.M. All statistical tests were performed using SigmaStat (version 3) software (Jandel Scientific). The effects of treatment were assessed by paired or unpaired Student's t-tests, where appropriate a $P < 0.05$ was regarded as significantly different and is shown in figures by an asterisk.

3.3 Results

3.3.1 The effects of high salt diet on basal cardiovascular variables and plasma catecholamines

The P_{DA} of control fish (23.8 ± 1.2 mmHg) was similar to previously reported values for rainbow trout (Thorarensen *et al.*, 1996). The fish fed a high salt diet exhibited a significantly higher P_{DA} than the control fish by approximately 37% (Table 3.1). There were no significant differences in R_s, V_b or f_H between the two groups of fish (Table 3.1).

The concentration of total catecholamines (noradrenaline plus adrenaline) in plasma did not differ between the control (5.5 ± 0.7 nmol l^{-1}) and salt-diet fed (4.1 ± 0.6 nmol l^{-1}) fish.

3.3.2 Plasma Na^+ and Cl^- levels

Plasma Na^+ and Cl^- concentrations were measured in control fish and fish fed a high-salt diet. Although there were trends for elevated levels of Na^+ (139.1 ± 7.1 *versus* 124.6 ± 5.3 mmol l^{-1}) and Cl^- (154.5 ± 10.6 *versus* 141.6 ± 4.0 mmol l^{-1}) in the fish fed a high-salt diet (Figure 3.1), the differences were not statistically significant. P-value for Na^+ and Cl^- comparison experiments are 0.124 and 0.247, respectively.

3.3.3 Real-time PCR analysis

α_{1A}- and α_{1D}-AR mRNA expression were examined in six tissues including EBA, ABA, CMA, VA, DA and spleen. Figure 3.2 shows that in DA, ABA and EBA, the expression of the α_{1D}-AR gene was significantly lower in fish being fed the high-salt diet compared with the control fish (decreases of 51% in DA, 50.1% in ABA and 35% in EBA). Although there was a similar trend for decreased α_{1D}-AR mRNA expression in the VA, the difference was not statistically significant. Similarly, an apparent increase in α_{1D}-AR mRNA in spleen in the high-salt fed fish was not statistically significant. Interestingly, the relative expression of the α_{1A}-AR gene was significantly increased (by 83%) in the VA of the salt fed fish (Fig. 3.3). α_{1A}-AR mRNA expression in DA, EBA and CMA was unaffected by salt feeding (Fig. 3.3). α_{1A}-AR mRNA was below the level of detection in the ABA. Table 3.2 summarizes the changes in the expression of the α_{1D}-AR and α_{1A}-AR gene in the various tissues.

3.3.4 Hypercapnia

Fish (n = 6) were exposed to acute increases in ambient Pco_2 to achieve (within 20 min) a stable level of hypercapnia of 6.3±0.4mm Hg (0.84±0.05 kPa). In the control fish, hypercapnia was associated with increases in P_{DA} and R_s of 11.0 ± 2.6 mm Hg and 0.38 ± 0.14 mm Hg ml^{-1} min^{-1} kg^{-1}, respectively (Fig. 3.4). The changes in P_{DA} (3.9 ± 0.7mm Hg) and Rs (0.11 ± 0.04 mm Hg ml^{-1} min^{-1} kg^{-1}) were significantly lower by approximately 70% in fish fed the high-salt diet (Fig. 3.4). The changes in V_b during hypercapnia were highly variable and thus there were no effects of the salt diet on this variable although hypercapnia significantly increased V_b in control group compared with their resting values. Similarly, f_H was decreased to a similar extent in the control and salt-fed groups (8.8 ± 2.6 min^{-1} and 7.9 ± 7.2 min^{-1}, respectively), although hypercapnia did not affect f_H compared with their resting level.

Table 3. 1. Basal cardiovascular variables in rainbow trout (*Oncorhynchus mykiss*) fed control or high salt (11% NaCl) diets.

	Control diet (10)	High salt diet (10)
P_{DA} (mm Hg)	23.8 ± 1.2	32.6 ± 1.4*
R_s (mm Hg ml^{-1} min^{-1} kg^{-1})	0.81 ± 0.02	0.96 ± 0.09
V_b (ml min^{-1} kg^{-1})	31.0 ± 0.8	35.1 ± 2.6
f_H (min^{-1})	76.0 ± 2.9	77.6 ± 2.7

Data are means ± 1 SEM; * indicates a significant difference ($P < 0.05$) from the corresponding value in the control group. The numbers of animals used is indicated in parentheses.

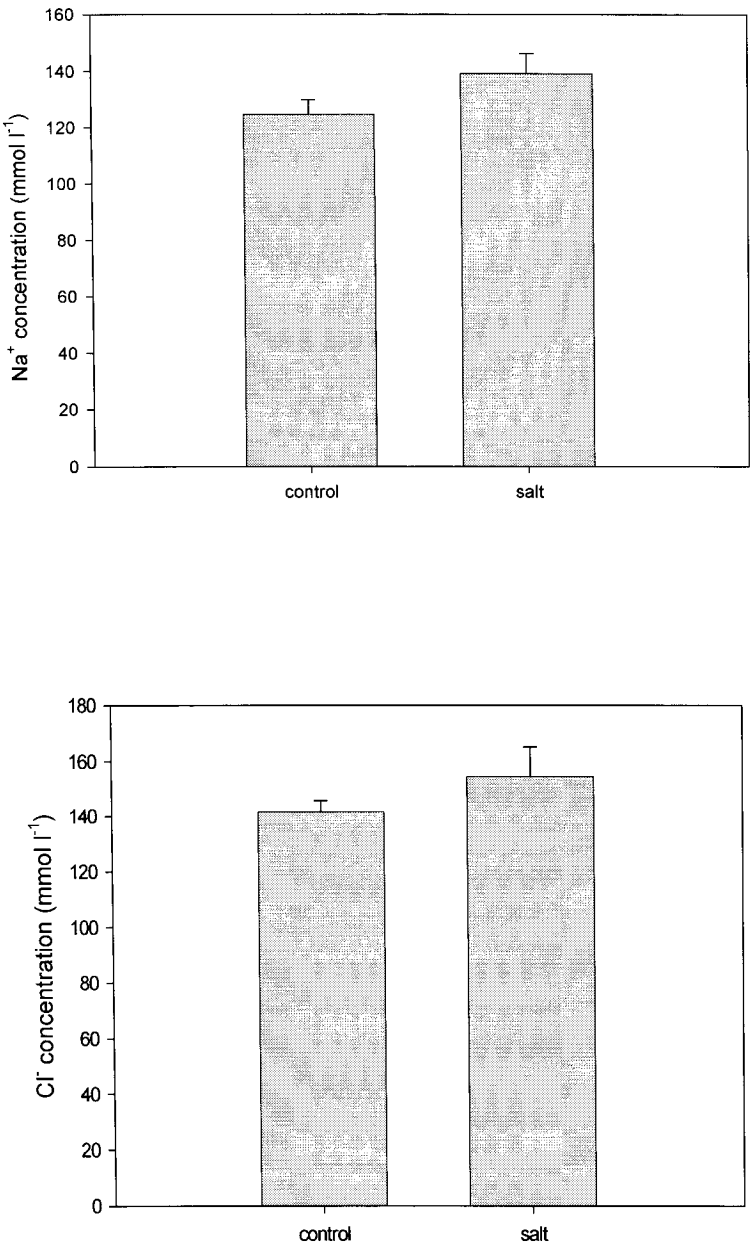

Figure 3.1. Plasma Na^+ (top) and Cl^- (bottom) concentrations in fish fed the control (n = 8) and high-salt (11% NaCl; n = 8) diets.

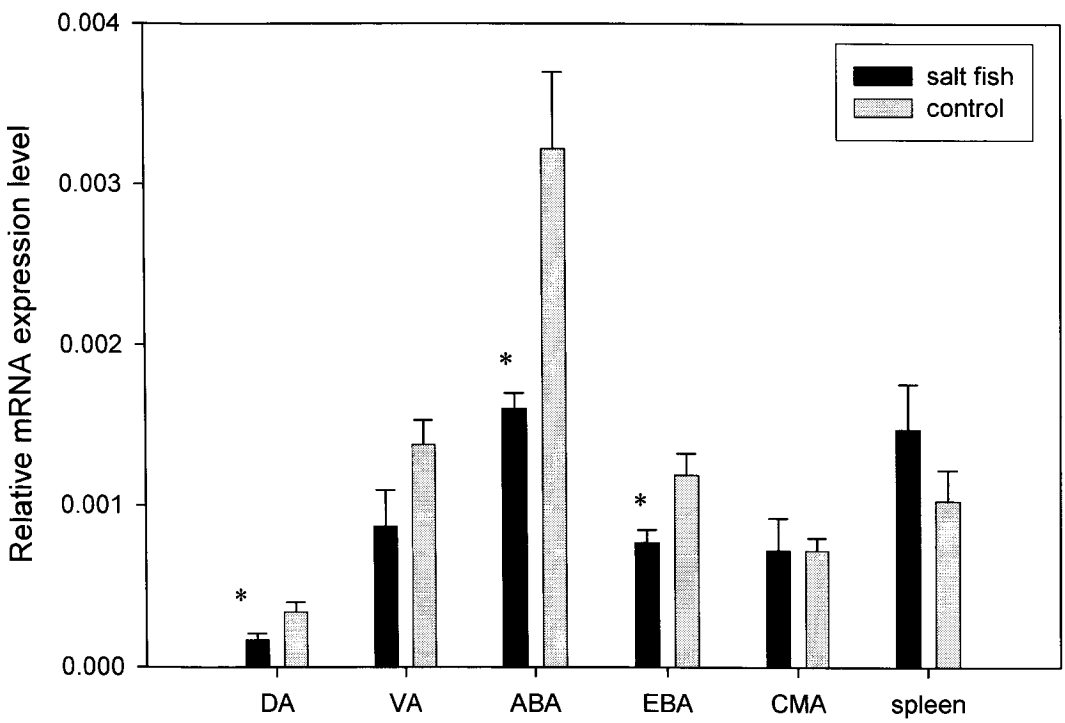

Figure 3.2. Relative levels of α_{1D}-AR mRNA in selected tissues of rainbow trout (*Oncorhynchus mykiss*) including DA (dorsal aorta), VA (ventral aorta), ABA (afferent branchial artery), EBA (efferent branchial artery), CMA (celiacomesenteric artery) and spleen in control fish (gray bars) and salt fed fish (black bars). Values are means ± 1 SEM; n = 8. * indicates a significant difference ($P < 0.05$).

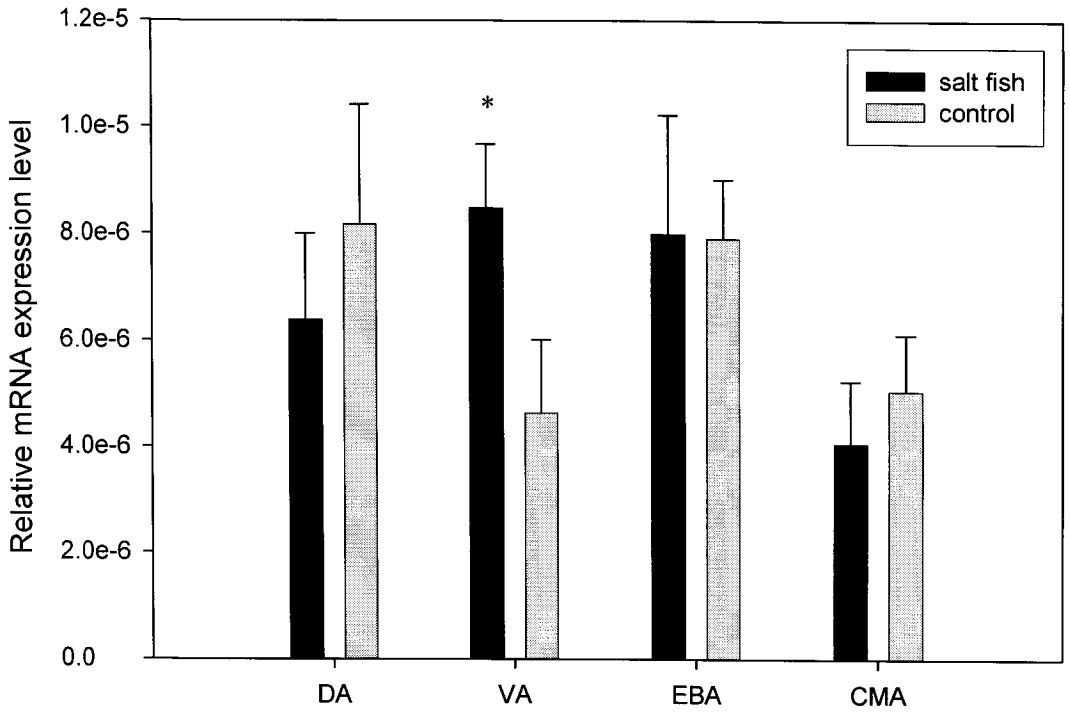

Figure 3.3. Relative levels of α_{1A}-AR mRNA in selected tissues of rainbow trout (*Oncorhynchus mykiss*) including DA (dorsal aorta), VA (ventral aorta), EBA (efferent branchial artery) and CMA (celiacomesenteric artery) in control fish (gray bars) and salt fed fish (black bars). Values are means ± 1 SEM; n = 8. * indicates a significant difference ($P < 0.05$).

Table 3.2 The change of α_{1D}-AR and α_{1A}-AR gene mRNA expression in high salt feeding fish compared with normal feeding fish in six tissues.

	α_{1D}-AR(8)	α_{1A}-AR(8)
DA	⇩*	no change
VA	no change	⇧*
CMA	no change	no change
EBA	⇩ *	no change
ABA	⇩ *	no α_{1A}-AR
Spleen	no change	

n number is showed in parentheses. * indicates significant difference.
DA (dorsal aorta), VA (ventral aorta), CMA (celiacomesenteric arteries), EBA (efferent branchial artery), ABA (afferent branchial artery).

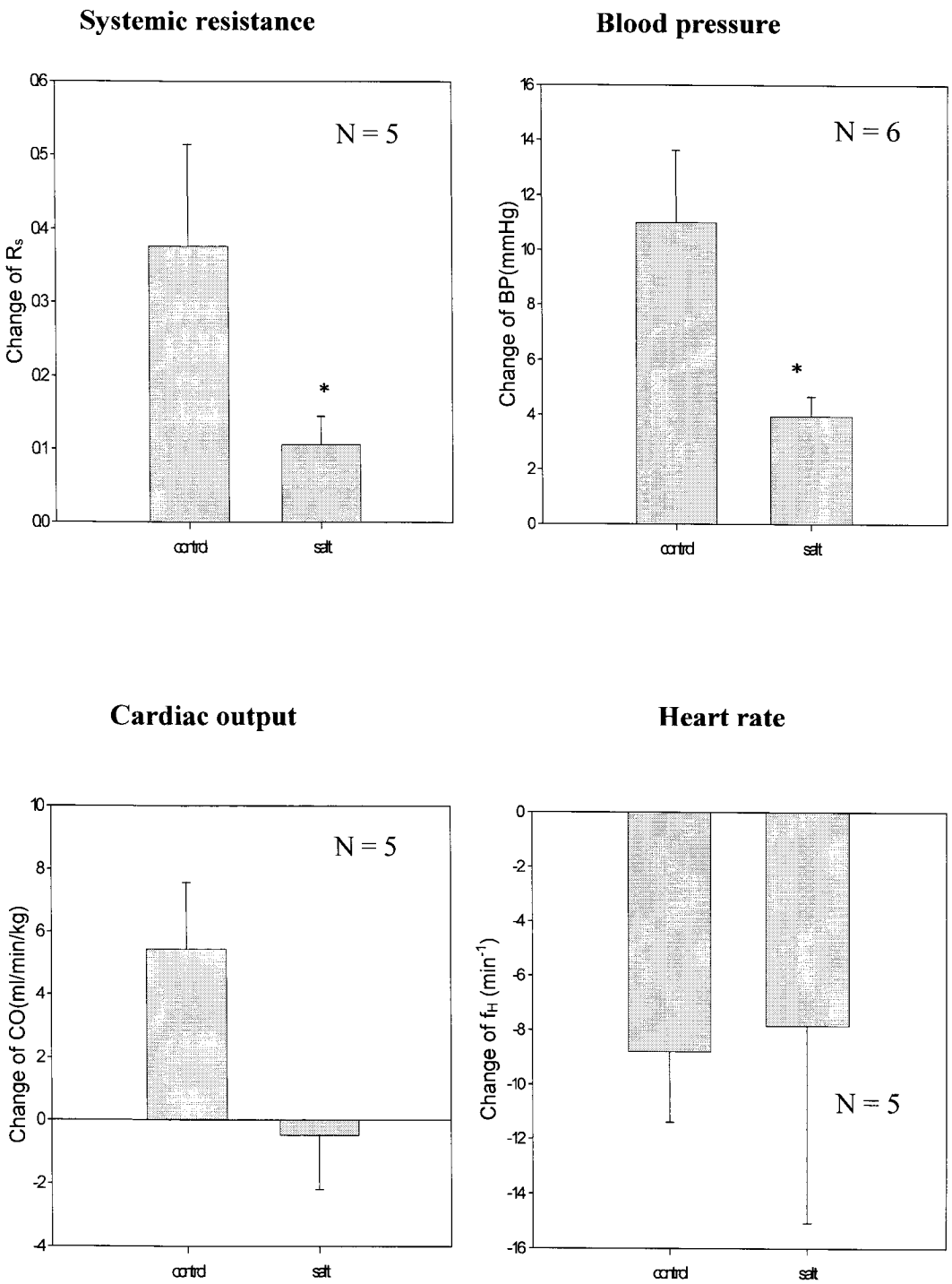

Figure 3.4. The effects of acute external hypercapnia on the absolute changes in selected cardiovascular variables including systemic resistance (R_s), blood pressure P_{DA}), cardiac output (V_b) and heart rate (f_H) in fish fed normal (gray bars) or high-salt (black bars) diets. * indicates a significant difference between the two diets ($P < 0.05$).

3.3.5 Catecholamine and prazosin injections *in vivo*

The potential role of α_1-ARs in salt-induced high BP was assessed by injecting α_1-AR agonists and an antagonist *in vivo*. The natural catecholamines, ADR and NOR were used as non-specific α_1-AR agonists to determine whether the chronic hypertension associated with the high-salt diet had affected the capacity of endogenous α_1-ARs to promote vasoconstriction. The non-specific α_1-AR antagonist, prazosin, was used as a tool to determine whether α-adrenergic vascular tone was affected by chronic hypertension.

The injection of catecholamines significantly increased P_{DA} and R_S in both groups of fish (Fig. 3.5). However the salt-fed fish displayed significantly smaller changes in P_{DA} (24 *versus* 68%) and R_S (28 *versus* 55%) after catecholamine injection in comparison with the control group. V_b and f_H were unaffected by catecholamine injection (Fig. 3.5).

The injection of prazosin caused similar decreases in P_{DA} and R_S in both groups of fish, although prazosin injection significantly decreased P_{DA} and R_S in both salt-fed and control groups compared with their resting values; however Vb and f_H were unaffected (Fig. 3.6). Heart rate was not changed compared with pre-prazosin values.

The catecholamine levels at rest in both the high-salt fed fish and normal fed fish were 4.14±0.60 nM and 5.54±0.74 nM, respectively. There were no differences between these two groups. Fifteen minutes after injection, the catecholamine levels in both high-salt and normal fed fish were 3.85±0.51 nM and 4.62±1.68 nM, respectively. There remains no difference between these two groups. Catecholamine levels did increase after the catecholamine injection, however plasma levels showed higher value in the salt-fed fish even though both groups received the same inject amount.

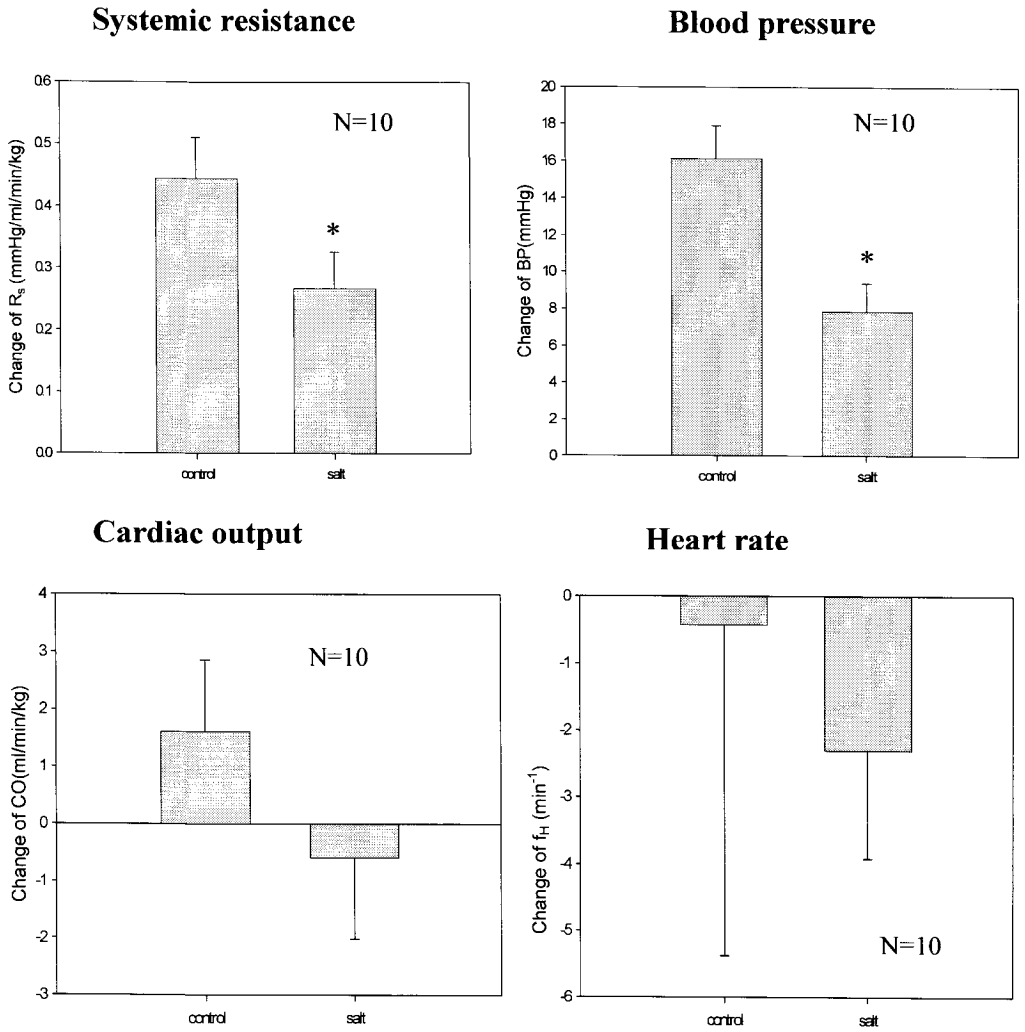

Figure 3.5. The effects of intra-arterial injection of catecholamines on the absolute changes in systemic resistance (R_S), blood pressure (P_{DA}), cardiac output (V_b) and heart rate (f_H) in control (gray bars) and salt-fed (black bars) fish * indicates a significant difference between the two groups of fish. The change was determined by the difference of the value at maximum response and the basal (pre-injection) value. Data are means ± 1 SEM.

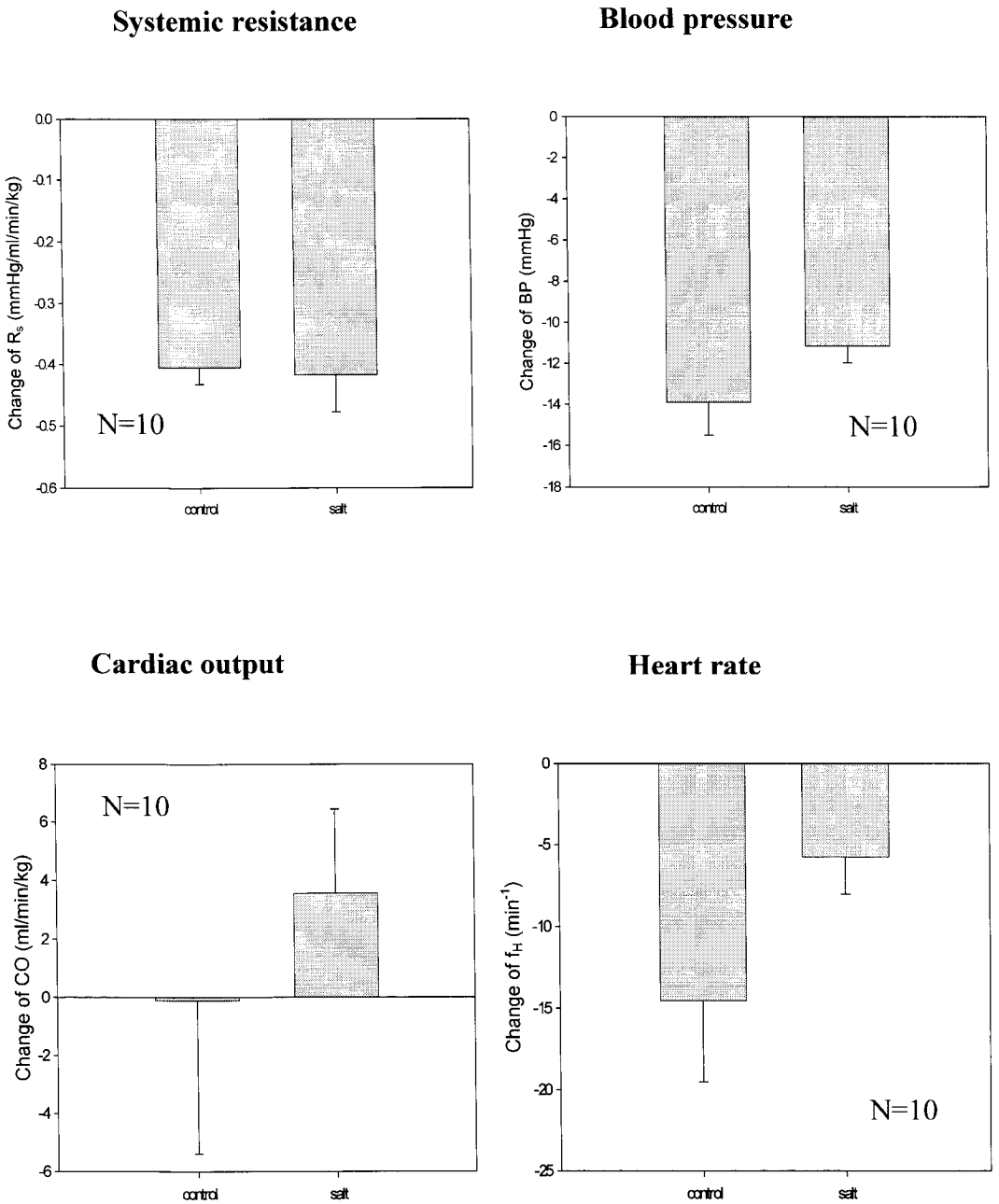

Figure 3.6. The effects of intra-arterial injection of prazosin on the absolute changes in systemic resistance (R_S), blood pressure (P_{DA}), cardiac output (V_b) and heart rate (f_H) in control (gray bars) and salt-fed (black bars) fish. * indicates a significant difference between the two groups of fish. The change was determined by the difference between the value at the maximum response and the basal (pre-injection) value. Data are means ± 1 SEM.

3.4 Discussion

The first goal of the present study was to develop a piscine model for salt-induced hypertension. The results clearly demonstrate that feeding rainbow trout a diet supplemented with 11% NaCl for 4 - 6 weeks resulted in a significant rise in arterial blood pressure. To our knowledge, this is the first study to demonstrate salt-induced hypertension in a non-mammalian vertebrate. The second goal was to determine whether the salt-induced hypertension was associated with any potentially compensatory adjustments to cardiovascular α_1-ARs. The results demonstrate a reduction in α_{1D}-AR mRNA in selected blood vessels and a corresponding reduction in the pressor responses mediated by exogenously administered catecholamines or by external hypercapnia. These results are in marked contrast to those obtained with mammals in which salt-induced hypertension is, in part, believed to reflect a heightened sensitivity of α_1-ARs to sympathetic stimulation (see review by Tanoue *et al.*, 2003).

3.4.1 A piscine model for salt-induced hypertension

Salman and Eddy (1988) demonstrated that rainbow trout fed a salt-enriched diet exhibited an increase in glomerular filtration rate (GFR). As a possible explanation, these authors proposed that salt feeding was promoting an elevation of blood pressure. The possibility of increased renal blood pressure contributing to elevated GFR is certainly feasible given that in the present study, dorsal aortic blood pressure was increased by approximately 37%. The intent of this study was to assess potential adrenergic adjustments associated with prolonged salt-induced hypertension rather than to specifically delineate the underlying mechanism of the hypertension. Nevertheless, it would appear that the increase in blood

pressure with salt feeding was the result of an increase in cardiac output rather than an increase in systemic resistance. In mammals with fully functional kidneys, the relationship between dietary salt load and blood pressure remains controversial (MacGregor, 1986; Chrysant *et al.*, 1999). However, there is general agreement that when occurring in mammals, salt-induced hypertension reflects an increase in systemic resistance (Fujita and Ando, 2004); this was not observed in the present study. The prolonged increase in cardiac output that was demonstrated in this study presumably reflected an uncompensated volume expansion of the vascular compartment owing to the movement of water into the NaCl-enriched plasma. As in mammals, water could be derived from the interstitial fluids and by drinking. In freshwater fish, drinking is probably not a major route of water entry although there is a report of an increase in drinking in trout fed a high-salt diet (Pyle *et al.,* 2003). Unlike mammals, freshwater fish possess an additional route for fluid entry into the blood, and that is trans-epithelial entry of water at the gill. Indeed, the passive movement of water across the gill constitutes a massive daily influx that is generally matched by the production of copious volumes of dilute urine (Perry *et al.*, 2003). The influx of water across the gills would be further increased owing to an increased osmotic gradient associated with salt loading. Although a previous study showed that urine flow rate increased by approximately 33% in trout fed a high-salt diet (Salman and Eddy 1988), this diuresis would not appear to be sufficient to restore vascular fluid volume and hence cardiac output. Thus, unlike in mammalian models that generally require experimentally induced renal dysfunction, salt-induced hypertension can be induced in fish possessing a fully functional kidney.

Although renal salt loss increases in salt-fed fish (Salman and Eddy, 1988), it nevertheless constitutes only a small fraction of whole body salt excretion. By far, the most

important site of salt balance in freshwater fish is the gill (Perry *et al.*, 2003) where, under normal conditions, there is a slight net gain of salt to balance losses at the kidney. In salt-fed fish (depending on the severity of the salt load), the gills become an important site for net Na^+ excretion owing to a decrease in Na^+ uptake and an increase in Na^+ efflux (Smith *et al.*, 1995; Pyle *et al.*, 2003). The mechanisms underlying the changes in unidirectional fluxes at the gill are unknown although they are accompanied by an increased number of branchial mitochondria-rich (MR) cells (chloride cells) and Na^+/K^+-ATPase activity (Salman and Eddy, 1987). These changes, which are typically observed when euryhaline fish are transferred from freshwater to saltwater, suggest that salt loading, regardless of its origin (e.g. seawater or dietary), may be causing similar alterations in the gill MR cells whereby they become more important in salt extrusion.

3.4.2 The consequences of prolonged hypertension on cardiovascular function and adrenergic responses

Resting vasomotor tone is high in rainbow trout and largely reflects the combined actions of basal sympathetic nerve activity and circulating catecholamines on α_1-AR's of the systemic vasculature (Perry *et al.*, 1999). Thus, intra-vascular injections of α-AR antagonists can evoke large reductions in systemic resistance and blood pressure (see Fig. 3.6). In the face of prolonged hypervolemic hypertension, we reasoned that trout might reduce α-adrenergic vascular tone to lower resistance and hence also lower blood pressure. This hypothesis was refuted, however, because α-AR blockade with prazosin caused identical reductions in systemic resistance and blood pressure in all fish regardless of diet and basal blood pressure. It is unclear as to why the marked increase in blood pressure observed in the

present study (37%) did not trigger a reduction in vasomotor tone *via* classical barostatic reflexes.

Two other protocols were employed to assess the impact of prolonged hypertension on the α-adrenergic responsiveness of the systemic vasculature; intra-arterial injection of catecholamines and exposure to environmental hypercapnia. The latter treatment is known to cause a pronounced increase in systemic resistance and blood pressure owing to the combined actions of increased sympathetic nerve activity and circulating catecholamine levels (Perry *et al* 1989). In teleost fish, systemic resistance is regulated by the opposing influences of α_1-AR-mediated vasoconstriction and β-AR-mediated vasodilation (Nilsson, 1983). Because of the greater impact of α_1-AR-mediated vasoconstriction, the net effect of increased sympathetic nerve activity or circulating catecholamine levels is an elevation of systemic resistance (Wood and Shelton, 1980a, b). The novel finding of the present study is that the fish experiencing salt-induced hypertension exhibited attenuated vasopressor responses to both catecholamine injection and hypercapnia. The most parsimonious explanation for these results is that peripheral α_1-ARs were either desensitized or reduced in number in the hypertensive fish. Although we were unable to directly quantify α_1-AR numbers in the present study, we did quantify the levels of α_1-AR mRNA in selected blood vessels. The results demonstrated a marked reduction of α_{1D}-AR mRNA in the dorsal aorta, afferent branchial artery and efferent branchial artery and an increase in α_{1A}-AR mRNA in the ventral aorta. Thus, while impossible to measure, it is tempting to speculate that the α_{1D}-AR mRNA levels were also decreased in the resistance vessels of the systemic circulation and that reduced levels of transcript would be accompanied by a reduction in the numbers of functional α_{1D}-ARs.

Although the vasopressor responses to exogenous catecholamines and hypercapnia were diminished in the hypertensive salt-fed fish, there was no reduction in basal vasomotor tone. At first glance, these results are in apparent conflict with my contention that the attenuated responses in the salt-fed fish reflect a loss of functional α_1-ARs. The simplest explanation is that vasomotor tone was being maintained in the face of reduced α_1-AR numbers by spatial recruitment of previously inactive nerve fibers. A second explanation is that only a sub-set of α_1-ARs was affected by salt loading. Conceivably, there could have been a reduction in the numbers of α_1-ARs not in contact with sympathetic nerve fibers and no change in α_1-AR numbers at post-ganglionic sympathetic synapses. If so, this could explain the reduced vasopressor responses during conditions of elevated circulating catecholamine levels (catecholamine injection and hypercapnia) without there being any effect on resting tone.

3.5 Conclusions

In summary, this is the first description of salt-induced high BP in an early branching vertebrate, the rainbow trout. This study suggests that resting sympathetic vasomotor tone was unaltered in the chronically hypertensive fish. In support of the idea of loss of function of vascular α-adrenoreceptors during chronic hypertension, the salt-fed fish exhibited significantly decreased levels of α_{1D} receptor mRNA in the dorsal aorta and the afferent and efferent branchial arteries; mRNA levels were unchanged in the ventral aorta and coeliacomesenteric artery.

This study also demonstrates that salt-induced hypertension can be elicited in rainbow trout possessing a fully functional kidney and that at least one cardiovascular consequence of

chronic hypertension in trout is a reduction in the α-adrenergic responsiveness of the systemic blood vessels. α_{1D}-ARs are, at least in part, involved in salt-induced high blood pressure in the rainbow trout at both molecular and physiological levels.

More experiments are needed to confirm this suggestion, including the examination of the numbers of α_1-ARs in certain arteries during the development of salt-induced high BP in trout.

The present study provides valuable information regarding the basis for salt-induced high BP in trout, particularly in the relationship between α_1-ARs and the elevated BP. Once the detailed information regarding subtype changes during high BP is available, the design of α_1-ARs subtype selective drugs that has no side effect may be possible.

Chapter 4. General Conclusions

It is only recently that α_1-ARs have been described and investigated in non-mammalian systems. Indeed, to my knowledge, this is only the second study to provide molecular information on α_1-ARs in fish. Prior to this thesis, there was a major knowledge gap that now has been partially filled. Thus, this thesis makes a significant contribution to a better understanding of the α_1-AR gene family and its potential role in the cardiovascular physiology of the rainbow trout.

In short, I successfully cloned three members of the α_1-AR gene family from the rainbow trout. Two partial α_{1A}-AR genes were identified that encode for proteins of 330 and 120 amino acids, respectively. Meanwhile, two partial putative α_{1B}-ARs were found, which encode for proteins of 103 and 100 amino acids, respectively. Only one partial α_{1D}-AR gene, encoding a protein of 468 amino acids, was obtained. Multiple sequence alignments of the trout α_1-AR genes with their mammalian counterparts revealed highly conserved regions within the seven TMDs, however the third intracellular loop and the C-terminal regions provide the α_1-ARs with their distinctive subtype characteristics. Interestingly, unlike mammals, within both the α_{1A}- and α_{1B}-ARs subtypes, one form appears to lack potential phosphorylation sites within the G-protein binding domain. This feature is consistent with the character of the β_2-AR gene family in rainbow trout (Nickerson *et al.*, 2001). This indicates that α_1-AR gene regulation in trout may be more complicated than in mammals and that different genes may function under different conditions.

Phylogenetic analysis provided further evidence that the three well-defined (at the molecular, pharmacological and physiological levels) α_1-AR subtypes in mammals all have homologs in teleost fishes. In certain cases, some of the mammalian α_1-AR subtypes may have multiple homologs in fish.

Real-time PCR analysis demonstrated that α_{1A}-AR and α_{1D}-AR mRNAs are expressed in many tissues with relatively high expression in the cardiovascular system; no α_{1A}-AR was detected in ABA. Although there is not always a direct relationship between mRNA expression and functional protein, this result nonetheless implicates the potential function of α_1-ARs in the cardiovascular system of rainbow trout. The importance of α_1-ARs was demonstrated by the results of experiments that, for the first time, used α_{1A}-AR and α_{1D}-AR selective antagonists *in vivo*. The results clearly showed that both α_{1A}-ARs and α_{1D}-ARs are involved in maintaining vascular tone in trout.

To better understand the role of α_1-ARs in the development of salt-induced high blood pressure (BP), trout were fed a high-salt diet for 4 – 6 weeks and BP was shown to be significantly elevated compared with control fed fish. Thus, I established a high BP model by feeding trout a high-salt diet. This model may prove to be useful for researchers studying physiological aspects that are related to salt loading.

Real-time PCR analysis showed that after 4 weeks of high-salt diet feeding, the α_{1D}-AR mRNA expression was significantly decreased in several arteries including DA, ABA and EBA. Because α_1-ARs play a key role in vascular contraction, the simplest explanation for this phenomenon is that α_1-ARs have a tendency to compensate for the elevated vascular strength that was caused by volume expansion. To examine the functional role of α_1-ARs in the development of high BP during high-salt diet feeding, several experiments were

performed. The goal of these experiments was to compare the cardiovascular responses of high-salt fed fish and control fish by stimulating or inhibiting α_1-ARs. Both hypercapnia and the injection of α_1-ARs agonists revealed that the high-salt diet fed fish have a lower cardiovascular response capacity than control fish. This finding supports the real-time PCR results and further indicates that α_1-ARs are down-regulated in the high-salt diet fed fish. In conclusion, these data strongly suggest that α_{1D}-ARs, at least in part, are involved in salt-induced high BP in trout.

This thesis presents the first molecular evidence of the α_1-AR gene family in rainbow trout and the results reveal that α_1-ARs play a role in the regulation of BP and may be involved in the development of salt-induced high BP. Despite the information provided by my study, many questions remain to be addressed. For instance, 1) are there additional α_1-ARs subtypes in trout? 2) which of the two α_{1A}-ARs and two α_{1B}-ARs genes are functionally expressed? 3) which α_1-AR subtypes play the main role in regulation of the cardiovascular system? 4) when one subtype changes, what happens to the other two?

Further work is required to clone the full length sequences of the α_1-ARs in trout, and characterize the α_1-AR numbers in specific tissues during the development of salt-induced high BP. More research needs to focus on the role of α_1-ARs at different stages of development of high BP.

In conclusion, it is obvious that my work provides important basic knowledge and potentially beneficial science that ultimately may be applicable in pharmacological and clinical fields.

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Appendix.1 Lung fish α_{1A}-AR nucleotide and amino acids sequence

Lungfish α_{1A}-AR

```
1          GGGGTGGCAGCAGCCGGCGCCGGAGGATGAAACCATCTGTAAAATTACAGAGGAGCCTGG
1            G  W  Q  Q  P  A  P  E  D  E  T  I  C  K  I  T  E  E  P  G

61         ATATGCTTTATTTTCTGCATTTGGTTCCTTCTATATCCCCCTGACGATTATTCTCTTTAT
21           Y  A  L  F  S  A  F  G  S  F  Y  I  P  L  T  I  I  L  F  M

121        GTACTGCAGAGTTTATGTTGTAGCCAAGAGAGAAACTAAAGACTTAAGCTCTGGACTGAA
41           Y  C  R  V  Y  V  V  A  K  R  E  T  K  D  L  S  S  G  L  K

181        GACAGAAAAATCTGACTCTGAAGAAGTAACTCTTAGAATCCATCGTAAAAATACTCCAGA
61           T  E  K  S  D  S  E  E  V  T  L  R  I  H  R  K  N  T  P  E

241        AGAAAGTGGATCAACACCAAATTCCAAAAATAAGACAATTTTTTCAATACGACTTCTCAA
81           E  S  G  S  T  P  N  S  K  N  K  T  I  F  S  I  R  L  L  K

301        GTTCTCCAGCGAGAAAAAAGCCGCCAAGAA
101          F  S  S  E  K  K  A  A  K
```

Appendix.2 Eel α_{1D}-AR nucleotide and amino acids sequence

Eel α_{1D}-AR

```
1          GGGTGGAAGCAACCGGCGCCGAAGGACGAAAGCATCTGCAGCATAACCGAAGAACCAGGC
1           G  W  K  Q  P  A  P  K  D  E  S  I  C  S  I  T  E  E  P  G

61         TATGCCCTCTTCTCTTCCCTGTTCTCGTTCTACCTCCCGCTCATGGTCATCTTAGTGATG
21          Y  A  L  F  S  S  L  F  S  F  Y  L  P  L  M  V  I  L  V  M

121        TATTTCAGGGTTTATGTAGAAGCCAGACGGACTACTAAGAGTCTTGAAGCGGGAGTCAAA
41          Y  F  R  V  Y  V  E  A  R  R  T  T  K  S  L  E  A  G  V  K

181        CGGGAAAGAAACAAGTCAATGGATGTAGTGCTCCGGATTCACTGCAGAAGTGTATTGGAG
61          R  E  R  N  K  S  M  D  V  V  L  R  I  H  C  R  S  V  L  E

241        GACTCCGCGGCAAGTACAAAGAGTAAAACCCATCCCTTCCGAAGTTCCCTCTCCGTGCGT
81          D  S  A  A  S  T  K  S  K  T  H  P  F  R  S  S  L  S  V  R

301        TTGATGAAATTCTCTCGCGAAAAAAAAGCCGCCAAAAG
101         L  M  K  F  S  R  E  K  K  A  A  K
```

Appendix.3 Bullhead α_{1B}-AR and α_{1A}-AR nucleotide and amino acids sequence

Bullhead α_{1B}-AR

```
1          GGGGTGGCAGCAGCCGGCGCCGAAGGATGAAACCGTTTGTCCAATAACAGAGGAGCCGTT
1            G  W  Q  Q  P  A  P  K  D  E  T  V  C  P  I  T  E  E  P  F

61         TTATGCGCTTTTTTCGTCTCTTGGCTCTTTTTACATTCCCCTAGCTGTGATTTTGGCCAT
21           Y  A  L  F  S  S  L  G  S  F  Y  I  P  L  A  V  I  L  A  M

121        GTACTGCCGCGTTTACATCGTCGCCAAAAGGACCACTAAGAATCTAGAAGCTGGAGTGAT
41           Y  C  R  V  Y  I  V  A  K  R  T  T  K  N  L  E  A  G  V  M

181        GAAGGAGCGCAATGACTCCAGTGAGCTCACACTGCGAGTGCATTACAAAGGCTCTCACAT
61           K  E  R  N  D  S  S  E  L  T  L  R  V  H  Y  K  G  S  H  I

241        CCAGGACGACTGCAGCAGCAGCACGAGCAAGGGCCAAATGAGGAGCTCGCTCACTGTGAA
81           Q  D  D  C  S  S  S  T  S  K  G  Q  M  R  S  S  L  T  V  K

301        ATTACTGAAGTTTTCTAGAGAAAAGAAAGCTGCCAAAACTCTTGGCGTTGTAGTAGGCAT
101          L  L  K  F  S  R  E  K  K  A  A  K  T  L  G  V  V  V  G  M

361        GTTCATCCTGTGCTGGTTACCGTTTTTCCTCGTACTGCCCATCGGTGCCTTCAATACCAA
121          F  I  L  C  W  L  P  F  F  L  V  L  P  I  G  A  F  N  T  N

421        CCTGCGTCCTCCAGAGACATTTTTCAAGGTGATCTTCTGGCTTGGCTACTTCAACAGCTG
141          L  R  P  P  E  T  F  F  K  V  I  F  W  L  G  Y  F  N  S  C

481        CCTGAACCCCATCATCTACCCATGCACCAGCCGTGAGTTTAAACAGGGATTTATCCGCAT
161          L  N  P  I  I  Y  P  C  T  S  R  E  F  K  Q  G  F  I  R  I

541        CCTGCGCTGTCAATGCCAGCAGAGGAAGGGCTGGAGACCGTACAACTACCGATCCCACTT
181          L  R  C  Q  C  Q  Q  R  K  G  W  R  P  Y  N  Y  R  S  H  L

601        GGGTCCCGCCAACCAGATCTACATGGACAACAGTTCAGTTTGCATGAACGGCAGTCACGC
201          G  P  A  N  Q  I  Y  M  D  N  S  S  V  C  M  N  G  S  H  A

661        TTCTTCCAGCCCTCGACTCTTCAGCAGAGGCACTGGCTCTCATACAGCATCGGGCCTCTT
221          S  S  S  P  R  L  F  S  R  G  T  G  S  H  T  A  S  G  L  L

721        ACCAGGCTGGAGTTCCTCCTCCACCTCTTTGTCTAGCAGTTCCTTCCCTAGGAAGTTCGT
241          P  G  W  S  S  S  S  T  S  L  S  S  S  S  F  P  R  K  F  V

781        TGCTAGCCCTGGACCAGTGTGTGAGAATTTAGACAAATCCAGCGGGATGACTCTTCTTAT
261          A  S  P  G  P  V  C  E  N  L  D  K  S  S  G  M  T  L  L  I

841        TCCTGATGGGGCTCCATCGAAAAAGCCCCAGCATGCCAATGGGCAGAACGGTAAAGACGA
281          P  D  G  A  P  S  K  K  P  Q  H  A  N  G  Q  N  G  K  D  E

901        AGTAGAATCAGAGACAAATTCCAGACTCATGCCTGAAATTAACTGTAATTGGGTACATGA
301          V  E  S  E  T  N  S  R  L  M  P  E  I  N  C  N  W  V  H  D

961        CAT
321          I
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Bullhead α_{1A}-AR

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1          GGGTGGAAGCAACCGGCGCCGAAGGACGAGTACGTGTGTAAAATCACAGAGGAACCTGGC
1           G  W  K  Q  P  A  P  K  D  E  Y  V  C  K  I  T  E  E  P  G

61         TACGCCATTTTTTCTGCAGTGGGCTCTTTCTACCTCCCCCTAGTGGTTATCCTGGTTATG
21          Y  A  I  F  S  A  V  G  S  F  Y  L  P  L  V  V  I  L  V  M

121        TATTGCCGTGTGTACGTGGTGGCCCGCAGGGAGACATACAGCCTTAGGAAAGGTCACAAG
41          Y  C  R  V  Y  V  V  A  R  R  E  T  Y  S  L  R  K  G  H  K

181        ACGGAGAAGGCACACCACGCAGAAGCAGTGACTTTGCGGATACACCGAGGCAACACCACT
61          T  E  K  A  H  H  A  E  A  V  T  L  R  I  H  R  G  N  T  T

241        GTGGCGGAGGATGAAACGTTGAGAAGCCGCACTCACTTCACAATCCGCCTACTTAAGTTC
81          V  A  E  D  E  T  L  R  S  R  T  H  F  T  I  R  L  L  K  F

301        TCCAGCGAAAAAAAGGCCGCCAAAAG
101         S  S  E  K  K  A  A  K
```