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PHYSIOLOGICAL SIGNIFICANCE OF THE 5-HT_{2B} AND 5-HT_{1B} RECEPTORS IN DEOXYCORTICOSTERONE ACETATE-SALT HYPERTENSION

By

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ABSTRACT

PHYSIOLOGICAL SIGNIFICANCE AND MECHANISMS OF THE UPREGULATION OF THE 5-HT$_{2B}$ AND 5-HT$_{1B}$ RECEPTORS IN DEOXYCORTICOSTERONE ACETATE-SALT HYPERTENSION

By

Amy Kissiah Lynn Banes

5-HT is a neurotransmitter which possesses a plethora of actions in the body including vasoconstrictor and mitogenic actions in the vasculature. The specific aims of this project were to determine the mechanisms of enhanced sensitivity to 5-HT observed in DOCA-salt hypertension and the physiological relevance of the 5-HT receptor subtypes, which mediate contraction to 5-HT in the vasculature. I tested the hypothesis that during the condition of DOCA-salt hypertension there is a switch from the 5-HT$_{2A}$ receptor to the 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors as the receptors which primarily mediate 5-HT-induced contraction in arteries from hypertensive rats.

To address this hypothesis we characterized the 5-HT receptor subtypes found in vascular smooth muscle with Southern analysis. There was mRNA detected for 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1F}$, 5-HT$_{2A}$, and 5-HT$_{2B}$ receptors. Contractility experiments with selective agonists of these receptors demonstrated no role for 5-HT$_{1D}$ and 5-HT$_{1F}$ receptors in arteries from normotensive sham and hypertensive DOCA-salt rats. 5-HT$_{1B}$ and 5-HT$_{2B}$ receptor agonists elicited contraction only in

arteries from DOCA-salt rats. Inhibition of both 5-HT_{2B} and 5-HT_{1B} receptors normalized the contractile response to 5-HT in arteries from DOCA-salt hypertensive rats compared to that observed in arteries from normotensive sham rats. This suggests that both the 5-HT_{1B} and 5-HT_{2B} receptors mediate the hyperresponsiveness to 5-HT observed in arteries from hypertensive rats.

The functional upregulation of these receptors under conditions of DOCA-salt hypertension were supported by Western analysis. We found a significant increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor protein levels in the aorta and mesenteric arteries from DOCA-salt hypertensive rats. However, we found no increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor mRNA levels. This finding suggests that the receptor upregulation is not occurring at the transcriptional level. Additional studies revealed that aldosterone *in vitro* increased the 5-HT_{1B} and 5-HT_{2B} receptor protein levels in a time and concentration-dependent manner. These effects were blocked by the mineralocorticoid receptor antagonist spironolactone. Further studies also demonstrated that prior to an increase in blood pressure, an increase in 5-HT_{2B} receptor function can be observed after 24 hr of treatment with DOCA and salt. Additional data from the time course studies while suggesting that down stream effectors in the signaling cascade may be altered also support the idea that the level of 5-HT_{1B} and 5-HT_{2B} receptor proteins is not the critical change which allows for functional receptor coupling to contraction. These data suggest that the 5-HT_{2B} and possibly the 5-HT_{1B} receptors may be important to the development and maintenance of hypertension.

Dedication

To my parents, James and Patricia, for teaching me to be grateful for the blessing that I have received and remembering what really matters.

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Finally, I want to express my sincere gratitude, appreciation and love to my family for their love and support, both personal and financial. I could not have done this without you.

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List of Abbreviations

AA	Arachidonic Acid
ACE	Angiotensin I converting enzyme
ADP	Adenosine diPhosphate
AMPK	AMP-Activated Protein Kinase
Ang II	Angiotensin II
bHLH	Basic Helix Loop Helix
BW723C86	1-[5(2-thienylmethoxy)-1H-3-indolyl]propan-2-amine hydrochloride
cAMP	Cyclic Adenosine monophosphate
Ca^{2+}	Calcium
CGRP	Calcitonin Gene Related Product
5-CT	5-Carboxamidotryptamine
c-fos	Finkel-Biskis-Jinkins Osteosarcoma oncogene homologue
CO	Cardiac Output
DAG	Diacylglycerol
DOC	Deoxycorticosterone
DOCA-salt	Deoxycorticosterone acetate-salt
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine Tetraacetic Acid
ET-1	Endothelin-1

eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal Regulated Kinase
GAPDH	Glyceraldehyde-Phosphate Dehydrogenase
G protein	GTP-dependent regulatory protein
GRE	Glucocorticoid Response Element
GTP	Guanosine triphosphate
5HIAA	5-hydroxyindole acetic acid
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen Peroxide
5-HT	5-hydroxytryptamine
IHC	Immunohistochemistry
iNOS	Inducible Nitric Oxide Synthase
5-NOT	5-Nonyloxytryptamine
IP_3	Inositol triphosphate
JNK	c-Jun N-terminal Kinase
LNAME	NG-nitro-L-arginine methylester
LNNA	N^{ω}-Nitro-L-Arginine
LY272015	6- methyl-1,2,3,4-tetrahydro-1-[3,4-dimethoxyphenyl)methyl]-9H-pyrido[3,4-b]indole hydrochloride
LY344864	(R)-N-[3Dimethylamino-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamine
MAP	Mean Arterial Pressure

MAPK	Mitogen Activated Protein Kinase
MRE	Mineralocorticoid Response Element
MAPKK	Mitogen Activated Protein Kinase Kinase
MUPP1	Multi-PDZ Domain Protein1
mRNA	Messenger Ribonucleic Acid
NAD(P)H Oxidase	Nicotinamide Adenine Dinucleotides (Phosphate) Hydride Oxidase
$NO^{\bullet}$	Nitric Oxide
O_2^-	Superoxide Anion
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDZ domain	PSD-95/Discs Large/ZO-1
PE	Phenylephrine
PI3-K	Phosphatidylinositol 3- Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PSS	Physiological Salt Solution
RAS	Renin-Angiotensin System
RGS4	Regulator of G-Protein Signaling Protein 4
ROS	Reactive Oxygen Species

SAPK	Stress Activated Protein Kinase
SBP	Systolic Blood Pressure
SIK	Salt-Inducible Kinase
SDS	Sodium Dodecyl Sulfate
SH-3	Src Homology-3
SHR	Spontaneously Hypertensive Rat
SNF1	Sucrose-Nonfermenting 1 Protein Kinase
SOS	Sodium Octyl Sulfate
SRF	Serum Response Element
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline + Tween
TCA	Trichloroacetic Acid
TPR	Total Peripheral Resistance

Introduction

I. Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) was isolated from bovine serum by Rapport and colleagues in 1948 (Rapport *et al*, 1948). Since its discovery, 5-HT has been a molecule which has generated much interest and controversy in cardiovascular as well as behavioral and neurochemical research fields. 5-HT possesses a plethora of vascular effects including vasoconstriction (Hoyer, 1989), vasodilation (Ellis *et al*, 1995) and mitogenic actions (Launay *et al*, 1996). These vascular effects have led to the implication of 5-HT in diseases such as migraine (Fozard, 1995), pulmonary hypertension (Keegan *et al*, 2001), atherosclerosis (Ishida *et al*, 2001), mineralocorticoid-induced hypertension (Watts and Fink, 1999), Raynaud's phenomenon (Coleiro *et al*, 2001, Igarashi *et al*, 2000) and as a contributor to liver injury from ischemia and reperfusion (Nakamura *et al*, 2001). 5-HT is involved in irritable bowel syndrome (Miwa *et al*, 2001), brain development (Witaker-Azmitia, 2001) and cardiac development (Nebigil and Maroteaux, 2001). Interestingly, 5-HT potentiates both agonist-induced contraction (Watts, 2000) and agonist-induced smooth muscle cell proliferation for other vasoactive substances such as endothelin (Watanabe *et al*, 2001). The wide variety of actions possessed by 5-HT necessitates an understanding of the role of 5-HT in normal and diseased state. Additionally, understanding the receptors that 5-HT interacts with to produce its myriad of effects in normal and diseased states is also necessary.

Biosynthesis and storage

5-HT is an indolethylamine and is produced both centrally by serotonergic neurons and peripherally in the enterochromaffin cells of the gut. The synthesis of 5-HT begins with the uptake of the amino acid L-tryptophan. L-tryptophan is converted by tryptophan hydroxylase to 5-hydroxytryptophan. 5-hydroxytryptophan is then decarboxylated by aromatic amino acid decarboxylase to 5-HT. 5-HT is metabolized by monoamine oxidase to form 5-hydroxyindoleacetaldehyde. This is then converted by the enzyme aldehyde dehydrogenase to 5-hydroxyindole acetic acid (5HIAA). In the human body 5HIAA is the primary metabolite excreted (Tyce, 1985). Both platelets and nerves store 5-HT and platelets serve as the primary source of 5-HT for the vasculature.

5-HT Receptors

5-HT acts at plasma membrane receptors to mediate its myriad of effects. 5-HT receptors have been divided into seven classes with subtypes in many of these classes. Most of the 5-HT receptors are classified as heptahelical, G-protein coupled receptors. The 5-HT_3 class of receptors is the one exception as the members of the 5-HT_3 class of receptors are ion channels. Among the known 5-HT receptors, there are five that have been suggested to mediate vascular smooth muscle contraction to 5-HT; the 5-HT_{2A}, 5-HT_{2B}, 5-HT_{1B}, 5-HT_{1D} and the 5-HT_{1F} receptors (Smith *et al*, 1999, Watts *et al*, 1996,Razzaque *et al*, 1999, VanDenBrink *et al*, 2000).

5-HT$_{2A}$ Receptor

Physiology

The 5-HT$_{2A}$ receptor has been characterized as a heptahelical G-protein coupled receptor (Roth *et al*, 1995). Specifically, the 5-HT$_{2A}$ receptor couples to the $G_{q/11}$ family of G-proteins (Li *et al*, 1997). 5-HT$_{2A}$ receptors couple to the extracellular signal regulated kinase (ERK)/ mitogen activated protein kinase pathway (MAPK) (Grewal *et al*, 1999). The ERK/MAPK pathway is one of the three recognized mitogen activated protein kinase pathway (MAPK) pathways. Of the three canonical MAPK pathways 5-HT, acting *via* the 5-HT$_{2A}$ receptor, appears to only activate the ERK/MAPK pathway. The c-Jun N-terminal kinase (JNK)/ stress activated protein kinase (SAPK) and p38 pathways are not activated by 5-HT *via* the 5-HT$_{2A}$ receptor (Banes *et al*, 2001). Activation of the ERK/MAPK pathway by the 5-HT$_{2A}$ receptor has been linked to generation of superoxide anion ($O_2^{\cdot}$) and H_2O_2 (Lee *et al*, 2001, Greene *et al*, 2000). This receptor couples to phospholipase C (PLC), protein kinase C (PKC), src and L-type calcium channels (Pandey *et al*, 1995, Watts *et al*, 1994, Banes *et al*, 1999) (figure 1). Furthermore, internalization of this receptor is dependent on dynamin and independent of arrestins (Bhatnagar *et al*, 2001). It has been suggested that this internalization may be involved in receptor resensitization (Gray et al, 2001). The 5-HT$_{2A}$ receptor is the only 5-HT receptor that has been characterized so thoroughly with regards to internalization and its effects on signaling mechanisms.

Location

The 5-HT_{2A} receptor mRNA has been located in vascular smooth muscle cells (Watts *et al*, 2001) and in regions of the brain and spinal cord (Cyr *et al*, 2000, Helton *et al*, 1994). Functionally the 5-HT_{2A} has been suggested to be in platelet membranes (Kagaya *et al*, 1990), renal messangial cells (Greene *et al*, 2000) and bovine pulmonary artery smooth muscle cells (Lee *et al*, 2001). 5-HT_{2A} receptors have been implicated in several tissues as mediators of contraction including: the rat aorta (Florian and Watts, 1998), rabbit mesenteric artery (Yildiz and Tuncer, 1995), rat trachea and guinea pig aorta (Baez *et al*, 1994), human umbilical artery (Lovren *et al*, 1999), rat pulmonary artery (MacLean *et al*, 1996) and human coronary artery (Nilsson *et al*, 1999a). Under conditions of normal blood pressure the 5-HT_{2A} receptor appears to be the predominate receptor which mediates contraction to 5-HT in the rat aorta and mesenteric arteries.

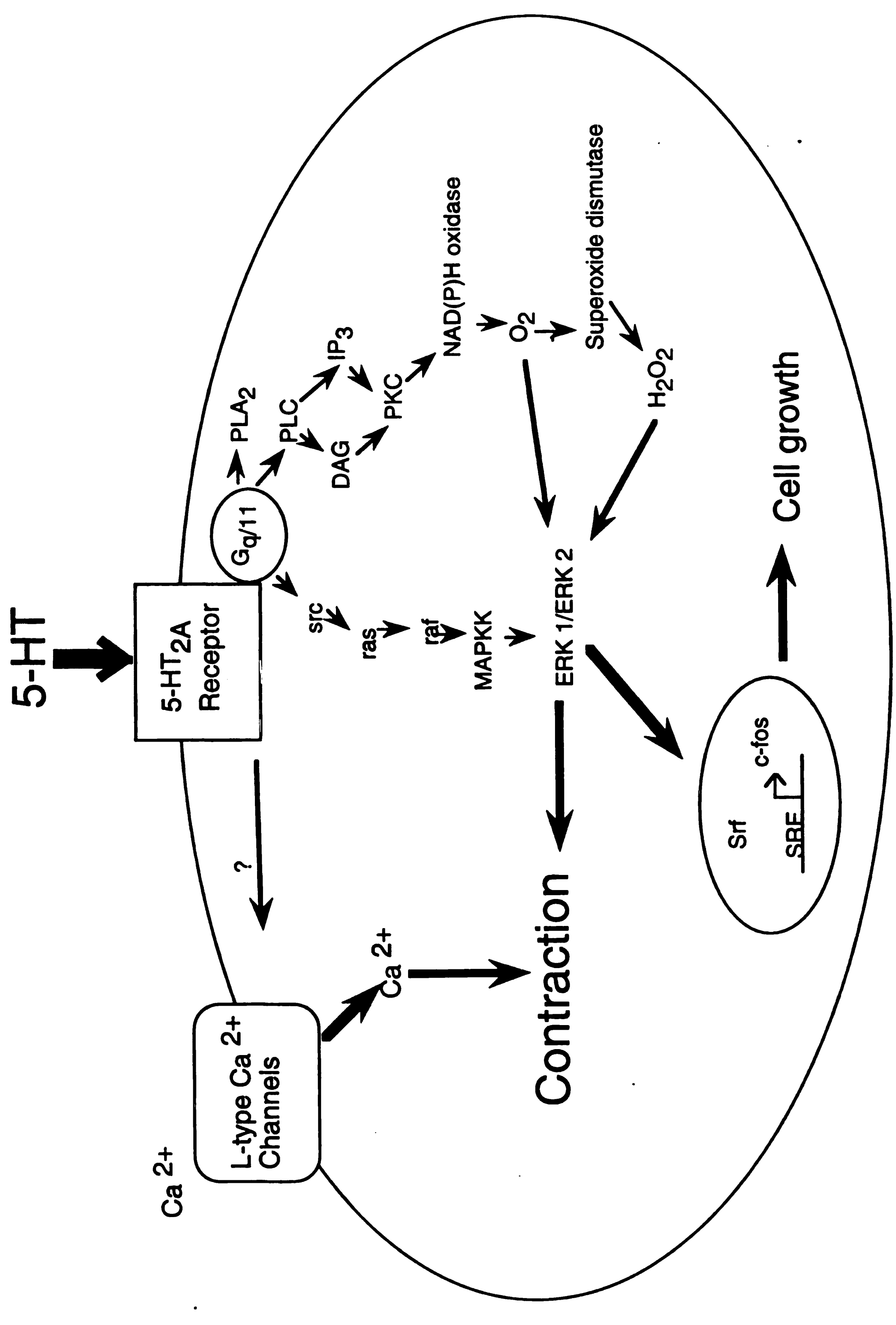
Regulation

Currently, there is very little known about the regulation of the 5-HT_{2A} receptor. This receptor has been cloned and assigned to chromosome 13q14-q21 in the human (Sparkes et al, 1991) and the promoter for the human gene has been under investigation. Currently, sequence analysis of the 5-HT_{2A} receptor promoter has revealed the presence of at least two promoter regions, a silencer, Sp1 sites, PEA3 sites, cyclic AMP response element (CRE) sequences and E-boxes (Zhu *et al*, 1995). However, the presence of these elements has not lead to any further characterization of the mechanisms by which expression of this gene is controlled or what substances may effect expression of this receptor. There have been several studies performed which involved receptor downregulation through agonist and antagonist exposure (Roth *et al*, 1990, Roth, 1999,Berry *et al*, 1996). Interestingly, changes in the levels of 5-HT_{2A} receptor mRNA have been noted to be both independent and dependent on protein kinase C (PKC) activation (Anji et al, 2001, Wohlpart and Molinoff, 1998). The decreases in 5-HT_{2A} receptor mRNA levels in the C6 glioma cell line implicate PKCα and/or PKCγ activation in response to 5-HT stimulation (Anji *et al*, 2001). In P11 cells, an immortalized cells line, an increase in 5-HT_{2A} receptor mRNA levels through PKC dependent and independent mechanisms has been demonstrated (Wohlpart and Molinoff, 1998). These increases appear to be due to an increased stability of the 5-HT_{2A} receptor mRNA.

Pharmacology

Pharmacologically, the 5-HT_{2A} receptor is characterized by stimulation with the agonists 5-HT, α-methyl 5-HT and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). Ketanserin is a widely utilized 5-HT_{2A} antagonist. Another 5-HT_{2A} antagonist, MDL100907, is considered the most selective 5-HT_{2A} antagonist based upon binding affinity (Kehne *et al*, 1996).

Figure 1. Diagram of the proposed signaling mechanisms utilized by the 5-HT_{2A} receptor. Abbreviations: PLC=Phospholipase C; PKC= Protein Kinase C, ERK=Extracellular signal regulated kinase; PLA_2= Phospholipase A_2; IP_3= Inositol triphosphate; DAG= Diacylglycerol; O_2^-= Superoxide; NAD(P)H oxidase= Nicotinamide adenine dinucleotides (phosphate) hydride; H_2O_2 = hydrogen peroxide; MAPKK= Mitogen activated protein kinase kinase; Srf= Serum response factor; SRE= Serum response element; c-fos= Finkel-Biskis-Jinkins osteosarcoma oncogene homologue.


5-HT
5-HT2A Receptor
Gq/11
PLA2
PLC
IP3
DAG
PKC
NAD(P)H oxidase
O2
Superoxide dismutase
H2O2
src
ras
raf
MAPKK
ERK 1/ERK 2
?
L-type Ca 2+ Channels
Ca 2+
Ca 2+
Contraction
Srf
SRE
c-fos
Cell growth

$5\text{-}HT_{2B}$ Receptor

Physiology

The 5-HT $_{2B}$ receptor, which was cloned in the rat stomach fundus (Foguet *et al*, 1992, Kursar *et al*, 1992), has been characterized as a heptahelical G-protein coupled receptor. The $5\text{-}HT_{2B}$ receptor specifically couples to the $G_{q/11}$ in smooth muscle cells and the Gz proteins in the rat stomach fundus (Wang *et al*, 1993). This receptor utilizes signal transduction pathways which include: L-type calcium channels (Cox and Cohen, 1995), the ERK/MAPK pathway (Nebigil *et al*, 2000a, Launay *et al*, 1996) and phospholipase A_2 (PLA_2) to induce the metabolism of arachadonic acid (AA) *via* cyclooxygenase (Tournois *et al*, 1998). These metabolites, as well as activation of ras and src family kinases, may play a role in the intracellular signaling cascades utilized by this receptor (Tournois *et al*, 1998, Launay *et al*, 1996) (figure 2). Interestingly, in both the rat stomach fundus and in the rat vasculature $5\text{-}HT_{2B}$ receptors fail to stimulate phosphoinositide (PI) hydrolysis (Cohen and Wittenauer, 1987, Ellis *et al*, 1995).

In cardiac myocytes the $5\text{-}HT_{2B}$ receptor has been linked to transactivation of the $ErbB_2$ and PDGF receptors (Nebigil *et al*, 2000b). The $5\text{-}HT_{2B}$ receptor has also been linked to PKC activation in the rat stomach fundus (Cox and Cohen, 1995), but does not appear to use phosholipase D or nitric oxide ($NO^{\cdot}$) in modifying contraction (Cox *et al*, 1999). Most of the literature indicates that $5\text{-}HT_{2B}$ receptor-mediated contraction is dependent upon influx of extracellular calcium. However, in human pulmonary arterial endothelial cells the $5\text{-}HT_{2B}$

receptor has been implicated in release of intracellular calcium from ryanodine-sensitive stores (Ullmer *et al*, 1996). In some endothelial cells 5-HT_{2B} receptors also stimulate release of $NO^{\bullet}$ (Ellis *et al*, 1995). Interestingly, 5-HT_{2B} receptors can activate nitric oxide synthases *via* its group I PDZ (PSD-95/discs large/ZO-1) motif located at the C-terminus (Manivet *et al*, 2000). This receptor-mediated stimulation of iNOS requires the 5-HT_{2B} receptor to be coupled to the $G\alpha_{13}$ (Manivet *et al*, 2000).

Additionally, the MUPP1 protein (multi-PDZ domain protein 1), a binding protein which has thirteen PDZ domains, interacts with the 5-HT_{2B} receptor (Bécamel *et al*, 2001). Many signaling molecules and enzymes, such as nitric oxide synthases, have PDZ-binding domains. The MUPP1 protein may serve as a scaffolding protein which facilitates signal transduction and protein-protein interactions involving the receptor and these intracellular signaling molecules and enzymes. Additionally, MUPP1 has been shown to induce clustering of the 5-HT_{2C} receptors at the cell surface (Bécamel *et al*, 2000). This suggests a potential role for MUPP1 in receptor dimerization. Furthermore, the PDZ domain has been implicated in the interaction between the N-methyl-D-aspartate receptor and potassium channel subunits (Kornau *et al*, 1995, Kim *et al*, 1995). These findings suggest that the PDZ domain and proteins such as MUPP1 may play an important role in signal transduction, an idea which merits further research. Further characterization of the signal transduction pathways, especially those utilized to cause contraction, also merits further studies. For example, unlike the 5-HT_{2A} receptor , there has been no investigation into the potential interaction of the 5-HT_{2B} receptor and the use of reactive oxygen species as signaling molecules. Thus, there are multiple pathways which the 5-HT_{2B} receptor may utilize in order to mediate contraction, vasorelaxation, regulation of cell-cycle progression and morphogenesis (Choi *et al*, 1997).

In addition to being involved in nitric oxide synthase activation and contraction, 5-HT_{2B} receptors have been implicated in mitogenesis (Launay *et al*, 1996), cell cycle progression (Nebigil *et al*, 2000a) and are crucial to proper craniofacial and cardiovascular morphogenesis (Lauder *et al*, 2000, Nebigil *et al*, 2001a). Mice that lack the 5-HT_{2B} receptor show abnormalities in migration of neural crest cells, sarcomeric organization of the subepicardial layer and an absence of the trabecular cell layer in the myocardium of the ventricle (Nebigil *et al*, 2001b). Interestingly, 5-HT_{2B} receptors have been implicated in human valvular heart disease associated with fenfluramine, methylsergide and ergotamine (Rothman *et al*, 2000). 5-HT_{2B} receptor expression and function in normal vascular tissues as well as in disease states has not yet been characterized.

Location

The 5-HT_{2B} receptor mRNA has been detected in the stomach fundus (Foguet *et al*, 1992, Kursar *et al*, 1992), cerebral vasculature (Schmuck *et al*, 1996), human small intestine (Borman and Burleigh, 1995), aorta from Sprague-Dawley rats (figure 4), mesenteric arteries from DOCA-salt hypertensive rats

(Watts *et al*, 1995) neural crest and myocardiac cells (Choi *et al*, 1997), enteric neurons (Fiorica-Howells *et al*, 2000), osteocytes and osteoblasts from chicken embryos (Westbroek *et al*, 2001), human liver, pancreas, cerebral cortex , spleen and kidneys (Bonhaus *et al*, 1995) as well as in some vascular endothelial cells (Ishida *et al*, 1998, Ullmer *et al*, 1996 Glusa and Pertz, 2000).

Regulation

The human 5-HT_{2B} receptor has been characterized structurally so that the residues which bind to 5-HT and those that form the aromatic box around 5-HT in the binding site are known (Manivet *et al*, 2002). The human 5-HT_{2B} receptor has also been mapped to chromosome 2, specifically to 2q36.3-2q37.1 (Horton *et al*, 1996, Le Coniat *et al*, 1996). Unfortunately, this is the extent of knowledge about the structure and the gene which encodes the 5-HT_{2B} receptor. No characterization of the 5-HT_{2B} promoter has been published. There is also nothing currently published which addresses regulation, post-transcriptional modifications, internalization, dimerization or transcriptional control of the 5-HT_{2B} receptor.

Pharmacology

Interestingly, 5-HT has approximately a three hundred times higher affinity for the 5-HT_{2B} receptor compared to the 5-HT_{2A} receptor (table 1). Therefore, activation of the 5-HT_{2B} receptor can occur at concentrations of 5-HT lower than that which are required to activate the 5-HT_{2A} receptor. The 5-HT_{2B} receptor is pharmacologically characterized by high affinity for the agonist 1-[5(2-

thienylmethoxy)-1H-3-indolyl]propan-2-amine hydrochloride, otherwise known as BW723C86 (Kennett et al, 1996). This receptor is further characterized by high affinity for the selective 5-HT_{2B} receptor antagonist 6-methyl-1,2,3,4-tetrahydro-1-[3,4-dimethoxyphenyl)methyl]-9H-pyrido[3,4-b]indole hydrochloride also known as LY272015 (Audia *et al*, 1995,Cohen *et al*, 1996, Watts, 1997). Other non-selective 5-HT_{2B} agonists are 5-HT and α-methyl-5-HT. Other 5-HT_{2B} receptor antagonists which have been used are SB204741, RS-127445 and SB200646 (Bonhaus *et al*, 1999,Kennett *et al*, 1994, Baxter, 1996).

Figure 2. Diagram of the proposed signaling pathways utilized by the 5-HT_{2B} receptor. Abbreviations: PLC=Phospholipase C; PKC= Protein Kinase C, ERK=Extracellular signal regulated kinase; PLA_2= Phospholipase A_2; AA= Arachidonic Acid; ROS= Reactive oxygen species; PLC=Phospholipase C; DAG= Diacylglycerol; $NO^{\bullet}$= Nitric oxide; MUPP1 = multi-PDZ domain protein 1; MAPKK= Mitogen activated protein kinase kinase; Srf= Serum response factor; SRE= Serum response element; c-fos= Finkel-Biskis-Jinkins osteosarcoma oncogene homologue.

5-HT2B
e C,
AA=
se C;
protein 1;
se factor,
coma

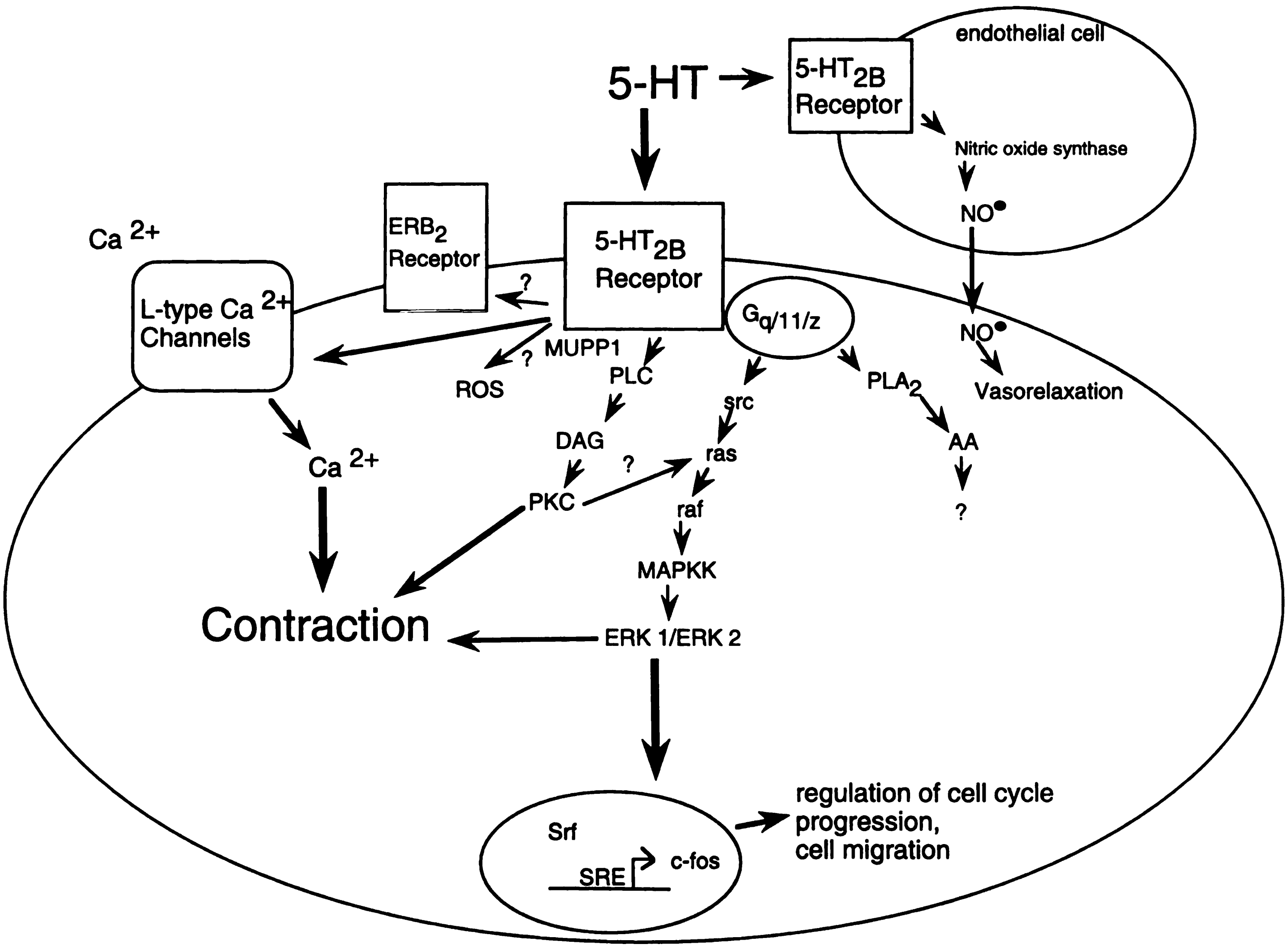


Table 1: Binding affinities obtained from the literature for the 5-HT_{1B} receptor, 5-HT_{1D} receptor, 5-HT_{1F} receptor, 5-HT_{2A} receptor and 5-HT_{2B} receptor. Values are reported as $pK_i = - \log K_i$ [M].

Literature Binding Affinity Table
pKi=-log Ki [M]

Compound		5-HT1B	5-HT1D	5-HT1F	5-HT2A	5-HT2B
Agonists						
5-HT	(1,2,3)	7.6	8.4	8.0	5.5R;7.2H	8.0R;9.1H
BW723C86	(4)	6.2	6.9	5.1	6.6	7.9R
RU24969	(5,6)	8.4R	7.3	NA	NA	7.2
BRL54443	(7)	6.9	7.2	8.9	5.9	7.0
Sumatriptan	(5,8)	6.3R;8.1H	8.4	7.6	3.7	< 5
LY344864	(9)	6.2	NA	8.2	NA	NA
PNU0142633	(10)	4.7	8.3	NA	NA	NA
CP93129	(11)	7.8	NA	NA	> 6	NA
Antagonists						
LY272015	(12)	< 7	< 7	< 7	7.5	9.1
Ketanserin	(1,13)	5.7	7.1	< 5	8.9	5.4
GR55562	(14)	7.3	6.3	5.6	5.6	NA

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$5\text{-}HT_{1D}$ and $5\text{-}HT_{1F}$ Receptors
Physiology

The $5\text{-}HT_{1D}$ and $5\text{-}HT_{1F}$ receptors are heptahelical G-protein coupled receptors, utilizing G_i as well as to G_o in signal transduction (Adham *et al*, 1993, Wurch and Pauwel, 2000). Members of this family of receptors couple to inhibition of adenylate cyclase. Until recently there has been much controversy as to the roles of the $5\text{-}HT_{1B}$ (also known as the $5\text{-}HT_{1D\beta}$ receptor) and $5\text{-}HT_{1D}$ (also known as the $5\text{-}HT_{1D\alpha}$ receptor) receptors due to a lack of specific pharmacological tools to discriminate between these two structurally similar receptors. Interestingly, $5\text{-}HT_{1D}$ receptors are found to be co-localized by double immunohistochemical staining with substance P in the rat trigeminal ganglion neurons and with substance P, calcitonin gene-related peptide (CGRP) and nitric-oxide synthases in neurons in humans (Ma *et al*, 2001, Hou *et al*, 2001). These studies provide correlative evidence for the theory that anitmigraine drugs which activate $5\text{-}HT_{1D}$ receptors may inhibit substance P and CGRP release.

Location

$5\text{-}HT_{1F}$ receptor mRNA has been localized to the human middle meningeal arteries (Razzaque *et al*, 1999), human trigeminal ganglia (Bouchelet *et al*, 1996), human coronary arteries (Bouchelet *et al*, 2000) and the rabbit saphenous vein (Cohen and Schenck, 1999). Additionally, $5\text{-}HT_{1F}$ receptor mRNA has also been localized to the guinea pig trigeminal ganglion neurons where they mediate 5-HT stimulated inhibition of neurogenic dural inflammation (Johnson *et al*, 1997). $5\text{-}HT_{1F}$ receptors decrease c-fos expression in the rat trigeminal nucleus caudalis (Mitsikostas *et al*, 1999). The 5-HT_{1D} receptor mRNA has been localized to human heart valve interstitial cells (Roy *et al*, 2000), porcine cerebral cortex (Bhalla *et al*, 2000) and as autoreceptors in the guinea pig mesencephalic raphe, hippocampus and frontal cortex (el Mansari and Blier, 1996). The 5-HT_{1D} receptor has been localized by immunohistochemical staining in rat trigeminal ganglion neurons (Ma *et al*, 2001) and human trigeminal ganglion (Hou *et al*, 2001).

Regulation

There is a dearth of information about the regulation of the 5-HT_{1D} and 5-HT_{1F} receptors. Most current research has been directed to the functional and pharmacological characterization of these receptors. No analysis of the promoters has been reported. Additionally, there have been no studies reported looking at regulators of these receptors. Signaling mechanisms utilized by these receptors are also not clearly defined.

Pharmacology

The 5-HT_{1F} receptor is pharmacologically characterized by the agonists BRL54443 and (R)-N-[3Dimethylamino-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide also known as LY344864 (Phebus *et al*, 1997). Currently, there are no 5-HT_{1F} receptor antagonists available. The 5-HT_{1D} receptor is characterized by the agonist PN0142633 (Gomez-Mancill *et al*, 2001). There are no selective 5-HT_{1D} antagonists available. All of the 5-HT_{1D} antagonists available (i.e. GR127935) also have a high affinity for the 5-HT_{1B} receptor.

5-HT$_{1B}$ Receptor

Physiology

The 5-HT$_{1B}$ receptor is a heptahelical G-protein coupled receptor which specifically utilizes G_i as well as to G_o for signal transduction (Wurch and Pauwels, 2000). As a member of the 5-HT$_1$ class of receptors it is coupled to inhibition of adenylate cyclase. Additionally, 5-HT acting via 5-HT$_{1B}$ receptors has been linked to stimulation of DNA synthesis in fibroblasts (Seuwen *et al*, 1998). Similar to the 5-HT$_{2B}$ receptors in human coronary artery endothelial cells the 5-HT$_{1B}$ receptor is positively coupled to NO$^\bullet$ production (Ishida *et al*, 1998). In the presence of elevated extracellular potassium concentrations, the 5-HT$_{1B}$ receptor activates phospholipase D (PLD) (Hinton *et al*, 1999). Additionally, in bovine endothelial cell cultures the 5-HT$_{1B}$ receptor has been linked to release of NO$^\bullet$ (McDuffie *et al*, 1999) as well as activation of the ERK/MAPK pathway (McDuffie *et al*, 2000) (figure 3).

Furthermore, 5-HT$_{1B}$ receptors have been linked to activation of Akt/Protein kinase B in BE(2)-C neuroblastoma cells. This activation is inhibited by the regulator of G-protein signaling protein 4 (RGS4) (Lione *et al*, 2000). Stimulation of 5-HT$_{1B}$ receptors in native smooth muscle and primary cultures of rabbit renal artery smooth muscle resulted in activation of phosphatidylinositol 3-kinase (PI3-kinase) as well as the MAPK pathway (Hinton *et al*, 2000). In chinese hamster ovary (CHO) cells activated 5-HT$_{1B}$ receptors stimulate p70 S6 kinase (Pullarkat *et al*, 1998). p70 S6 kinase participates in the phosphoinositide

3-kinase (PI3-K) signaling pathway and in cell proliferation (Romanelli *et al*, 1999). Human 5-HT_{1B} receptors transfected into CHO cells have also demonstrated synergy with G_q coupled receptors as sumatriptan synergistically enhanced P_2U purinoceptor stimulated [^{3}H] inositol phosphate accumulation through a pertussive toxin-sensitive mechanism (Dickenson and Hill, 1998). To date, contractile signaling pathways for this receptor has not been completely elucidated in vascular smooth muscle. Additionally, 5-HT_{1B} receptors are "unmasked" in the tail artery from rats with normal blood pressure by a depolarizing stimuli (Craig and Martin, 1993). This "unmasking" of a silent 5-HT_{1B} receptor has also been described for the rabbit ear artery (Movahedi and Purdy, 1997) and rabbit femoral artery (Chen *et al*, 2000). The mechanisms for this "unmasking" of silent receptors is not completely clear yet. However, it seems to entail the sensitization of the contractile myofilaments to calcium by the influx of calcium through voltage gated calcium channels activated by the depolarizing stimulus (Hill *et al*, 2000).

Location

The 5-HT_{1B} receptor mRNA has been localized to: mouse striatum (Knobleman et al, 2000), rat trigeminal ganglion cells (Wotherspoon and Priestley, 2000), human coronary artery (Nilsson *et al*, 1999a), human cerebral artery (Nilsson *et al*, 1999b), human heart valve interstitial cells (Roy *et al*, 2000), human umbilical artery (Lovren *et al*, 1999) and human temporal artery (Verheggen *et al*, 1998).

Regulation

Interestingly, 5-HT_{1B} receptors form both homodimers as well as heterodimers with the 5-HT_{1D} receptors (Lee *et al*, 2000, Xie *et al*, 1999). Furthermore, posttranscriptional modifications that have been described for the 5-HT_{1B} receptor include phosphorylation and palmitoylation (Ng *et al*, 1993). The presence or absence of these posttranscriptional modifications and dimers, homo or hetero, may affect the function of the 5-HT_{1B} receptors.

While the human 5-HT_{1B} gene has a naturally occurring Phe-124-Cys variant which changes the pharmacological properties, G-protein coupling and second messenger formation (Kiel *et al*, 2000) of this receptor very little else is known about the gene and promoter of this receptor. Currently, there is a dearth of information about regulators and mechanisms of regulation of transcription of this receptor. There is also no published information about a characterization of the promoter, internalization and the ability to of this receptor be resensitized and recycled to the membrane. It is also not clear why in some tissues the 5-HT_{1B} receptor requires “unmasking” and in other tissues this “unmasking” is not necessary to observe a functional response.

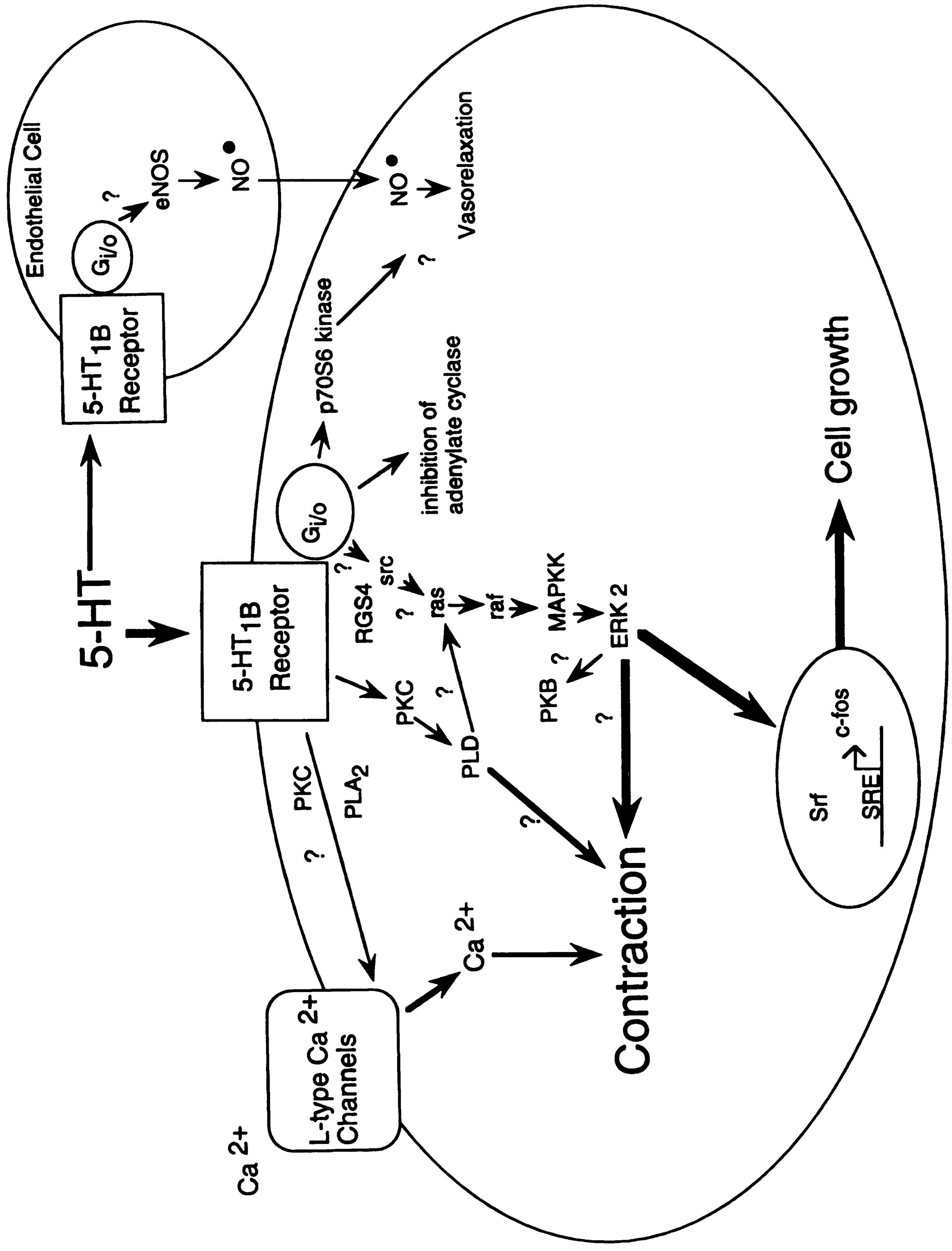
Pharmacology

The 5-HT_{1B} receptor is characterized by the agonists: sumatriptan, CP93129, RU24969, CGS12066B, 5-carboxamidotryptamine (5-CT) and 5-nonyloxytryptamine (5-NOT). The antagonists that are used to characterize this receptor are: isamoltane, GR55562, GR127935 and SB216641. The binding data reported for these compounds, with the exception of CP93129, were determined primarily at the human receptor. This is problematic as there is significant variability in this receptor subtype between species. Interestingly, this pharmacological variation between the rodent and human receptors is due to a single amino acid difference, a switch between a threonine at residue 355 in the human to an asparagine in the rodent 5-HT_{1B} receptor (Oksenberg *et al*, 1992).

II. Hypertension

The role of 5-HT in hypertension remains controversial. Much of this controversy stems, in part, from prior studies in which patients with hypertension were treated with the 5-HT_{2A} antagonist ketanserin. These studies were inconclusive due to ketanserin's ability to act as both a 5-HT_{2A} receptor antagonist as well as an α_1 adrenergic receptor antagonist (Balasubramaniam *et al*, 1993, Vanhoutte, 1991, van Zwieten *et al*, 1992). Hypertension is defined by the Sixth Report of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure as an average systolic blood pressure >140 mmHg, an average diastolic blood pressure >90 mmHg or current antihypertensive therapy (JNC VI, 1997). This condition is a major risk factor for end-stage renal disease, myocardial infarction, peripheral vascular disease, stroke and congestive heart failure. There is substantial evidence to demonstrate that lowering blood pressure reduces cardiovascular related mortality. However,

Figure 3. Diagram of the proposed signaling pathways utilized by the 5-HT_{1B} receptor. Abbreviations: PLC=Phospholipase C; PKC= Protein Kinase C, ERK=Extracellular signal regulated kinase; PLA_2= Phospholipase A_2; PLC=Phospholipase C; MAPKK= Mitogen activated protein kinase kinase; DAG= Diacylglycerol; eNOS= endothelial nitric oxide synthase; PKB =Protein kinase B; $NO^{\bullet}$= Nitric oxide; Regulator of G-protein signaling protein 4 = RGS4; Srf= Serum response factor; SRE= Serum response element; c-fos= Finkel-Biskis-Jinkins osteosarcoma oncogene homologue.



5-HT
5-HT$_{1B}$ Receptor
Endothelial Cell
$G_{i/o}$
eNOS
NO•
Vasorelaxation
p70S6 kinase
inhibition of adenylate cyclase
RGS4
src
ras
raf
MAPKK
ERK 2
PKB
PKC
PLD
PLA$_2$
Ca 2+
L-type Ca 2+ Channels
Contraction
Cell growth
Srf
c-fos
SRE

the goals of antihypertensive therapy are no longer simply to lower the blood pressure. The goals now include preventing or reducing target organ damage as well as reducing concomitant risk factors (Messerli and Laragh, 2000).

Hypertension is a complex and heterogeneous disease with two diagnostic categories, essential or primary hypertension and secondary hypertension. The diagnosis of secondary hypertension is applied when the elevation in blood pressure has a definable cause. This is a condition often caused by a change in hormone secretion or renal function. With the appropriate treatment of the cause, secondary hypertension is generally curable. The diagnosis of essential or primary hypertension is applied when the cause of the increased pressure is undetermined. Due to the involvement of several physiological systems in the regulation of blood pressure, determining the cause is often impossible. The course of treatment is often a combination of lifestyle changes (diet, exercise, weight loss *ect.*) as well as pharmacological intervention. Essential hypertension may be the result of multiple abnormalities in the systems which regulate blood pressure. These abnormalities may be genetic in origin or may be environmentally related (Messerli and Laragh, 2000).

Hemodynamics

Blood pressure is generally thought of as being determined by two factors, cardiac output (CO) and total peripheral resistance (TPR). Blood pressure can be calculated by the following formula: MAP=TPR x CO. Total peripheral resistance can be calculated as the mean arterial pressure (MAP) divided by the cardiac output: TPR=MAP/CO (Messerli and Laragh, 2000). The cardiac output is determined by the stroke volume (SV) and the heart rate (HR): CO=SV x HR. While the sympathetic nervous system plays a large role in determining overall blood pressure the total peripheral resistance is mainly determined in the small muscular arteries. Vascular smooth muscle cells and endothelial cells are primarily responsible for maintenance of the vessel diameter. This contractile tissue regulates flow and resistance by changing the diameter of the vessels via contraction or relaxation. Therefore, factors which affect the vascular smooth muscle, either to cause relaxation or contraction, will have an effect on the blood pressure. Hypertrophy and hyperplasia of vascular smooth muscle also contributes to hemodynamic changes seen in hypertension. By encroaching on the lumen of the vessel the new growth creates a narrowing of the diameter and thus an increase in resistance.

Mineralocorticoid hypertension

Hypertension can be created experimentally and it is an established protocol to administer exogenous mineralocorticoids such as aldosterone or deoxycorticosterone (DOC) to create experimental models of hypertension. Mineralocorticoid excess, also known as primary aldosteronism, is a well-known form of secondary hypertension in humans. Aldosterone is produced by the enzyme aldosterone synthase in glomerulosa of the adrenal cortex as well as locally in the vasculature (Takeda *et al*, 1995). Aldosterone is a component of the renin-angiotensin system (RAS). Renin, an aspartyl protease, is produced by the granular cells of the juxtaglomerular apparatus of the kidney. Renin's substrate angiotensinogen is produced in the liver and circulates in the blood. Renin cleaves angiotensinogen to form angiotensin I. Angiotensin I circulates to the lung where it is enzymatically converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II has many effects, one of which is to act on the adrenal glomerulosa to cause an increase in aldosterone synthesis.

Mineralocorticoids exert their effects by initially binding to mineralocorticoid receptors, a type of steroid hormone receptor. These are intracellular receptors, generally located in the cytosol. They are bound by heat shock proteins, which serve to anchor the receptors in the cytosol. After the hormone binds to the receptor, the receptor dissociates from the heat shock proteins and dimerizes with another steroid receptor. This dimer then translocates to the nucleus where it binds to steroid response elements in the DNA. Mineralocorticoids have been implicated in affecting the expression of the human sodium/potassium-ATPase β1 gene promoter (Derfoul *et al*, 1998), K-ras (Stockand *et al*, 1999), and the 5-HT_{1A} receptor in the rat dentate gyrus (Meijer *et al*, 1994). Additionally, aldosterone induces methylation of ras in renal epithelial cells (Al-Baldawi *et al*, 2000). This suggests that aldosterone not only changes the level of protein expression but also the state of activation of those proteins. Important to the changes seen in hypertension, activation of renal mineralocorticoid receptors will lead to cellular changes which result in salt and water retention by the kidney. Ultimately there is a suppression of the renin-angiotensin system due to the inhibitory effects of high sodium on renin secretion. Mineralocorticoids are also known to act in other tissues to modulate both gene expression as well as to exert nongenomic effects. In vascular smooth muscle, aldosterone increases levels of cAMP within one minute of exposure (Christ *et al*, 1999). Other nongenomic effects of aldosterone are rapid activation of the sodium/proton exchanger and the inositol 1,4,5-triphosphate/calcium pathway, both of which occur within one to two minutes (Wehling *et al*, 1992).

An interesting substrain of rats, the Wistar and Wistar-Furth rats, are used to study mineralocorticoid hypertension. In this model the Wistar-Furth rats are insensitive to the hypertensive effects of DOCA-salt treatment (Kayes *et al*, 1996, Ullian *et al*, 1997, Sciotti and Gallant, 1987). The mechanisms for this are still under investigation. These animals do become hypertensive with renal artery stenosis (Sciotti and Gallant, 1987) and 5/6th nephrectomy (Fitzgibbon *et al*, 1999). Wistar-Furth rats appear to be resistant to end-organ damage associated with mineralocorticoids even though there is a measurable increase in circulating level of mineralocorticoids. (Sciotti and Gallant, 1987). In the Wistar-Furth rat model, there are increases in responsiveness, as measured by isometric contractile force, to both serotonin and the 5-HT$_{2B}$ agonist BW723C86 (Watts and Harris, 1999, Bruner, 1992). Additionally, in the NG-nitro-L-arginine methyl ester (LNAME) model of hypertension where nitric oxide synthase is inhibited, the mineralocorticoid antagonist spironolactone has been reported to lower blood pressure (Mandarim-de-Lacerda *et al*, 2001). The thoracic aorta from another rat model of nitric oxide synthase inhibition, the LNNA (N^{ω}-Nitro-L-Arginine) hypertensive rats, demonstrate an increased contraction to BW723C86 as well (Russell and Watts, 1999). The endogenous levels of aldosterone have not been reported in the LNAME or LNNA hypertensive rats. However, when considered together, these results suggest that increases in the level of mineralocorticoids as well as pressure may play regulatory roles in 5-HT_{2B} receptor gene expression in hypertension.

Additionally, the roles of an increase in pressure and elevated levels of salt in modulating gene expression and vascular reactivity can not be ignored. High salt treatment increases the expression in the adrenal gland of the salt-inducible kinase (SIK) which is a member of the myocardial sucrose-nonfermenting 1 protein kinase (SNF1) /AMP-activated protein kinase (AMPK) family of serine/threonine protein kinases (Feldman *et al*, 2000). High salt has been implicated in vascular changes independent of an increase in pressure. These changes include alterations in the extracellular matrix in conduit arteries (Safar *et al*, 2000), membrane depolarization of both conduit and resistance arteries in salt loaded spontaneously hypertensive rats (SHR) (Fujii *et al*, 1999), impaired dilation of skeletal muscle resistance arteries (Weber and Lombard, 2000) and generation of reactive oxygen species (Lenda *et al*, 2000), specifically through the xanthine oxidase pathway (Swei *et al*, 1999).

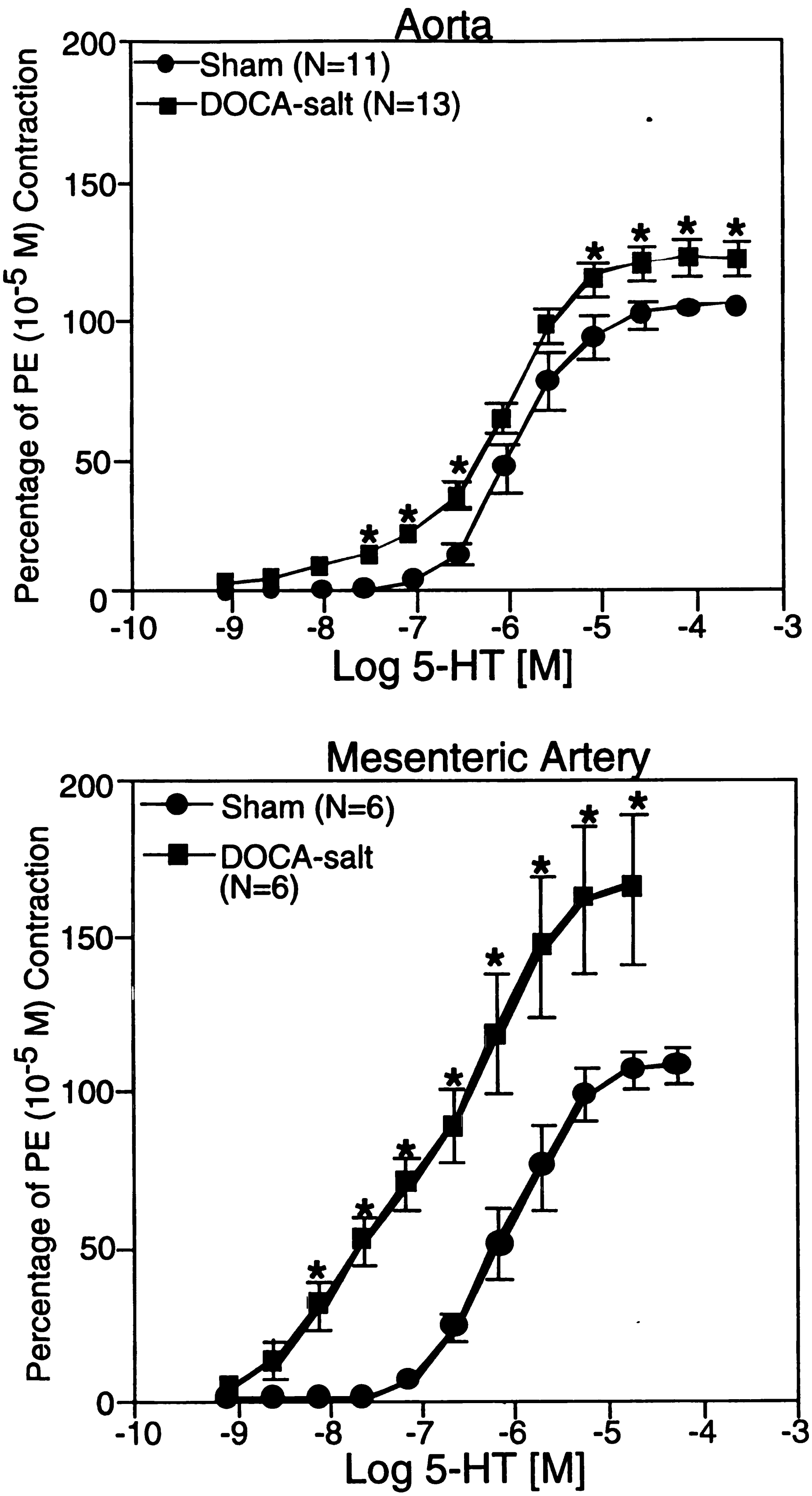
Increased vascular pressure is often modeled as increases in shear stress, mechanical stretch and mechanical strain. In endothelial cells exposed to mechanical strain, there was a sequential activation of PKC and ERK activation (Cheng *et al*, 2001). Pulmonary artery endothelial cells subjected to increased sheer stress increased expression of endothlin-1 but decreased expression of the adrenomedullin receptor and urotensin II (Dschietzig *et al*, 2001). Interestingly, elevated perfusion pressure increases expression of endothelin-1 and the endothelin (ET) B receptor in rabbit carotid arteries (Lauth *et al*, 2000). The mechanisms of changes in receptor expression by increased pressure has not yet been characterized. The effects of increased pressure on 5-HT receptor subtypes has never been examined.

Increased responsiveness

Arteries in many models of experimental hypertension and in other disease states display an increased responsiveness to 5-HT (Tompson and Webb, 1987, Ito *et al*, 1995, Miwa *et al*, 1994, Watts, 1998, Uematsu *et al*, 1987, Roson *et al*, 1990). Increased responsiveness is defined as an increase in potency, a decrease in threshold of activation and/or an increase in the maximal contraction elicited to an agonist. Increased responsiveness to 5-HT is pronounced in the DOCA-salt model of hypertension (figure 4). Mesenteric arteries, which control approximately 25 % of the cardiac output (Berne and Levy, 1997) and are therefore important contributors to total peripheral resistance, demonstrate a greater degree of hyperresponsiveness to 5-HT than does the thoracic aorta (figure 4). This hyperreactivity to 5-HT may be physiologically relevant in that it may play a role in the increased total peripheral resistance observed in hypertension.

Our laboratory and collaborators have published pharmacological data which suggest that the 5-HT-induced contraction in vascular smooth muscle in the DOCA–salt hypertensive and sham normotensive animals is mediated predominantly by the 5-HT $_{2B}$ and 5-HT $_{2A}$ receptors, respectively. (Watt and Fink, 1999, Watts, 1997, Watts and Webb, 1994). The contraction in arteries from normotensive sham rats is sensitive to inhibition by ketanserin. This suggests that this contraction is mediated by the 5-HT $_{2A}$ receptor. This is not the case with the tissue from DOCA-salt treated animals. The 5-HT concentration response curve in the arteries from DOCA-salt hypertensive rats is not sensitive to inhibition by ketanserin at the lower concentrations. As the data in table 1 demonstrate, 5-HT$_{2B}$ and 5-HT$_{1B}$ receptors vary significantly in their pharmacology. Ketanserin, a 5-HT $_{2A/2C}$ receptor antagonist, has a very low binding affinity for the 5-HT $_{2B}$ receptor. Therefore, ketanserin will not be able to inhibit an effect mediated by the 5-HT$_{2B}$ receptor. The lack of inhibition of 5-HT-induced contraction by ketanserin in arteries from hypertensive DOCA-salt rats suggest that the 5HT$_{2A}$ receptor is not mediating this response. However, these data are consistent with the hypothesis that the ketanserin-insensitive 5-HT$_{2B}$ receptor does mediate the contraction to 5-HT in the arteries from DOCA-salt hypertensive rats.

Figure 4. Top: Effect of 5-HT in the endothelium-denuded thoracic aorta from normotensive sham and DOCA-salt hypertensive rats. Bottom: Effect of 5-HT in the endothelium-denuded superior mesenteric artery from normotensive sham and hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the sham and DOCA-salt responses.


Aorta
Sham (N=11)
DOCA-salt (N=13)
Percentage of PE (10^{-5} M) Contraction
Log 5-HT [M]
Mesenteric Artery
Sham (N=6)
DOCA-salt
(N=6)
Percentage of PE (10^{-5} M) Contraction
Log 5-HT [M]

Other pharmacological data which provide support for a change in the predominate receptor subtype which mediates contraction are the effects of the 5-HT_{2B} receptor agonist BW723C86 and the 5-HT $_{2B}$ antagonist LY272015. BW723C86 does not contract an artery from a normotensive sham rat. However, BW723C86 produces a significant contraction in the arteries from the DOCA-salt hypertensive animal (Watts and Fink, 1999, Watts and Harris, 1999). The 5-HT $_{2A/2C}$ antagonist ketanserin did not reduce the BW723C86–induced contraction in the artery from the DOCA-salt treated animal (Watts and Harris, 1999). Moreover, the 5-HT $_{2B}$ antagonist LY272015 produced a significant inhibition of the 5-HT $_{2B}$ agonist BW723C86-induced contraction of the mesenteric artery from the DOCA-salt hypertensive rat (Watts and Harris, 1999).

A physiological role for this receptor subtype switch is supported by the data which show that LY272015 reduced the blood pressure of only hypertensive DOCA-salt treated rats (Watts and Fink, 1999). This indicates that the 5-HT_{2B} receptor is endogenously activated and plays a role in the maintenance of the elevated blood pressure in these rats. While these functional data suggest a change in the expression of the 5-HT_{2B} receptor the levels of 5-HT_{2B} receptor protein have never been measured.

Surprisingly, there has been no thorough characterization of the 5-HT receptors, neither those linked to contraction nor those linked to growth pathways, in arteries from hypertensive rats. 5-HT has the potential to play a role in the pathology of hypertension both in the hypertrophy and hyperplasia seen in arteries from hypertensive rats as well as in its capacity as a vasoconstrictor.

Collectively, these pharmacological data all support the ideas that the 5-HT-induced contraction in a vessel from a normotensive sham rat is mediated by a 5-HT $_{2A}$ receptor and that the contractions in the vessels from the DOCA-salt hypertensive rats are mediated primarily through 5-HT $_{2B}$ receptors with the 5-HT$_{2A}$ receptor being activated at the higher concentration of 5-HT. This proposed change in the receptor subtypes mediating contraction to 5-HT in hypertension has both functional and physiological effects. Functionally, the arteries from DOCA-salt hypertensive rats are much more sensitive to 5-HT compared to the arteries from the normotensive sham rats (figure 4). An increased sensitivity to 5-HT means that it takes less endogenous 5-HT to activate the 5-HT $_{2B}$ receptor than the 5-HT$_{2A}$ receptor; potentially a small increase due to platelet dysfunction, or a thrombotic event could result in an increase in 5-HT sufficient to activate the 5-HT $_{2B}$ receptor (Kamal *et al*, 1984, Baudouin-Legros *et al*, 1985, Guicheney *et al*, 1985, Carrascol *et al*, 1998).

Hypothesis

I hypothesize that during normotensive conditions the 5-HT $_{2A}$ receptor is the predominant 5-HT receptor which mediates contraction in vascular smooth muscle. Under conditions of increased vascular pressure and/or in the presence of DOCA there is a change in the receptor subtype(s) which mediate contraction in vascular smooth muscle, specifically a change from only the 5-HT $_{2A}$ receptor to the 5-HT$_{1B}$ and 5-HT $_{2B}$ receptors in addition to the 5-HT$_{2A}$ receptor.

My specific aims were to determine the mechanism of enhanced serotonergic sensitivity in DOCA-salt hypertension and the physiological relevance of the 5-HT receptor subtypes, which mediate the contraction in the vasculature. To do this I tested the following four specific hypotheses:

Subhypothesis #1: The 5-HT$_{1D}$ and the 5-HT $_{1F}$ receptors are not involved in the contraction of the aorta and superior mesenteric artery from normotensive and DOCA-salt hypertensive rats. 5-HT $_{1B}$ receptors are involved in the contraction of the aorta and superior mesenteric artery from DOCA-salt hypertensive rats.

Subhypothesis #2: There is an increase in the level of the smooth muscle 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors in the vasculature of DOCA-salt hypertensive rats.

Subhypothesis # 3: Elevated pressure and mineralocorticoid(s) treatment independently increase the levels of the 5-HT$_{1B}$ and 5-HT $_{2B}$ receptor proteins.

Subhypothesis #4: The level of free 5-HT circulating in the blood is increased in the DOCA-salt hypertensive rat.

Methods

I. General Animal Methods:

Animals

All animal procedures were followed in accordance with the institutional guidelines of Michigan State University. Male Sprague-Dawley rats were purchased from Charles River (Portage, MI). Until surgery, animals were kept in clear plastic boxes with free access to standard rat chow (Teklad) and tap water.

Mineralocorticoid Hypertension:

Male Sprague-Dawley rats (250-300g), under isoflurane (IsoFlo®, Abbott Laboratories, North Chicago, IL.) anesthesia, were uninephrectomized and deoxycorticosterone acetate (DOCA, 200mg/kg in silicone rubber) was implanted subcutaneously. Postoperatively, rats were given a solution of 1% NaCl and 0.2 % KCl for drinking. Normotensive sham rats were uninephrectomized, received no DOCA and drank normal tap water. Animals remained on this regimen for four weeks (unless otherwise specified) prior to use.

Blood Pressure Measurement:

Systolic blood pressure of rats was determined in the conscious state by the tail cuff method (pneumatic transducer). Briefly, the rat was placed in a plastic pail with wood chip bedding covering the bottom. This pail was placed on a heating pad and the rat was contained by placing a steel cage over it. A warming light was placed over the steel cage. The rat was warmed in this manner for 6 minutes. Warming the rat serves to vasodilate the tail artery which facilitates the measurement of the blood pressure. The rat was then placed into a restraint and the blood pressure cuff was slipped onto the tail. The balloon transducer was placed onto the ventral side of the tail and secured with tape. The blood pressure was monitored until a stable pulse pressure was observed. The blood pressure was measured utilizing a sphygmomanometer in conjunction with the pulse transducer. Blood pressure measurements were taken three times to obtain an average systolic blood pressure.

II. Isolated Smooth Muscle Contractility Measurement in Aorta and Superior Mesenteric Arteries:

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the tissues were dissected and removed. Tissues were placed in physiologic salt solution consisting of (in mM) NaCl, 103; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \bullet 7H_2O$, 1.17; $CaCl_2 \bullet 2H_2O$, 1.6; $NaHCO_3$, 14.9; dextrose, 5.5; and $CaNa_2EDTA$, 0.03. The aorta and superior mesenteric artery were cleaned of fat and connective tissue, cut into helical strips, and mounted on stainless steel holders in tissue baths (50 ml) for isometric tension recordings using Mac Lab (Chart 3.4 software) and transducers. Strips were placed under optimum resting tension (1500 mg for aorta, 600 mg for superior mesenteric determined previously), and strips from normotensive and hypertensive rats were placed in the same bath, thereby controlling for experimental variations. Tissue baths were filled with warmed (37°C), aerated (95% O_2, 5% CO_2), physiological salt solution (PSS). Endothelium was removed by gently rubbing the luminal face of the vessel with a moistened cotton swab. Functional integrity of the endothelial cells was evaluated by testing endothelium-dependent relaxation of acetylcholine (1 μM) in strips contracted with phenylephrine (10 μM). Removal of the endothelium was to simplify evaluation of vascular smooth muscle cell response by removing a potential source of complicating modulators (i.e. nitric oxide). Cumulative concentration response curves to agonists were performed. Antagonists, inhibitors or vehicle were incubated with vessels for one hour prior to experimentation.

III. Depolarization Protocol:

Tissues were incubated for 1 hour with the 5-HT_{2A} receptor antagonist ketanserin (10 nM). This concentration of ketanserin does not block 5-HT_{1B} or 5-HT_{2B} receptors. Tissues were then depolarized with 15 mM KCl. This contraction was allowed to plateau (15-20% of maximal PE 10 μM contraction) and then cumulative concentration response curves to agonists were performed.

IV.Aldosterone Incubation Protocol:

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). The thoracic aorta from male Sprague-Dawley rats with normal blood pressure was removed and cleaned of fat and connective tissue, cut into helical strips, and cut into four equal pieces. The aortic tissues were incubated under tissue culture conditions (37 °C, 5 % CO_2) in Dulbecco's Modified Eagle Media (DMEM) (Gibco- BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2% penicillin/streptomycin and 2% L-glutamine (Gibco-BRL). Aldosterone (Sigma-

RBI, St. Louis, MO) or vehicle was added to the media. The mineralocorticoid antagonist spironolactone (10 μM, Sigma-RBI) was added thirty minutes prior to the addition of aldosterone. Tissues were removed from media and taken through the protein isolation procedure listed below.

V. Tissue Homogenization for Protein Work:

Aorta and superior mesenteric arteries from sham and DOCA-salt treated rats were removed, cleaned, denuded of endothelium and cut into helical strips. The tissue was frozen in liquid nitrogen, pulverized in a liquid nitrogen-cooled mortar and pestle and solubilized in a lysis buffer (0.5 mol/L Tris HCl [pH 6.8], 10% SDS, 10% glycerol) with protease inhibitors (0.5 mmol/L PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Homogenates were centrifuged (11,000 x g for 10 minutes, 4°C) and supernatant total protein measured (BCA, Sigma).

VI. Western Blotting:

Tissue homogenate supernatant (4:1 in denaturing sample buffer, boiled for 5 minutes), was separated on SDS-polyacrylamide gels (10% or 12 %) and transferred to Immobilon–P membrane. Membranes were blocked [4-6 hours in Tris-buffer saline + Tween-20 (0.1%; TBS-T) containing 4% chick egg ovalbulmin and 0.025% sodium azide] and probed overnight (4°C) with primary antibody. The following antibodies were used: mouse anti-serotonin 2B receptor (5-HT_{2BR}) monoclonal antibody (0.5 μg/ml, PharMingen, San Diego, CA) and guinea pig anti-serotonin 1B receptor (5-HT_{1B}) polyclonal antibody (1:1000, Chemicon, Temecula, CA). Blots were washed three times with TBS-T (30 minutes, 5 minutes, 5 minutes) and once with TBS (5 minutes). An antimouse horseradish peroxidase linked secondary antibody (1:10,000, Amersham Laboratories, Arlington Heights, IL) or an antiguinea pig horseradish peroxidase linked secondary antibody (1:10,000, Chemicon) was added for one hour and incubated with blots at 4°C. Blots were washed using the described regimen. Enhanced chemiluminescence was performed using standard reagents (Amersham Laboratories). Each blot was washed and redeveloped using an α-smooth muscle actin antibody (1:400, Oncogene Research Products, Boston, MA; anti-mouse secondary antibody, 1:5,000, Amersham Laboratories). Equal lane loading of protein was ensured by comparing α-smooth muscle actin densitometry.

VII. Real Time RT-PCR:

Total RNA was isolated from tissues using standard procedures and TRI reagent (Molecular Research Center). Concentration/purity/integrity of RNA was ascertained spectrophotometrically (A_{260}/A_{280}) and by running a qualitative 1% agarose gel to visualize all samples with ethidium bromide (18S/28S check). Samples were diluted to 50 ng/µl RNA and taken through reverse transcription on a Perkin Elmer GeneAmp 5700 for Real-time PCR in nuclease-free buffer containing appropriate reagents and Reverse Transcriptase (45 °C, 30 min.). Samples from sham and DOCA-salt tissues were run at the same time and run with samples that did not contain reverse transcriptase. After reverse transcription, the appropriate primer [synthesized by the Biochemistry department at MSU]; 5-HT_{1B} forward primer = 5'-CACTAGGCCAGGTGGTCTGC-3', reverse 3'-GCAGCGAAATCGAGATGGAG; 5-HT_{2B} forward primer = 5'-ACAGAAAGGCGAATGGCTTC-3', reverse = 5'-CGGCAGTCTGCTTCATTTCC -3'; annealing temperature of 60°C for 60 seconds, 35 cycles] and SYBR Green master mix was added for PCR. Samples were normalized to a GAPDH signal (GAPDH primers purchased from PE Applied Biosystems).

<u>VIII Measurement of Plasma 5-HT</u>:

Blood samples from both sham and DOCA-salt hypertensive rats were collected into 5 ml syringes coated with heparin (2000 units/mL) and anticoagulant solution [consisting of (in mM) citric acid, 13; sodium citrate, 12.6; D-glucose, 11; and 150 µl of 4% EDTA per tube] from the hepatic vein. After blood collection, the contents of the syringes was carefully mixed with anticoagulant solution (.1mL/mL of blood). This mixture was centrifuged for ten minutes at 160 x g and 4°C to obtain platelet rich plasma. Plasma (2 ml) was removed and a 1:1 dilution was performed with EDTA (0.4 M) and these samples were centrifuged again for 20 minutes at 1350 x g and 4°C to obtain platelet poor plasma. The remaining platelet rich plasma was resuspended in 1 ml of platelet buffer (pH =7.4) consisting of [(in mM) NaCl, 145; KCl, 5; $CaCl_2$, 1; $MgSO_4$, 1; D-glucose, 10], adenosine diphosphate (ADP) (1.0 uM/L) was added and then samples were vortexed and allowed to remain on ice for 15 minutes. The samples were deproteinized with trichloroacetic acid (0.5 M) for thirty minutes and then centrifuged at 4500 x g for twenty minutes at 4°C. The supematant was centrifuged again at 100,000 x g at 4°C for two hours to remove all proteins. The monoamine oxidase inhibitor parglyine (10 μM) and ascorbic acid (10 μM) were added to the blood after it was initially placed into anticoagulant solution and was added again after each centrifugation step as well as to the supernatant after deproteinization. The concentration of trichloroacetic acid used was chosen after a concentration response curve experiment was performed with one ng standards of 5-HT to ensure that no degradation occurred. Measurements were made using high performance liquid chromatography (HPLC). The chromatography column was packed with 6 μm spherical c18 bonded to silica gel particle. (Waters, Division of Millipore, Milford, MA). A precolumn Nova Pack c18 (Waters) was used. The mobile phase used was: EDTA 0.1 M, 0.03% sodium octyl sulfate (SOS), 0.05M $NaPO_4$ and 15% methanol.

IX. Data Analysis and Statistics:

Data are presented as means ± standard error of the mean for the number of animals in parentheses. Contraction was reported as a percentage of response to maximum contraction of phenylephrine (10 μM). EC_{50} values (agonist concentration necessary to produce a half-maximal response) were determined using non-linear regression analysis and was reported as the mean of the negative logarithm (-log) of the EC_{50} value (M). When comparing two groups, the appropriate Student's t test was used. ANOVA followed by a Student-Newman-Kuels post hoc test was performed when comparing three or more groups. In all cases, a p value less than or equal to 0.05 was considered statistically significant. Band density was quantified using the program NIH

Image. HPLC data was normalized for the plasma volume of the samples. Results from the Real-Time PCR experiments were reported as values normalized by GAPDH or as ΔC_T (threshold values)

Results

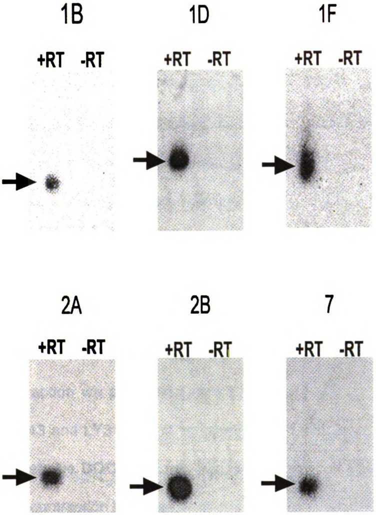
Hypothesis I:

Profiling of 5-HT Receptors

Which 5-HT receptors comprise the complete complement of 5-HT receptors in the vasculature of the normotensive and hypertensive rats is currently undetermined. We have data from RT-PCR in endothelium-denuded rat aorta from a normotensive rat (figure 5) which examines which 5-HT receptor mRNA are expressed in aortic smooth muscle cells. While all of the known 5-HT receptors were not probed for in this experiment, those probed for are receptors linked to contraction or reported to modulate vascular tone in other vascular beds. These data show the presence of the following receptor's mRNA: 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{1D}$, 5-HT$_{1B}$, 5-HT$_{1F}$ and 5-HT$_{7}$ receptors. The mRNA for the 5-HT$_{1E}$ (not shown) receptor was not detected. These findings suggest that in addition to the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors, 5-HT $_{1B}$, 5-HT$_{1D}$ and 5-HT $_{1F}$ receptors may also be involved in mediating 5-HT-induced vascular smooth muscle contraction because their message is present. Of the five receptors for which mRNA message was detected, four have been suggested to be involved in mediating vascular smooth muscle cell contraction to 5-HT (Smith et al, 1999, Watts *et al*, 1996,Razzaque *et al*, 1999, VanDenBrink *et al*, 2000). These contractile receptors are the 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{1F}$ receptors. The 5-HT$_{7}$ receptor is known to couple to activation of G$_{s}$ and thereby mediate vascular relaxation (Adham *et al*, 1998, Cushing *et al*, 1996).

Figure 5: Results of RT-PCR analysis and Southern blotting of rat thoracic aorta denuded of endothelium for 5-HT receptors. Data shown for the 5-HT_{2A}, 5-HT_{2B}, 5-HT_{1B}, 5-HT_{1F}, 5-HT_{1D} and 5-HT_7 receptors. Data for the 5-HT_{1E} receptor not shown. Experiments were preformed at Eli Lily, Indianapolis, IN in collaboration with Dr. Mel Baez. Abbreviations: +RT = Reverse Transcriptase present; -RT= Reverse Transcriptase absent.

RT-PCR of 5-HT Receptors in Rat Aorta



Based on these data, we next moved to contractile experiments to determine which of the receptors for which message was detected were involved in mediating 5-HT-induced contraction. The first receptor addressed was the 5-HT_{1D} receptor. We performed contractile experiments utilizing the selective 5-HT_{1D} agonist PNU014633 (10^{-9} to 10^{-5} M) in the aorta from both normotensive sham and hypertensive DOCA-salt rats (figure 6). The average systolic blood pressure for the sham rats utilized in the following studies was 123 $\pm$ 2 mm Hg. The average systolic blood pressure for the DOCA-salt rats used in the following studies was 194 $\pm$ 10 mm Hg. There was no contraction observed in the aorta from either the normotensive sham or the hypertensive DOCA-salt rats. Based on the contractile data we conclude that the 5-HT_{1D} receptor does not mediate contraction in rat aortic vascular smooth muscle either under conditions of normal or high blood pressure.

To address the involvement, if any, of the 5-HT_{1F} receptor in mediating 5-HT-induced contraction we performed contractile experiments using the 5-HT_{1F} agonists BRL54443 and LY344864 in aorta from both normotensive sham (figure 7,top) and hypertensive DOCA-salt rats (figure 7,bottom). LY344864 (10^{-9} to 10^{-5} M) did not elicit a contraction in the aorta from either the normotensive sham or the hypertensive DOCA-salt rats. BRL54443 (10^{-9} to 10^{-5} M) did cause a contraction in the aorta and the mesenteric artery from both the normotensive sham (figure 8,

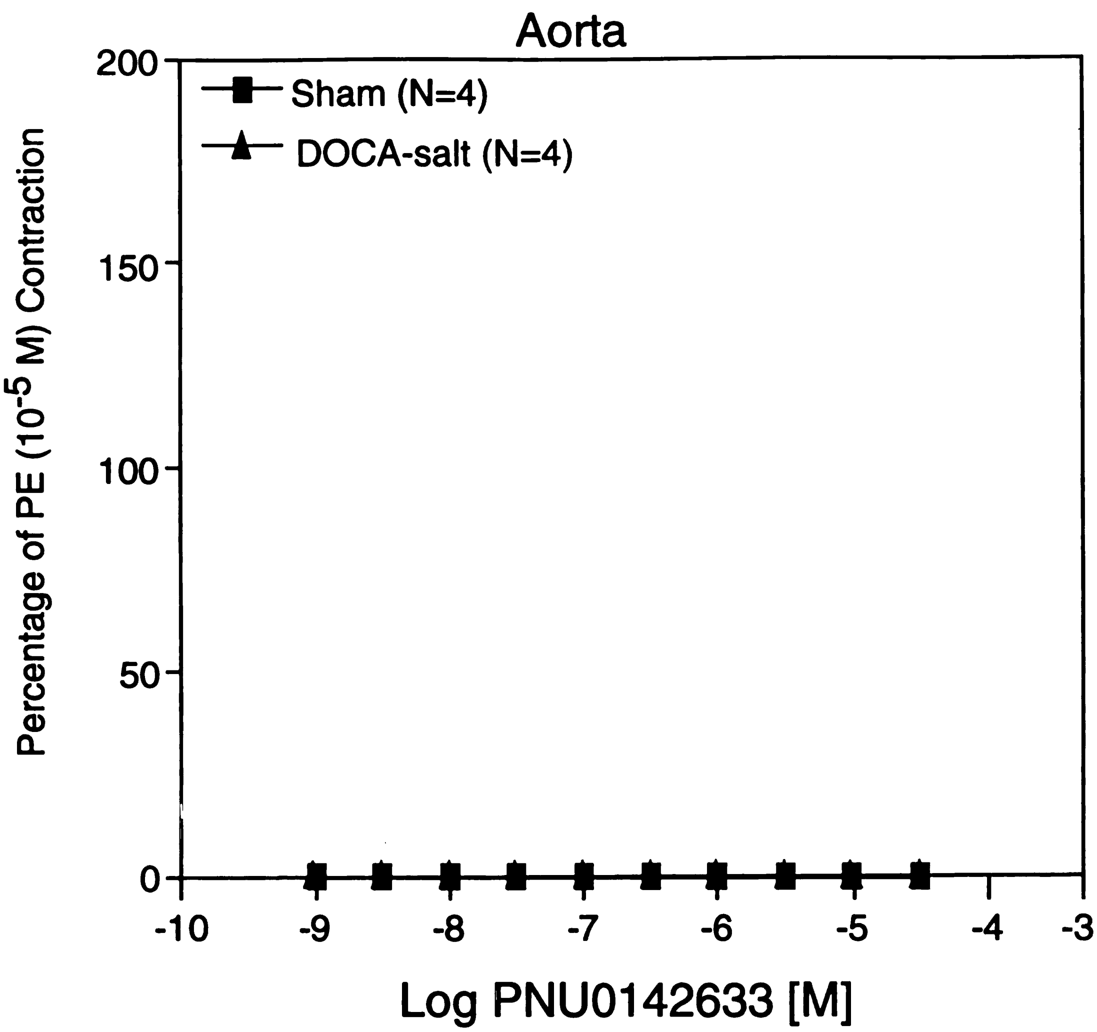
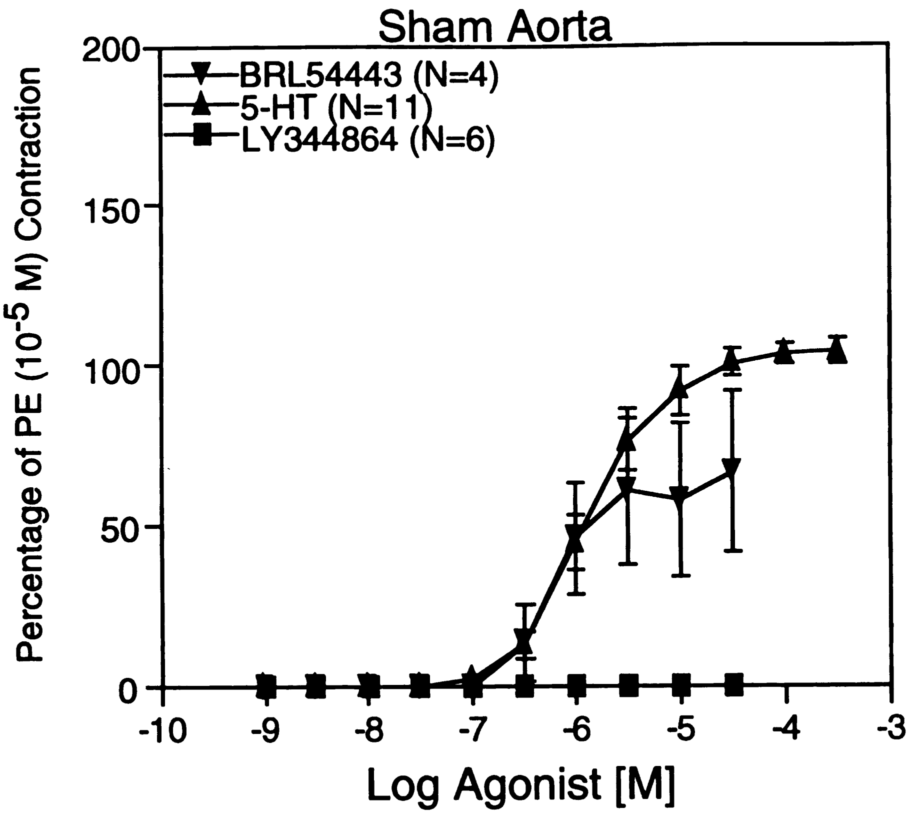


Figure 6. Effect of the 5-HT_{1D} receptor agonist PNU0142633 in endothelium-denuded thoracic aorta from normotensive sham and hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.

Figure 7. Top: Effect of the 5-HT_{1F} receptor agonists BRL54443 and LY344864 in endothelium-denuded thoracic aorta from normotensive sham rats. Bottom: Effect of the 5-HT_{1F} receptor agonists BRL54443 and LY344864 in endothelium-denuded thoracic aorta from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.


Sham Aorta
BRL54443 (N=4)
5-HT (N=11)
LY344864 (N=6)
Percentage of PE (10-5 M) Contraction
Log Agonist [M]
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3

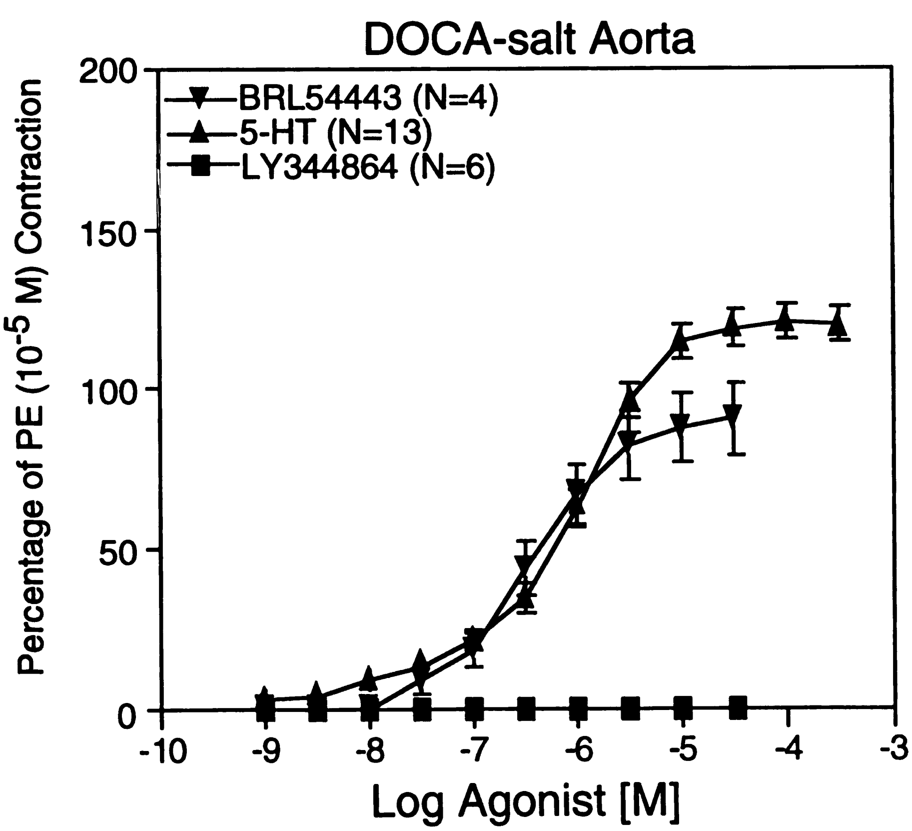
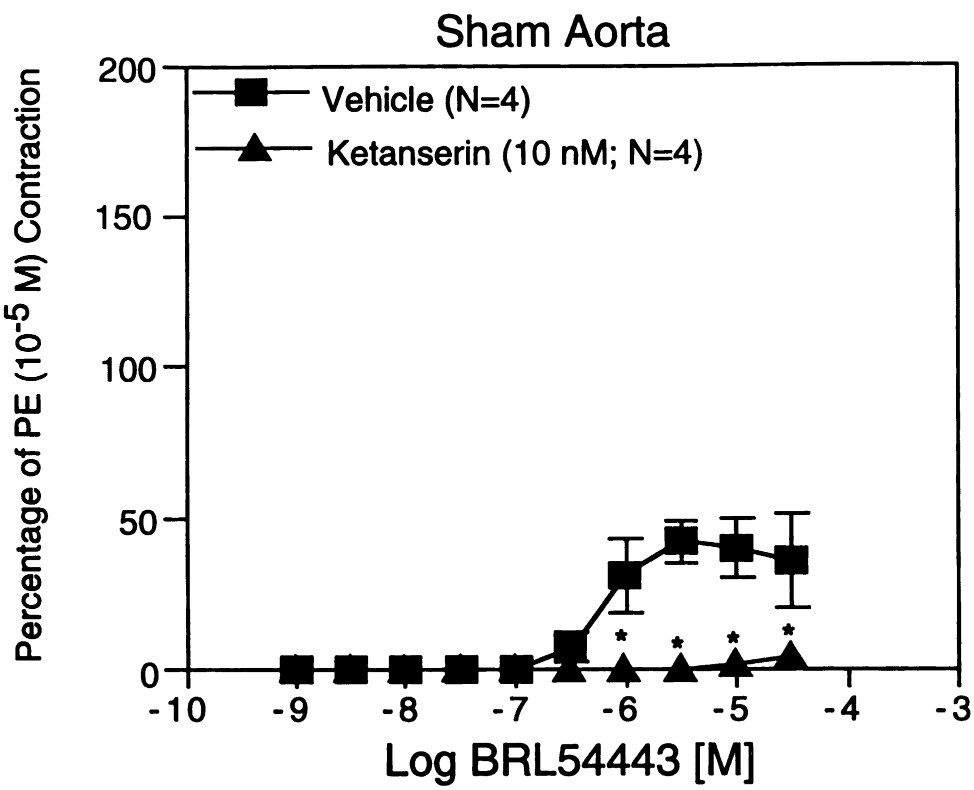

DOCA-salt Aorta
BRL54443 (N=4)
5-HT (N=13)
LY344864 (N=6)
Percentage of PE (10-5 M) Contraction
Log Agonist [M]
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3

Figure 8. Top: Effect of the 5-HT_{2A} receptor antagonist ketanserin (10 nM) on the 5-HT_{1F} receptor agonist BRL54443-induced contraction in endothelium-denuded thoracic aorta from normotensive sham rats. Bottom: Effect of the 5-HT_{2A} receptor antagonist ketanserin (10 nM) on the 5-HT_{1F} receptor agonist BRL54443-induced contraction in endothelium-denuded thoracic aorta from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the vehicle-incubated and ketanserin-incubated responses.


Sham Aorta
Vehicle (N=4)
Ketanserin (10 nM; N=4)
Percentage of PE (10^{-5} M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log BRL54443 [M]

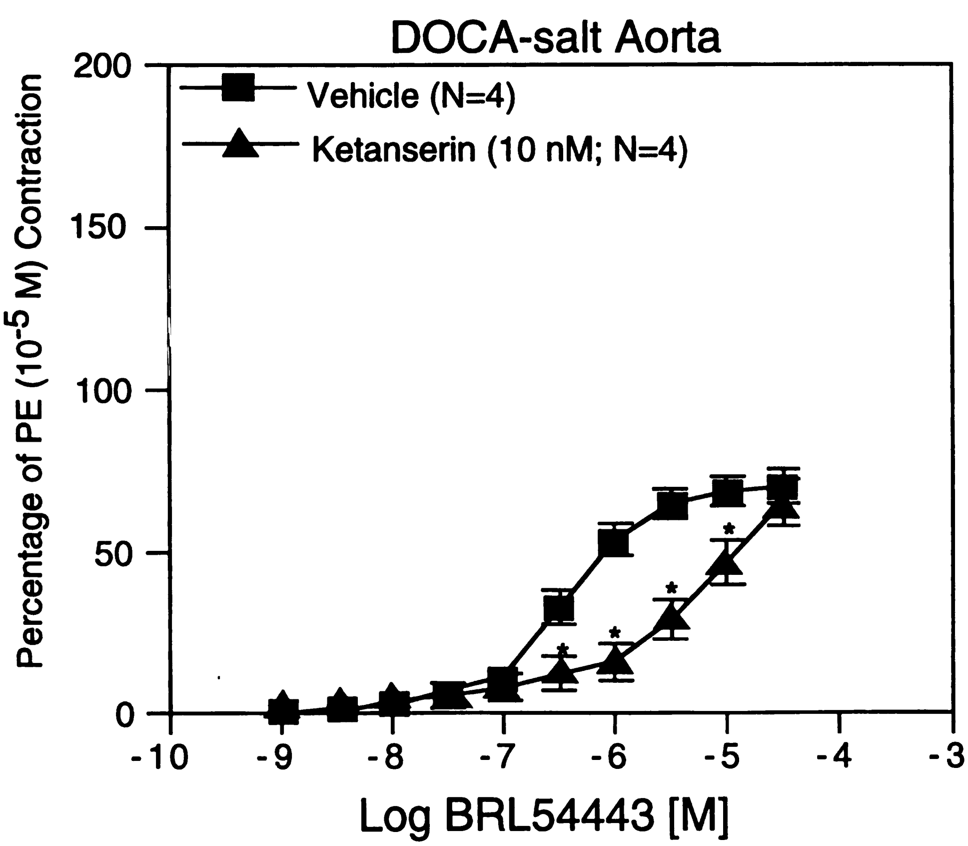
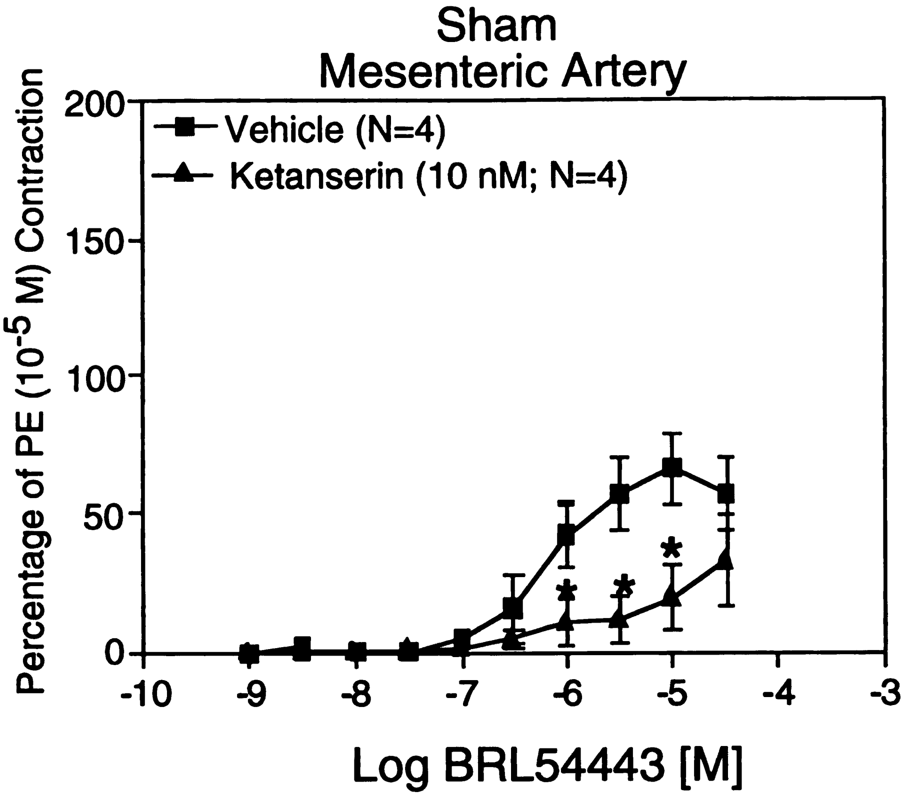
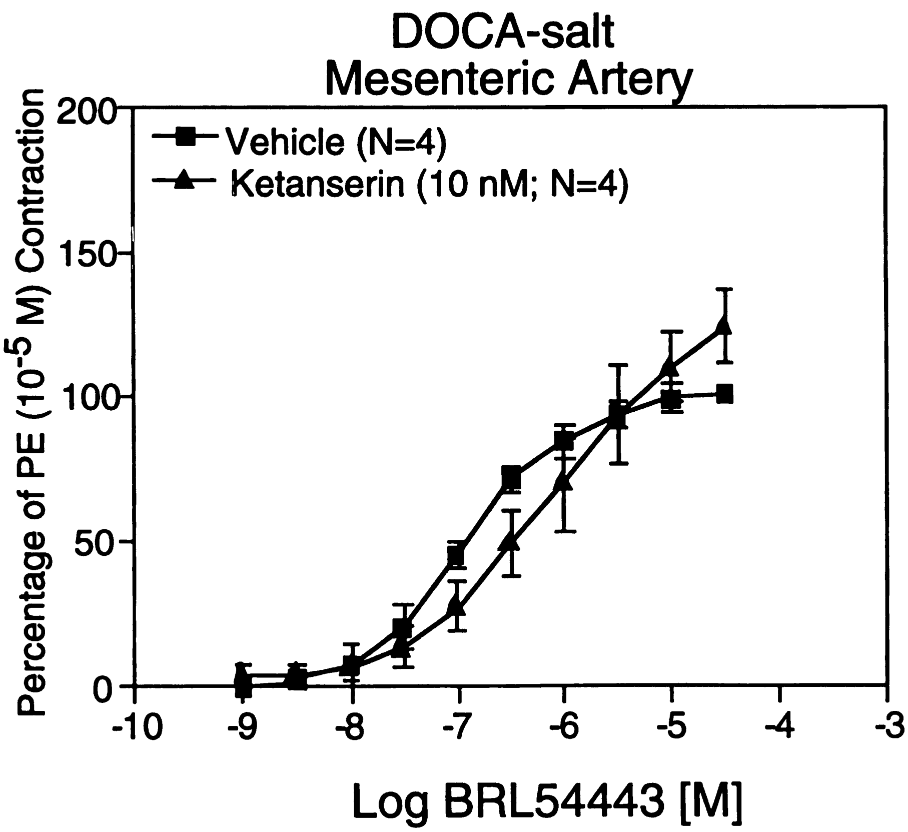

DOCA-salt Aorta
Vehicle (N=4)
Ketanserin (10 nM; N=4)
Percentage of PE (10^{-5} M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log BRL54443 [M]

Figure 9. Top: Effect of the 5-HT_{2A} receptor antagonist ketanserin (10 nM) on the 5-HT_{1F} receptor agonist BRL54443-induced contraction in endothelium-denuded superior mesenteric artery from normotensive sham rats. Bottom: Effect of the 5-HT_{2A} receptor antagonist ketanserin (10 nM) on the 5-HT_{1F} receptor agonist BRL54443-induced contraction in endothelium-denuded superior mesenteric artery from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the vehicle-incubated and ketanserin-incubated responses.



Sham
Mesenteric Artery
Vehicle (N=4)
Ketanserin (10 nM; N=4)
Percentage of PE (10^{-5} M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log BRL54443 [M]



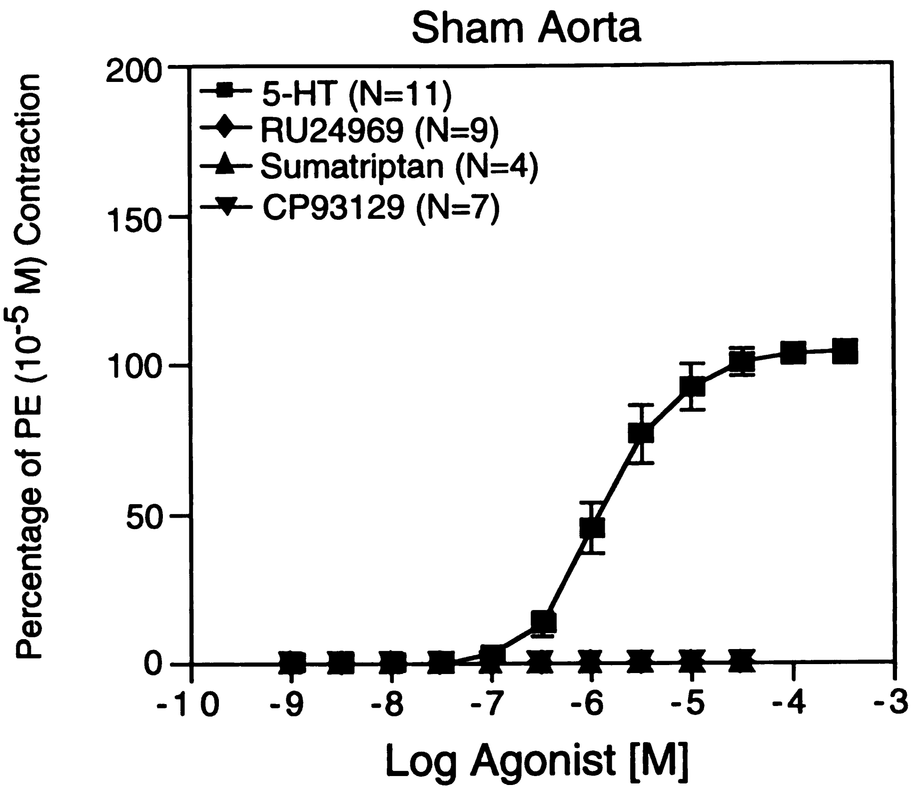
DOCA-salt
Mesenteric Artery
Vehicle (N=4)
Ketanserin (10 nM; N=4)
Percentage of PE (10^{-5} M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log BRL54443 [M]

top, figure 9,top) as well as the hypertensive DOCA-salt rats (figure 8, bottom, figure 9,bottom). However, the contraction elicited by BRL54443 in the aorta and mesenteric artery from the sham rat appears to be due to activation of the 5-HT_{2A} receptor as it is almost completely inhibited in the presence of the 5-HT_{2A} receptor antagonist ketanserin (figure 8, top, figure 9, top). In the superior mesenteric arteries from DOCA-salt treated rats ketanserin has no effect on the BRL54443-induced contraction (figure 9, bottom). This is a markedly different response from that seen in the thoracic aorta, where ketanserin inhibited BRL54443-induced contraction (-log EC_{50} value (M) 6.42 $\pm$ 0.04 and 5.26 $\pm$ 0.11, vehicle- and ketanserin-treated, respectively) (figure 8, bottom). These data suggest that BRL54443 is acting through the 5-HT_{2A} receptor in the aorta from sham and DOCA-salt hypertensive rats as well as in the mesenteric artery from the sham rats. Furthermore, these data suggest that the BRL54443-induced contraction in the superior mesenteric arteries of the DOCA-salt hypertensive rats is not mediated predominately by the 5-HT_{2A} receptor but rather either by the 5-HT_{2B} receptor or the 5-HT_{1B} receptor. BRL54443 has affinity for both of these receptors (table 1). Further contractile studies with the 5-HT_{2B} antagonist LY272015 and the 5-HT_{1B} antagonist GR55562 would determine which of these two possible receptors is mediating the BRL54443-induced contraction in the arteries from hypertensive DOCA-salt rats. However, based on the data, using multiple 5-HT_{1F} agonists, it is appropriate to rule out the involvement of the 5-HT_{1F} receptor in mediating contraction in arteries from normotensive and DOCA-salt hypertensive rats.

Lastly, we have contractile data demonstrating that the 5-HT_{1B} receptor agonists sumatriptan (10^{-9} to 10^{-5} M), RU24969 (10^{-9} to10^{-5} M) and the rodent selective 5-HT_{1B} agonist CP93129 (10^{-9} to10^{-5} M) have no effect in arteries from normotensive rats (figure 10, top). The presence of the 5-HT_{1B} antagonist GR55562 (100 nM) produced no effect on the 5-HT-induced contraction in the aorta or superior mesenteric artery from normotensive sham rats (figure 11, top left, bottom left). This suggests that under conditions of normal blood pressure, the 5-HT $_{1B}$ receptor does not mediate contraction in vascular smooth muscle.

However, these 5-HT_{1B} receptor agonists all produced a contraction in the aorta from hypertensive DOCA-salt rats (figure 10,bottom). This contraction was only a partial contraction compared to that elicited by 5-HT. The 5-HT_{1B} receptor antagonist GR55562 (100 nM) did produce a small, but significant inhibition of the 5-HT-induced contraction in both the thoracic aorta (-log EC_{50} values [M] 6.26 $\pm$ 0.08 and 5.88 $\pm$ 0.04, vehicle- and GR55562-treated, respectively) and superior mesenteric artery (-log EC_{50} values [M] 6.90 $\pm$ 0.10 and 6.29 $\pm$ 0.13, vehicle- and GR55562-treated, respectively) from DOCA-salt hypertensive rats (figure 11, top right, bottom right). The affinity of GR55562 is currently unknown at the 5-HT_{2B} receptor. Therefore, to determine the selectivity of GR55562 and the resultant response seen, we tested GR55562 (100 nM) against the 5-HT_{1B} rodent receptor selective agonist CP93129. In the mesenteric artery of normotensive sham rats CP93129 (10^{-9} to 10^{-5} M) and the combined treatment of GR55562 and CP93129 had no effect. In the mesenteric artery of hypertensive DOCA-salt rats, GR55562 (100 nM) produced a small but significant inhibition of the CP93129-induced contraction (-log EC_{50} values [M] 6.73 $\pm$ 0.07 and 5.68 $\pm$ 0.05, vehicle- and GR55562-treated, respectively) (figure 12, bottom). Interestingly, when the 5-HT_{1B} antagonist GR55562 (100 nM) and the 5-HT_{2B} receptor antagonist LY272015 (10 nM) are incubated with the mesenteric artery from hypertensive DOCA-salt rats simultaneously the 5-HT-induced contraction is shifted rightward (-log EC_{50} values [M] 6.79 $\pm$ 0.08 and 5.81 $\pm$ 0.07, dual vehicle- and GR55562 and LY272015 combined treatment, respectively) (figure 13, bottom). This response is normalized to the response seen from arteries from sham rats leftward (-log EC_{50} values [M] 5.99 $\pm$ 0.03 and 5.81 $\pm$ 0.07, sham dual vehicle- and DOCA-salt GR55562 and LY272015 combined, treatment, respectively) (figure 13, bottom). Neither the 5-HT_{1B} receptor antagonist GR55562 nor the 5-HT_{2B} receptor antagonist LY272015 had any significant effect on the 5-HT-induced contraction in the superior mesenteric arteries from the normotensive rats (figure 13, top). The variability of the response seen in the tissues from the sham rats is due to the different concentrations of dimethyl sulfoxide (DMSO) used as the vehicles for the antagonists. The one vehicle contains either 25 µl or 50 µl of DMSO. The two or dual vehicle conditions contain 75 µl of DMSO. These data suggest that 5-HT_{1B} receptors mediate, at

Figure 10. Top: Effect of the 5-HT_{1B} receptor agonists in endothelium-denuded thoracic aorta from normotensive sham rats. Bottom: Effect of the 5-HT_{1B} receptor agonists in endothelium-denuded thoracic aorta from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.


Sham Aorta
5-HT (N=11)
RU24969 (N=9)
Sumatriptan (N=4)
CP93129 (N=7)
Percentage of PE (10-5 M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log Agonist [M]

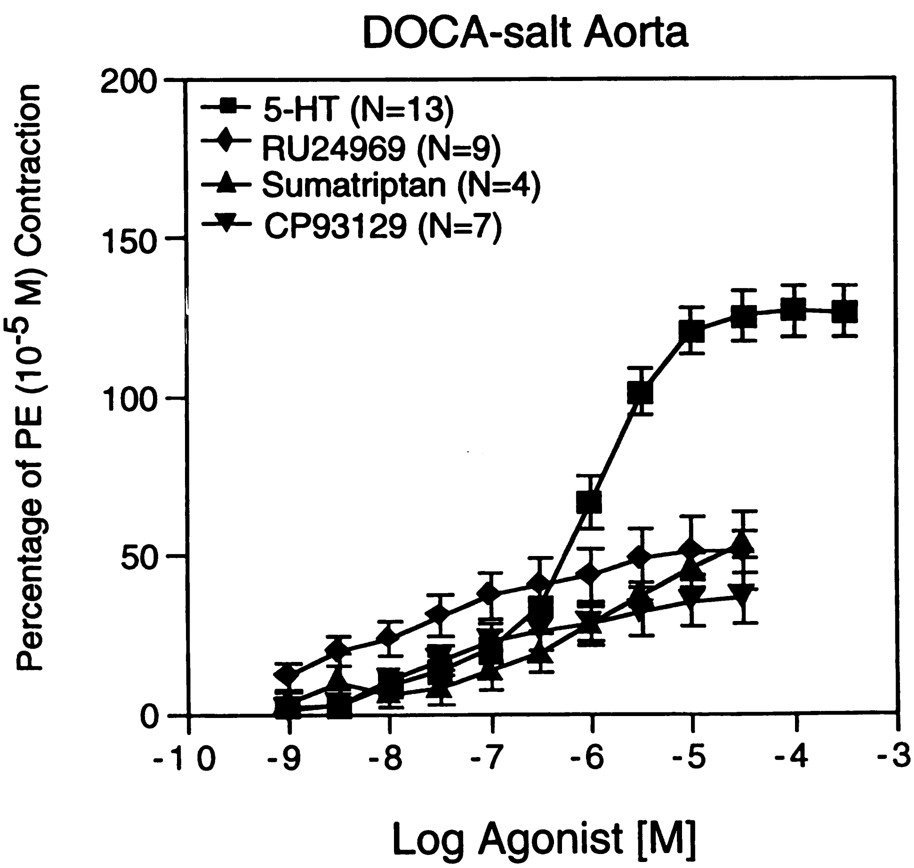

DOCA-salt Aorta
5-HT (N=13)
RU24969 (N=9)
Sumatriptan (N=4)
CP93129 (N=7)
Percentage of PE (10-5 M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log Agonist [M]

Figure 11. Top: Effect of the 5-HT_{1B} receptor antagonist GR55562 (100 nM) on 5-HT-induced contraction in the endothelium-denuded thoracic aorta from normotensive sham (left) and hypertensive DOCA-salt rats (right). Bottom: Effect of 5-HT_{1B} receptor antagonist GR55562 (100 nM) on 5-HT-induced contraction in endothelium-denuded superior mesenteric artery from normotensive sham (left) and hypertensive DOCA-salt rats (right). Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the vehicle-incubated and GR55562-incubated responses.

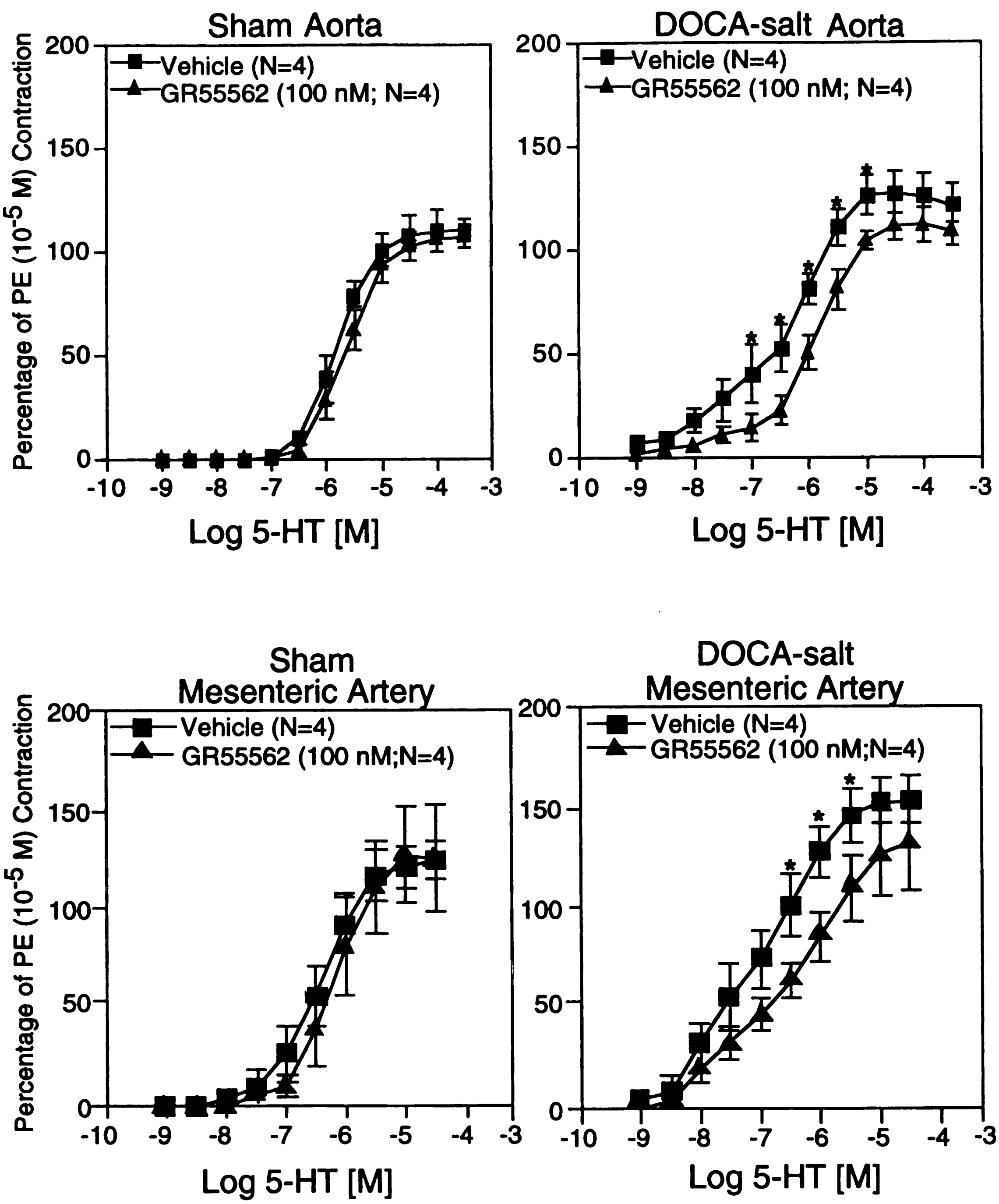
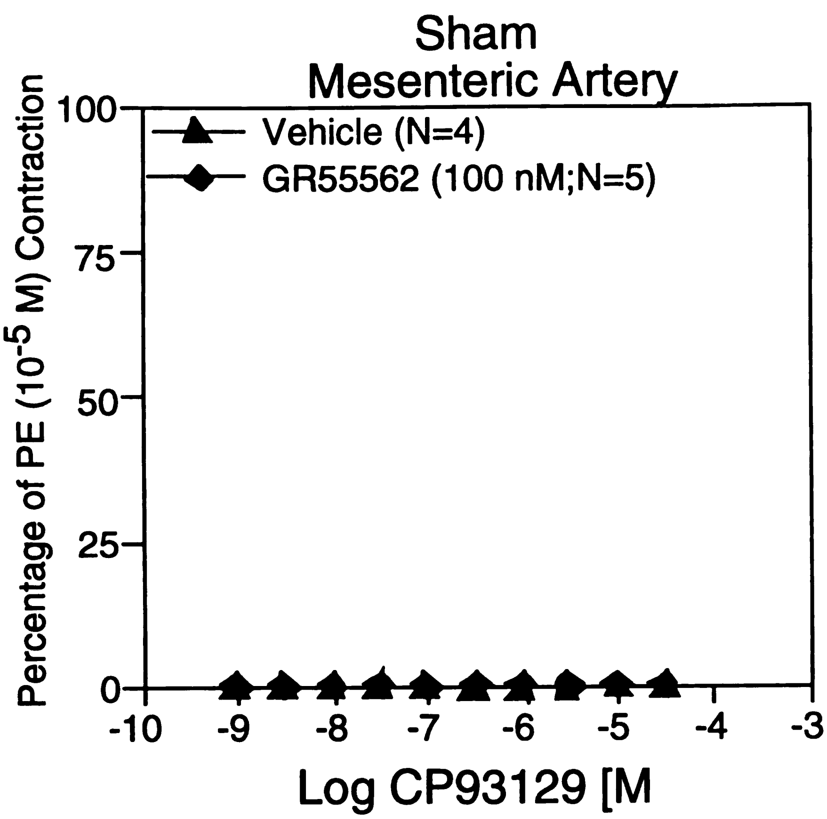
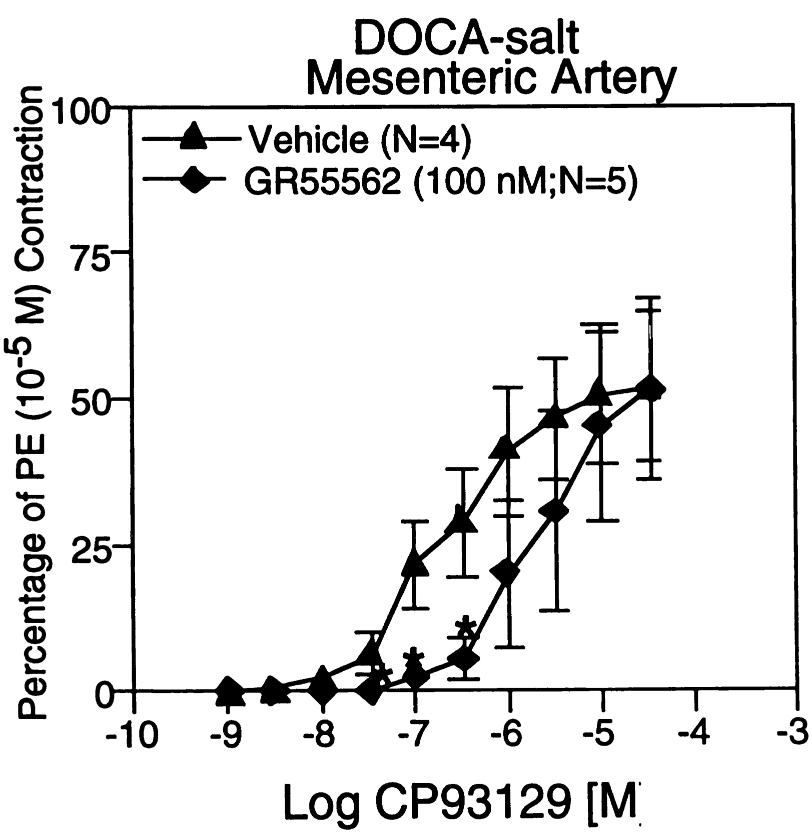

Sham Aorta
Vehicle (N=4)
GR55562 (100 nM; N=4)
Percentage of PE (10-5 M) Contraction
Log 5-HT [M]
DOCA-salt Aorta
Vehicle (N=4)
GR55562 (100 nM; N=4)
Log 5-HT [M]
Sham
Mesenteric Artery
Vehicle (N=4)
GR55562 (100 nM;N=4)
Percentage of PE (10-5 M) Contraction
Log 5-HT [M]
DOCA-salt
Mesenteric Artery
Vehicle (N=4)
GR55562 (100 nM;N=4)
Log 5-HT [M]

Figure 12. Top: Effect of the 5-HT_{1B} receptor antagonist GR55562 (100 nM) on the 5-HT_{1B} receptor agonist CP93129-induced contraction in endothelium-denuded superior mesenteric artery from normotensive sham rats. Bottom: Effect of the 5-HT_{1B} receptor antagonist GR55562 (100 nM) on the 5-HT_{1B} receptor agonist CP93129-induced contraction in endothelium-denuded superior mesenteric artery from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the vehicle-incubated and GR55562-incubated responses.

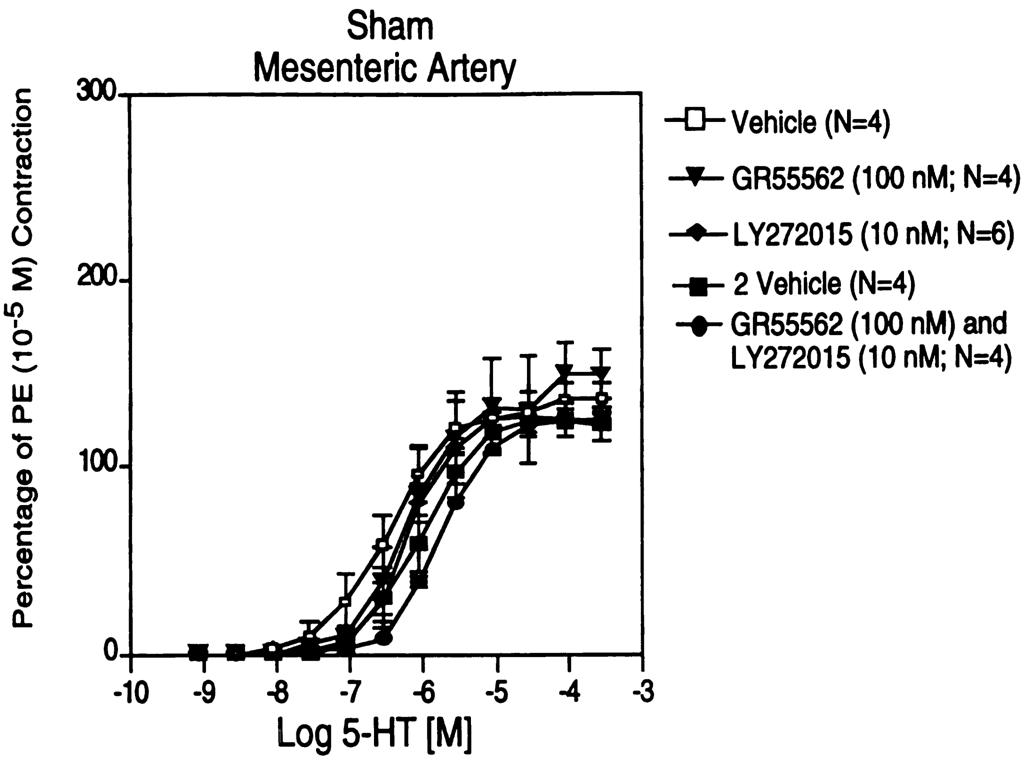


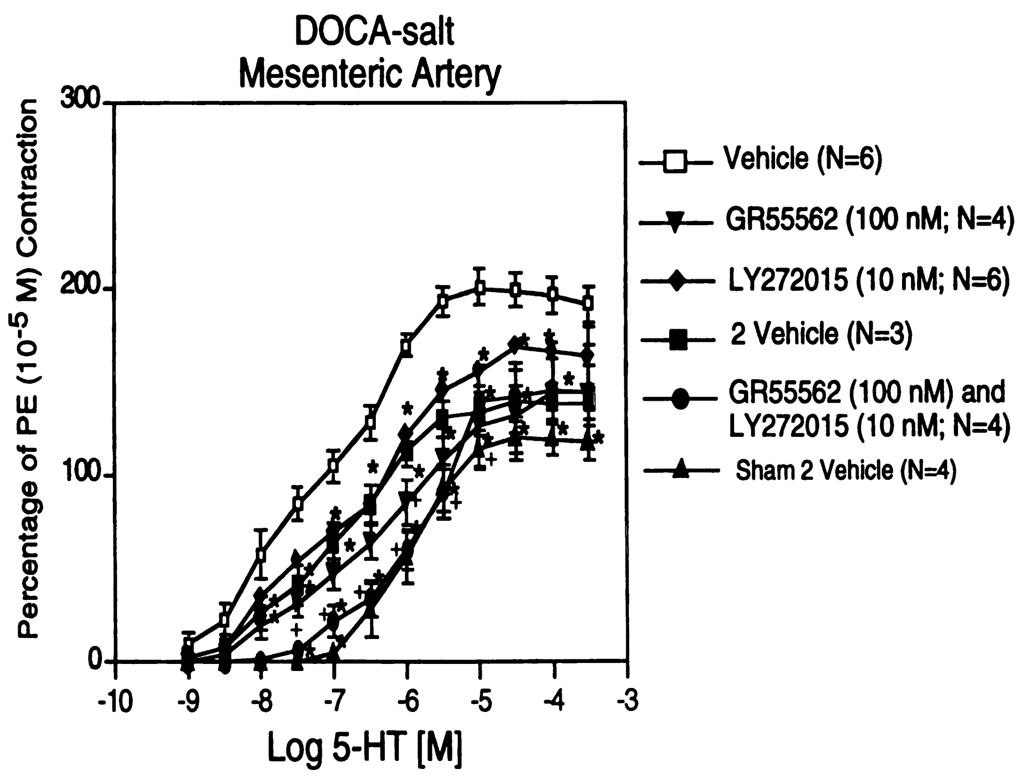
Sham
Mesenteric Artery
Percentage of PE (10^{-5} M) Contraction
100
75
50
25
0
Vehicle (N=4)
GR55562 (100 nM;N=5)
-10
-9
-8
-7
-6
-5
-4
-3
Log CP93129 [M



DOCA-salt
Mesenteric Artery
Percentage of PE (10^{-5} M) Contraction
100
75
50
25
0
Vehicle (N=4)
GR55562 (100 nM;N=5)
*
*
*
-10
-9
-8
-7
-6
-5
-4
-3
Log CP93129 [M

Figure 13. Top: Effect of the 5-HT_{1B} receptor antagonist GR55562 (100 nM) and the 5-HT_{2B} receptor antagonist LY272015 (10 nM) on 5-HT-induced contraction in endothelium-denuded superior mesenteric artery from normotensive sham rats. Bottom: Effect of the 5-HT_{1B} receptor antagonist GR55562 (100 nM) and 5-HT_{2B} receptor antagonist LY272015 (10 nM) on the 5-HT-induced contraction in endothelium-denuded superior mesenteric artery from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant different ($p<0.05$) from the DOCA-salt vehicle-incubated response. + represents statistically significant difference ($p<0.05$) from the combined vehicle (2 Vehicle) response.


Sham
Mesenteric Artery
Percentage of PE (10^{-5} M) Contraction
300
200
100
0
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]
Vehicle (N=4)
GR55562 (100 nM; N=4)
LY272015 (10 nM; N=6)
2 Vehicle (N=4)
GR55562 (100 nM) and
LY272015 (10 nM; N=4)


DOCA-salt
Mesenteric Artery
Percentage of PE (10^{-5} M) Contraction
300
200
100
0
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]
Vehicle (N=6)
GR55562 (100 nM; N=4)
LY272015 (10 nM; N=6)
2 Vehicle (N=3)
GR55562 (100 nM) and
LY272015 (10 nM; N=4)
Sham 2 Vehicle (N=4)

least partially, the 5-HT-induced contraction in both the thoracic aorta and superior mesenteric artery of DOCA-salt hypertensive rats.

Unmasking "Silent Receptors"

It has been reported in some forms of hypertension that vascular smooth muscle exhibits a slightly depolarized resting membrane potential (Kwan and Grover, 1983). This may be due to defects in the handling of calcium or a loss of potassium channel function (Pamnami *et al*, 1985, Hermsmeyer, 1993, Kwan, 1985, Borges *et al*, 1999, Yuan *et al*, 1998). It has also been reported that the 5-HT_{1B} receptor may require a depolarizing stimulus in order to become activated (Craig and Martin, 1993, Movahedi and Purdy, 1997). There is almost unanimous agreement among investigators that in order to be enabled, the 5-HT_{1B} receptor must be activated following a stimulus which either activates or increases the probability of opening of L-type calcium channels. This 5-HT_{1B}-mediated contraction is dependent on the influx of external calcium (Hill *et al*, 2000). These "silent receptors" have been found in several different arteries of normotensive subjects including human coronary artery, human pulmonary arteries, rabbit ear artery and tail artery of the rat (Nilsson *et al*, 1999, Morecroft *et al*, 1999, Movahedi and Purdy, 1997, Craig and Martin, 1993).

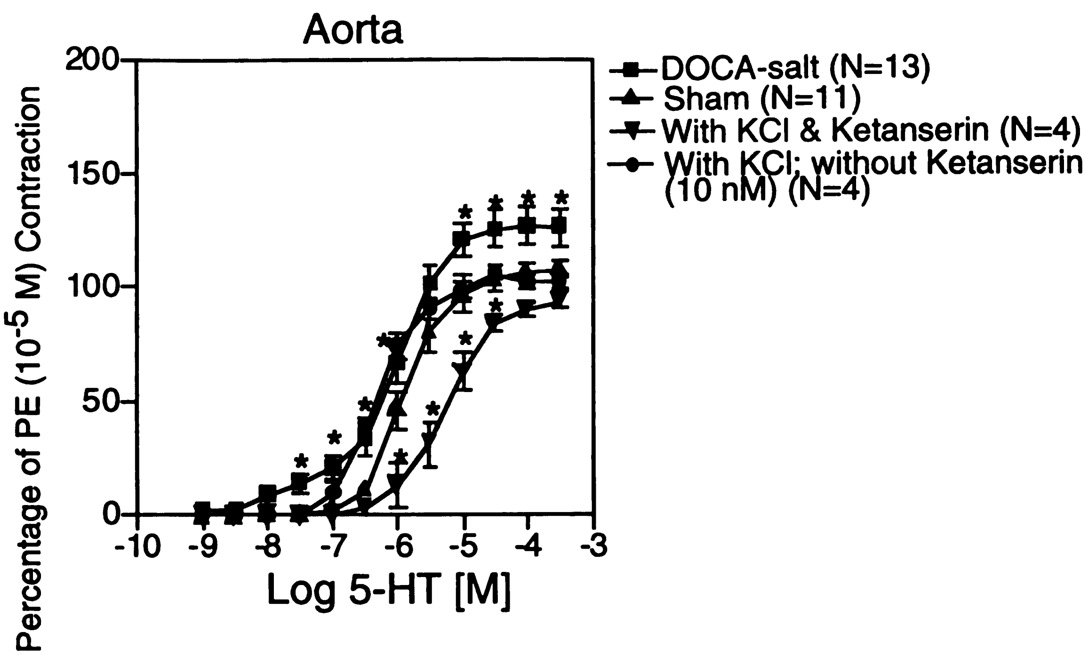
In order to determine if the 5-HT_{1B} receptors in the rat thoracic aorta and mesenteric artery requires a depolarizing stimulus, we performed isolated tissue bath experiments on normal Sprague-Dawley rat thoracic aorta in the presence of KCl (15 mM) and the 5-HT_{2A} receptor antagonist ketanserin (10 nM). KCl was chosen as a primary stimulus due to the fact that it activates L-type calcium channels and thereby acts as the depolarizing stimulus; this is similar to the published experimental protocols investigating "silent receptors". The 5-$HT_{2A/2C}$ receptor antagonist ketanserin (10 nM) was used to inhibit the contribution of the 5-HT_{2A} receptor to the 5-HT-induced contraction. For comparison values the responses of arteries from hypertensive DOCA-salt rats and sham rats with normal blood pressures have been included. These data demonstrate that acute depolarization alone was not sufficient to result in an increase in the maximal contraction to 5-HT in the aorta or mesenteric artery from the normal Sprague-Dawley rats (figure 14, top, figure 15, bottom). The observed leftward shift in potency (-log EC_{50} value [M] 6.31 $\pm$ 0.05 and 5.87 $\pm$ 0.05, with KCl and without KCl, respectively in aorta) (-log EC_{50} value [M] 6.55 $\pm$ 0.08 and 5.91 $\pm$ 0.03, with KCl and without KCl, respectively in mesenteric artery) may be due to activation of "silent" 5-HT_{2A} receptors. "Silent" 5-HT_{2A} receptor have been previously noted in the rabbit ear artery (Smith et al, 1999).

This notion is support by the data obtained in the presence of ketanserin. In the presence of KCl and ketanserin the 5-HT-induced contraction is rightward shifted (-log EC_{50} value [M] 6.55 $\pm$ 0.08 and 5.24 $\pm$ 0.03, with KCl and without ketanserin and with KCl and with ketanserin, respectively in mesenteric artery) (figure 15, top). This response is very similar to that seen in the thoracic aorta where the enhanced contraction to 5-HT is also sensitive to inhibition by ketanserin (-log EC_{50} value [M] 6.31 $\pm$ 0.05 and 5.24 $\pm$ 0.03, with KCl and without ketanserin and with KCl and with ketanserin, respectively) (figure 14, top). Ketanserin's ability to inhibit the 5-HT-induced contraction suggests that this is a 5-HT_{2A} mediated response. Additionally, the 5-HT-induced response in arteries from hypertensive DOCA-salt rats is insensitive to inhibition by ketanserin (30 nM) (figure 15, bottom). The lack of inhibition by ketanserin indicates that the 5-HT-induced contraction is not mediated by the 5-HT_{2A} receptor. Therefore, even in the presence of a depolarizing stimulus, the arteries from rats with normal blood pressure do not mimic the response seen in arteries taken from hypertensive DOCA-salt rats (figure 14, top, figure 16, top).

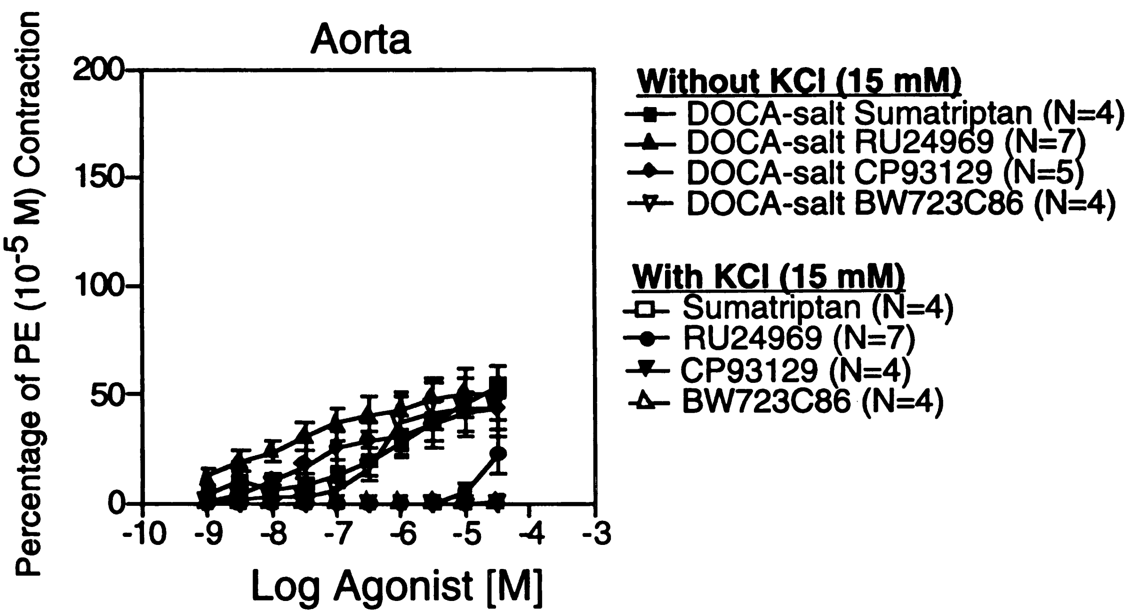
To determine if this enhanced response was selective for 5-HT we repeated these studies in the mesenteric artery with the α_1 adrenergic receptor agonist phenylephrine. This appears to be a selective enhancement of the 5-HT response as there was no change in the phenylephrine-induced contraction in the presence of the KCl (15 mM) (figure 16, bottom). The concentration of ketanserin is also selective for the 5-HT_{2A} receptor, not the α_1 receptor, at the concentration used as the phenylephrine-induced contraction was unaffected in the presence of ketanserin (figure 16, bottom).

Additional acute depolarization data was obtained by performing contractile studies using the 5-HT_{1B} agonists RU24969, CP93129 and sumatriptan as well as the 5-HT_{2B} agonist BW723C86. Even in the presence of the depolarizing stimulus, the 5-HT_{2B} receptor agonist BW723C86 and the 5-HT_{1B} receptor agonist sumatriptan did not elicit a contraction in the thoracic aorta from a Sprague-Dawley rat with normal blood pressure (figure 14, bottom). The 5-HT_{1B} agonist RU24969 only caused a very small contraction at the very highest concentrations (figure 14, bottom). CP93129 also did not cause a contraction in the mesenteric artery in the presence of the KCl (15 mM) (figure 16, top). These additional data do not support the role of "silent 5-HT_{1B} receptors" in the rat thoracic aorta or mesenteric artery.

Figure 14.Top: Effect of KCl (15 mM) depolarization on 5-HT-induced contraction in the presence and absence of the 5-$HT_{2A/2C}$ receptor antagonist ketanserin (10 nM) in the thoracic aorta from normal Sprague-Dawley rats. Sham and DOCA-salt responses were obtained in the absence of KCl (15 mM). Bottom: Effect of KCl (15 mM) depolarization on the 5-HT_{2B} receptor agonist BW723C86-induced response and the 5-HT_{1B} receptor agonists sumatriptan, CP93129 and RU24969-induced responses in the thoracic aorta from normal Sprague-Dawley rats. Responses obtained the thoracic aorta from hypertensive DOCA-salt rats were obtained in the absence of KCl. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham aorta. Abbreviations: w/= with; w/o= without.

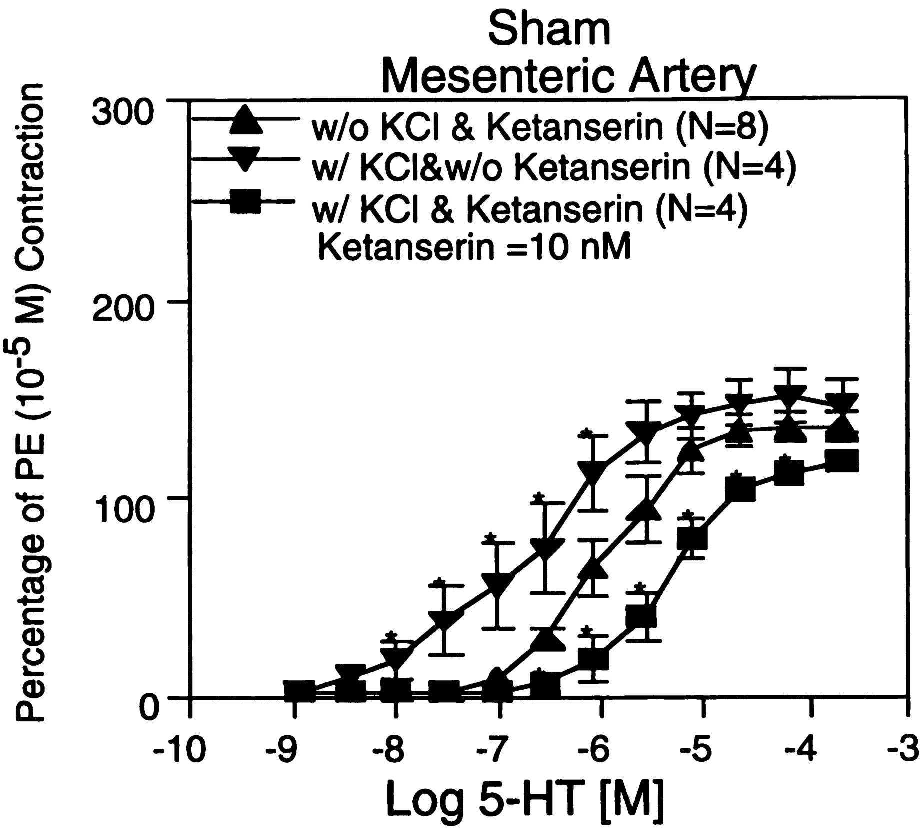


Aorta
Percentage of PE (10^-5 M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]
DOCA-salt (N=13)
Sham (N=11)
With KCl & Ketanserin (N=4)
With KCl; without Ketanserin (10 nM) (N=4)



Aorta
Percentage of PE (10^-5 M) Contraction
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Log Agonist [M]
Without KCl (15 mM)
DOCA-salt Sumatriptan (N=4)
DOCA-salt RU24969 (N=7)
DOCA-salt CP93129 (N=5)
DOCA-salt BW723C86 (N=4)
With KCl (15 mM)
Sumatriptan (N=4)
RU24969 (N=7)
CP93129 (N=4)
BW723C86 (N=4)

Figure 15. Top: Effect of KCl (15 mM) in the presence and absence of the $5\text{-HT}_{2A/2C}$ receptor antagonist ketanserin (10 nM) on the 5-HT-induced contraction in mesenteric artery from rats with normal blood pressure. Bottom: Effect of the $5\text{-HT}_{2A/2C}$ receptor antagonist ketanserin (30 nM) on the 5-HT-induced contraction in the endothelium-denuded superior mesenteric artery from hypertensive DOCA-salt rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery. Abbreviations: w= with; w/o= without.


Sham
Mesenteric Artery
Percentage of PE (10-5 M) Contraction
300
200
100
0
w/o KCl & Ketanserin (N=8)
w/ KCl&w/o Ketanserin (N=4)
w/ KCl & Ketanserin (N=4)
Ketanserin =10 nM
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]

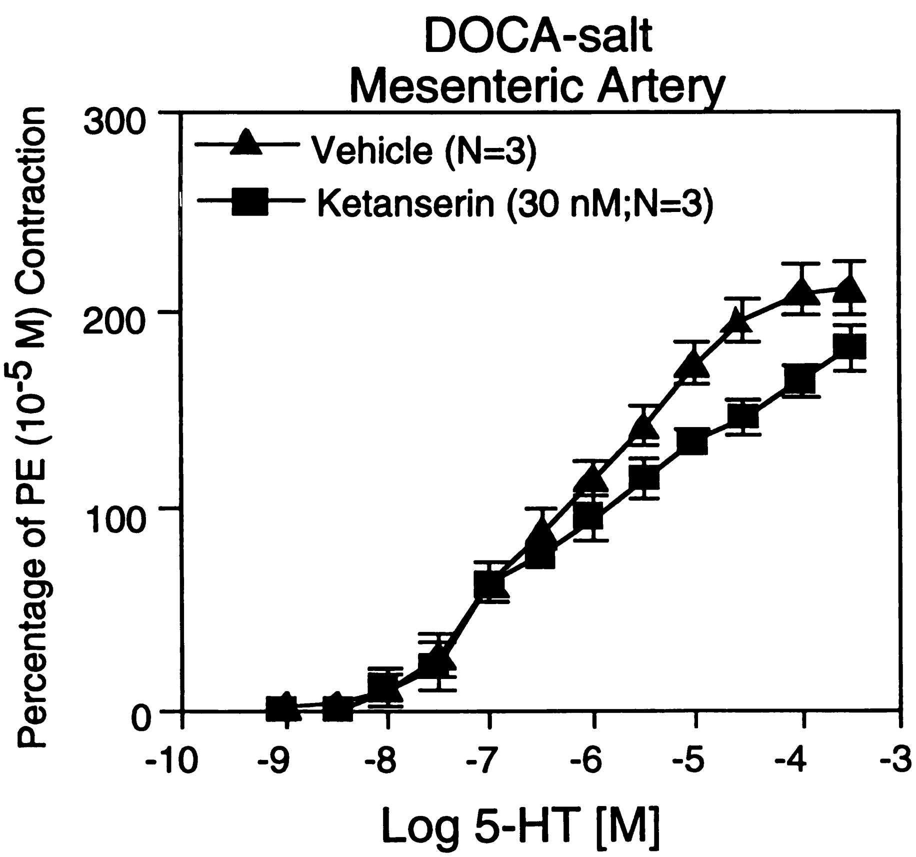
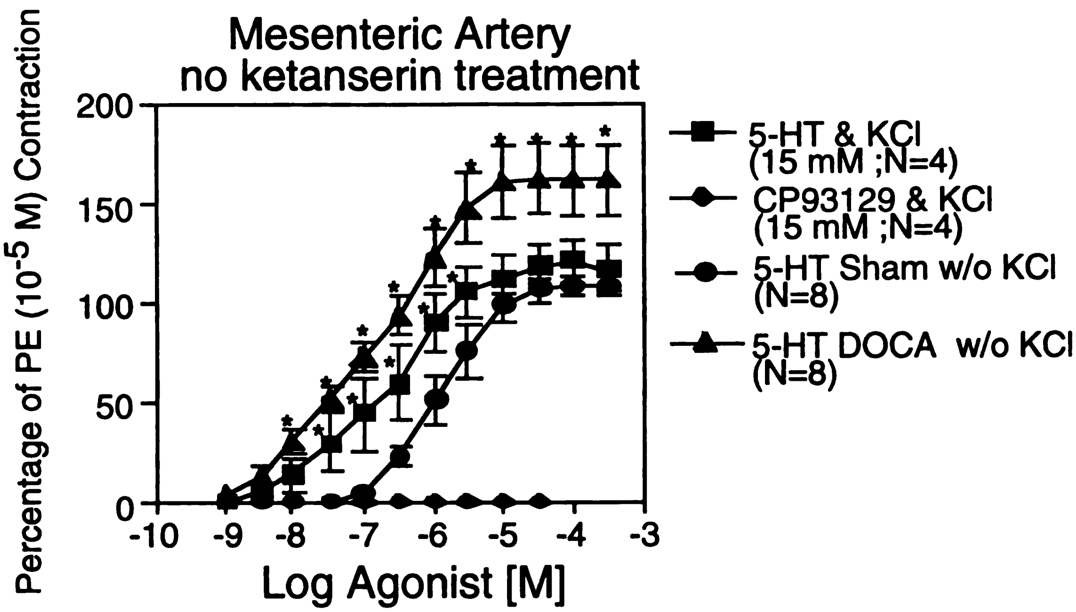
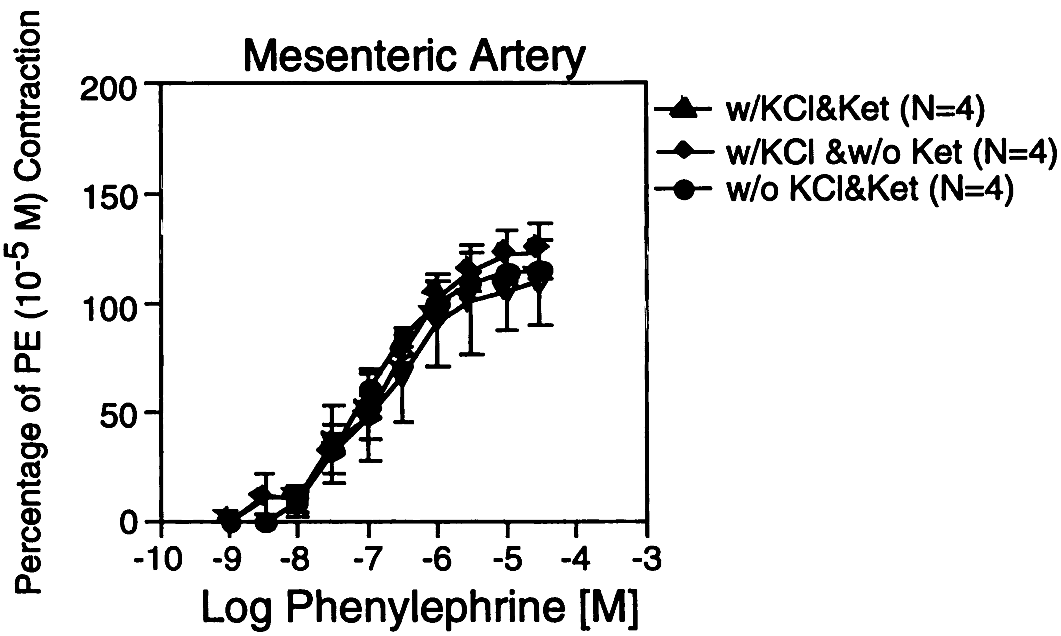

DOCA-salt
Mesenteric Artery
Percentage of PE (10-5 M) Contraction
300
200
100
0
Vehicle (N=3)
Ketanserin (30 nM;N=3)
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]

Figure 16. Top: Effect KCl (15 mM) on the 5-HT_{1B} receptor agonist CP93129- and 5-HT-induced contraction in the endothelium-denuded mesenteric artery from rats with normal blood pressure. Responses obtained the thoracic aorta from hypertensive DOCA-salt rats were obtained in the absence of KCl. Bottom: Effect of KCl (15 mM) depolarization on the α_1 adrenergic receptor agonist phenylephrine-induced contraction in the presence and absence of the 5-$HT_{2A/2C}$ receptor antagonist ketanserin (10 nM) in the mesenteric artery of normal Sprague-Dawley rats. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery. Abbreviations: w/= with; w/o= without.


Mesenteric Artery
no ketanserin treatment
Percentage of PE (10^-5 M) Contraction
200
150
100
50
0
-10 -9 -8 -7 -6 -5 -4 -3
Log Agonist [M]
5-HT & KCl (15 mM ;N=4)
CP93129 & KCl (15 mM ;N=4)
5-HT Sham w/o KCl (N=8)
5-HT DOCA w/o KCl (N=8)


Mesenteric Artery
Percentage of PE (10^-5 M) Contraction
200
150
100
50
0
-10 -9 -8 -7 -6 -5 -4 -3
Log Phenylephrine [M]
w/KCl&Ket (N=4)
w/KCl &w/o Ket (N=4)
w/o KCl&Ket (N=4)

Hypothesis II

Receptor Protein Levels

All of the pharmacological data from functional studies implicates the 5-HT_{2B} and 5-HT_{1B} receptors in the hyperresponsiveness to 5-HT observed in arteries from hypertensive DOCA-salt rats. These experiments do not address the mechanism by which the hyperresponsiveness to 5-HT occurs. Therefore, we proposed the hypothesis that the 5-HT_{2B} and 5-HT_{1B} receptor protein levels were increased under the conditions of DOCA-salt hypertension. The increased levels of receptor proteins would, at least partially, explain why there was no contraction to 5-HT_{2B} receptor agonist and 5-HT_{1B} receptor agonists under conditions of normal blood pressure but why there was contraction to these agonists under conditions of hypertension. We speculated that under conditions of normal blood pressure there were not enough 5-HT_{2B} and 5-HT_{1B} receptors to couple efficiently with either the G-proteins and/or effector molecules to mediate 5-HT-induced contraction.

To address this hypothesis we utilized Western analysis and antibodies to 5-HT_{1B} and 5-HT_{2B} receptors to determine if the change in contractility to 5-HT_{1B} receptor and 5-HT_{2B} receptor agonists was due to an increase in the levels of the receptor protein. 5-HT_{1B} and 5-HT_{2B} receptor proteins were both increased approximately 2-fold in aorta from Sprague-Dawley DOCA-salt hypertensive rats as compared to controls (figure 17). These data are similar to those obtained from the mesenteric artery where 5-HT_{1B} and 5-HT_{2B} receptor proteins were both increased approximately 2-fold in the mesenteric artery from Sprague-Dawley DOCA-salt hypertensive rats as compared to controls (figure 18). These data suggest that an increase in the level of receptor protein may be at least partially responsible for the changes observed to 5-HT_{1B} receptor and 5-HT_{2B} receptor agonists.

Interestingly, when the mRNA for the 5-HT_{2B} receptor in the thoracic aorta from normotensive sham and hypertensive DOCA-salt was examined by Real Time PCR there was no difference in the levels of mRNA (figure 19, bottom). The C_T values were 23.82 $\pm$ 0.35 for the sham and 24.02 $\pm$ 0.35 for the DOCA-salt (ΔC_T values of 6.93 $\pm$ 0.65 and 7.71 $\pm$ 0.62, sham and DOCA-salt, respectively). The mRNA for the 5-HT_{1B} receptor in the thoracic aorta from sham rats and hypertensive DOCA-salt rats also showed no difference in levels of mRNA (figure 19, top). The C_T values were 24.53 $\pm$ 0.40 for the sham and 23.39 $\pm$ 0.27 for the DOCA-salt (ΔCT values of 7.01 $\pm$ 0.31 and 6.92 $\pm$ 0.76, sham and DOCA-salt, respectively). The C_T values for GAPDH were similar for the sham and DOCA-salt (C_T values 16.89 $\pm$ 0.62 and 16.311 $\pm$ 0.40, sham and DOCA-salt, respectively). These data, while demonstrating an increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor proteins, suggest that the increase is not due to an increase in transcription. This would suggest post-transcriptional or translational modifications and/or a decrease in the degradation of these receptors. However, as we did not perform any RNAse protection assays and did not investigate the stability and rate of degradation of the 5-HT_{1B} and 5-HT_{2B} receptor mRNA we can not conclusively rule out a transcriptional regulation of these receptors.

Figure 17. Top: Measurement of 5-HT_{1B} receptor protein levels in thoracic aorta from normotensive sham and hypertensive DOCA-salt rats. Bottom: Measurement of 5-HT_{2B} receptor protein levels in thoracic aorta from normotensive sham and hypertensive DOCA-salt rats. Units are reported as arbitrary densitometry units. Blots are representative of four experiments. Total protein loaded in each lane was 25 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham aorta

Thoracic Aorta

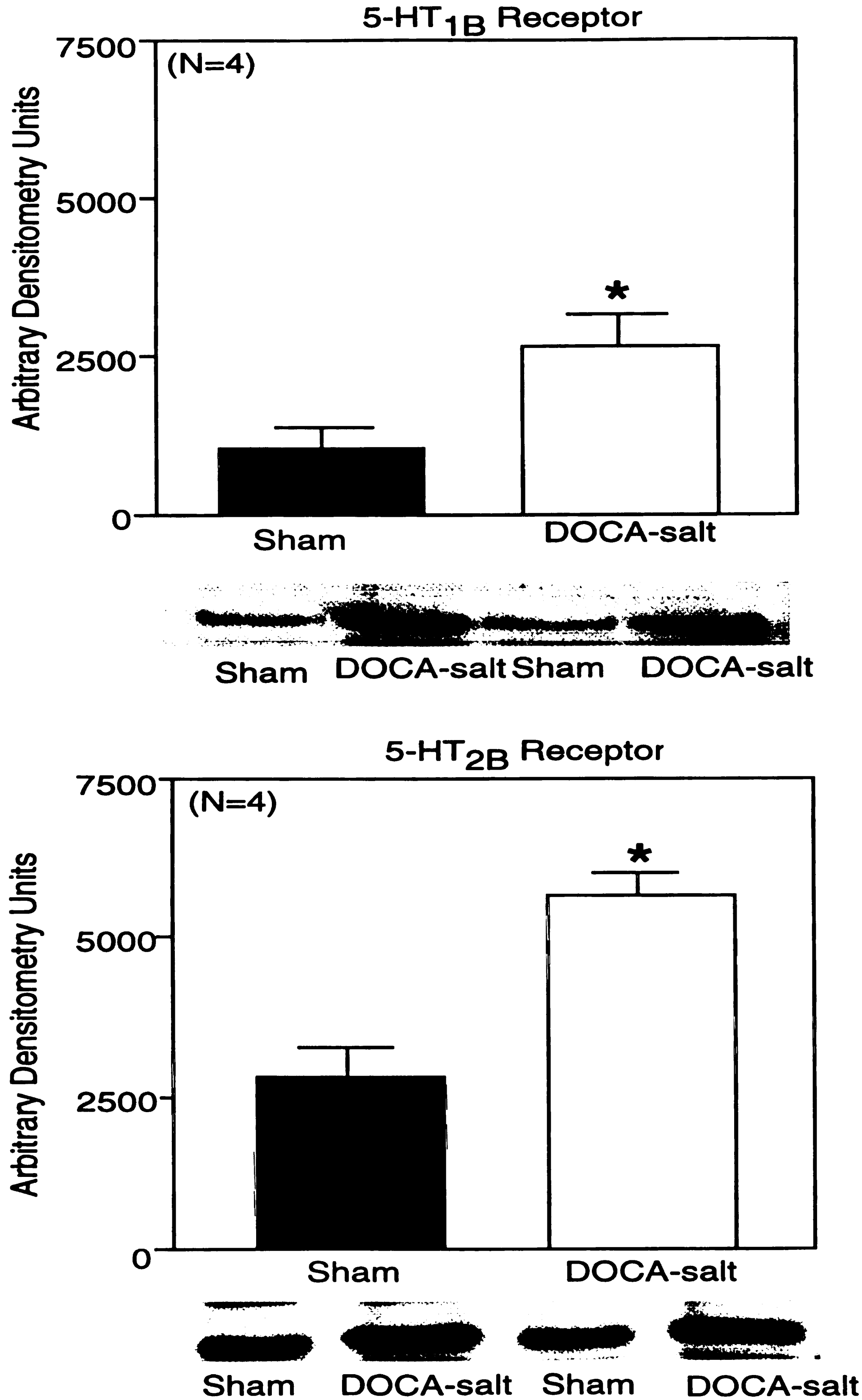
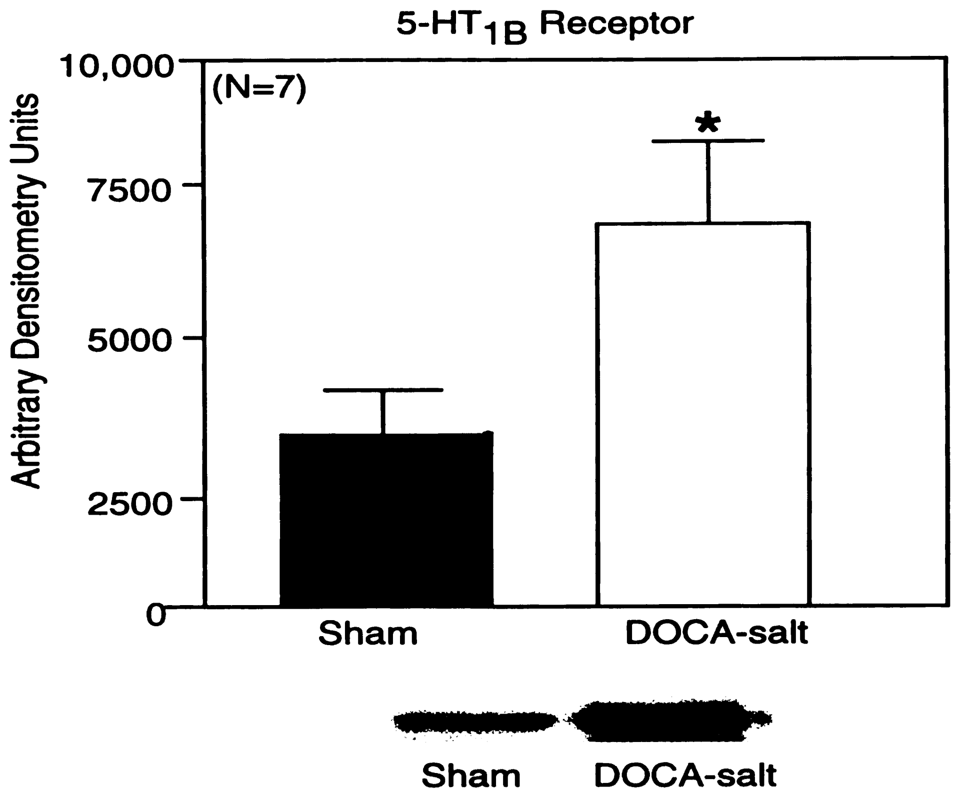


Figure 18. Top: Measurement of 5-HT_{1B} receptor protein levels in the superior mesenteric artery from normotensive sham and hypertensive DOCA-salt rats. Bottom: Measurement of 5-HT_{2B} receptor protein levels in the superior mesenteric artery from normotensive sham and hypertensive DOCA-salt rats. Units are reported as arbitrary densitometry units. Blots are representative of four experiments. Total protein loaded in each lane was 25 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery

Mesenteric Artery



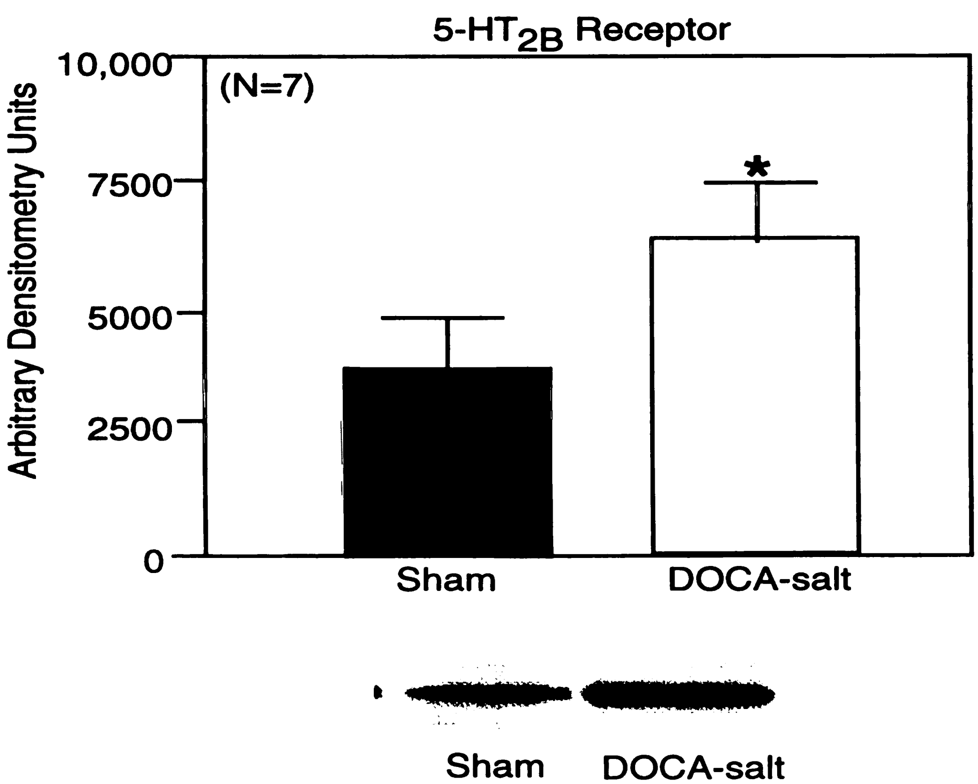
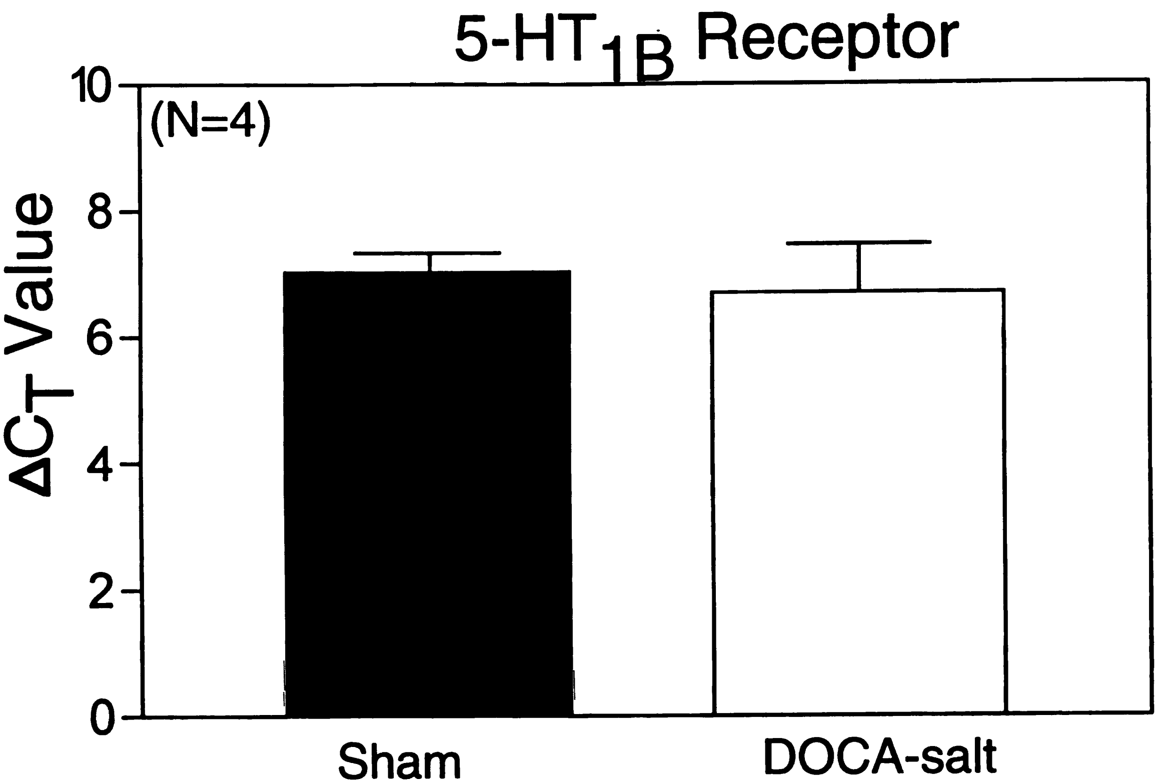
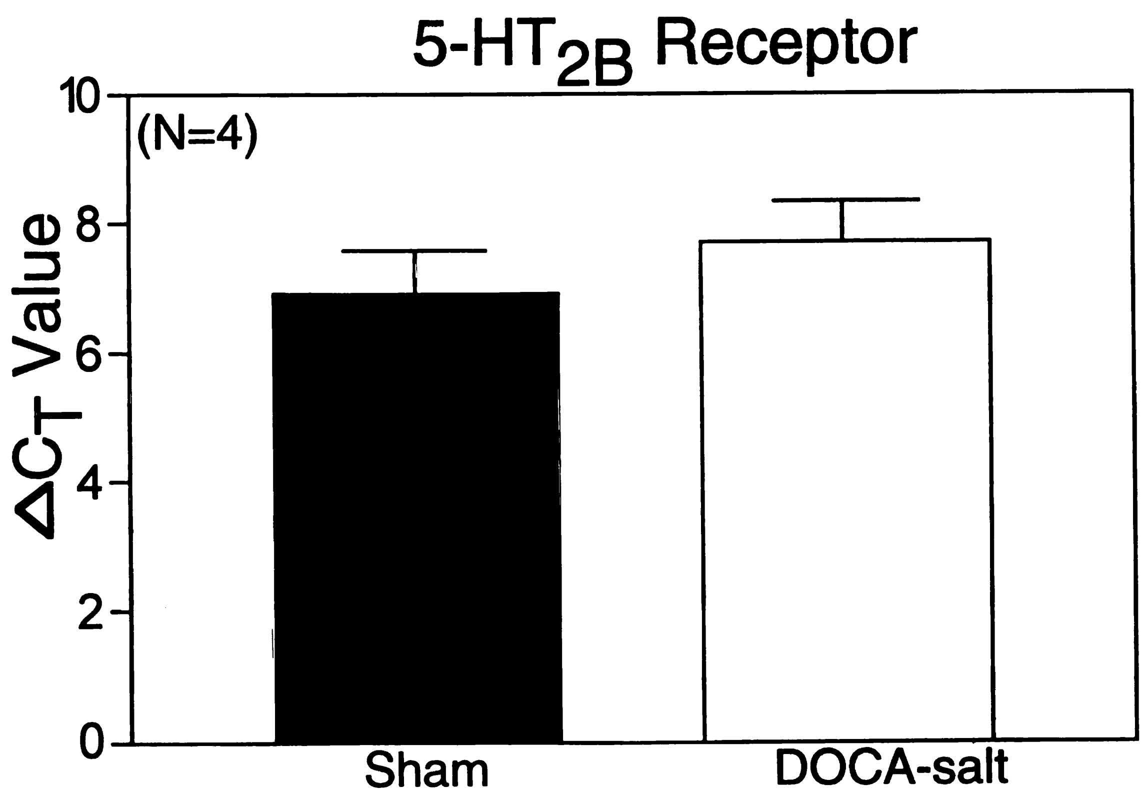


Figure 19. Top: Real Time PCR measurement of 5-HT_{1B} receptor mRNA in the thoracic aorta from normotensive sham and hypertensive DOCA-salt rats 28 days after initial surgery. Bottom: Real Time PCR measurement of 5-HT_{2B} receptor mRNA in the thoracic aorta from normotensive sham and hypertensive DOCA-salt rats. Units are reported as ΔC_T values, where C_T values for 5-HT_{1B} and 5-HT_{2B} receptor mRNA are corrected for GAPDH mRNA expression. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses.


5-HT1B Receptor
(N=4)
ΔCT Value
10
8
6
4
2
0
Sham
DOCA-salt


5-HT2B Receptor
(N=4)
ΔCT Value
10
8
6
4
2
0
Sham
DOCA-salt

Hypothesis III

Mechanisms of Receptor Regulation

There is little information currently available about the regulation of the 5-HT $_{2B}$ and 5-HT$_{1B}$ receptors. Previous studies in our laboratory with Wistar and Wistar-Furth rats (Watts and Harris, 1999) have suggested a role for both DOCA treatment and increased vascular pressure as variables which may regulate the expression of the 5-HT $_{2B}$ receptor. However, separation of these two factors is difficult to achieve *in vivo*. Due to the commonality of mineralocorticoids in the model of DOCA-salt hypertension and Wistar-Furth rats treated with DOCA and salt, we speculated that mineralocorticoids would have the ability to upregulate 5-HT$_{2B}$ and 5-HT$_{1B}$ receptors. Previous studies have demonstrated aldosterone's ability to increase expression of the Na-K-ATPase in vascular smooth muscle cells (Oguchi *et al*, 1993). Investigation of the promoters for the rat genes for the 5-HT$_{1B}$ receptor and the 5-HT$_{2B}$ receptor revealed that they contain mineralocorticoid response elements (MRE)(Foguet *et al*, 1993, Hamblin *et al*, 1992). Therefore, we tested the hypothesis that aldosterone-incubation would cause an increase in the levels of 5-HT$_{1B}$ receptor and 5-HT$_{2B}$ receptor proteins, independent of an increase in blood pressure.

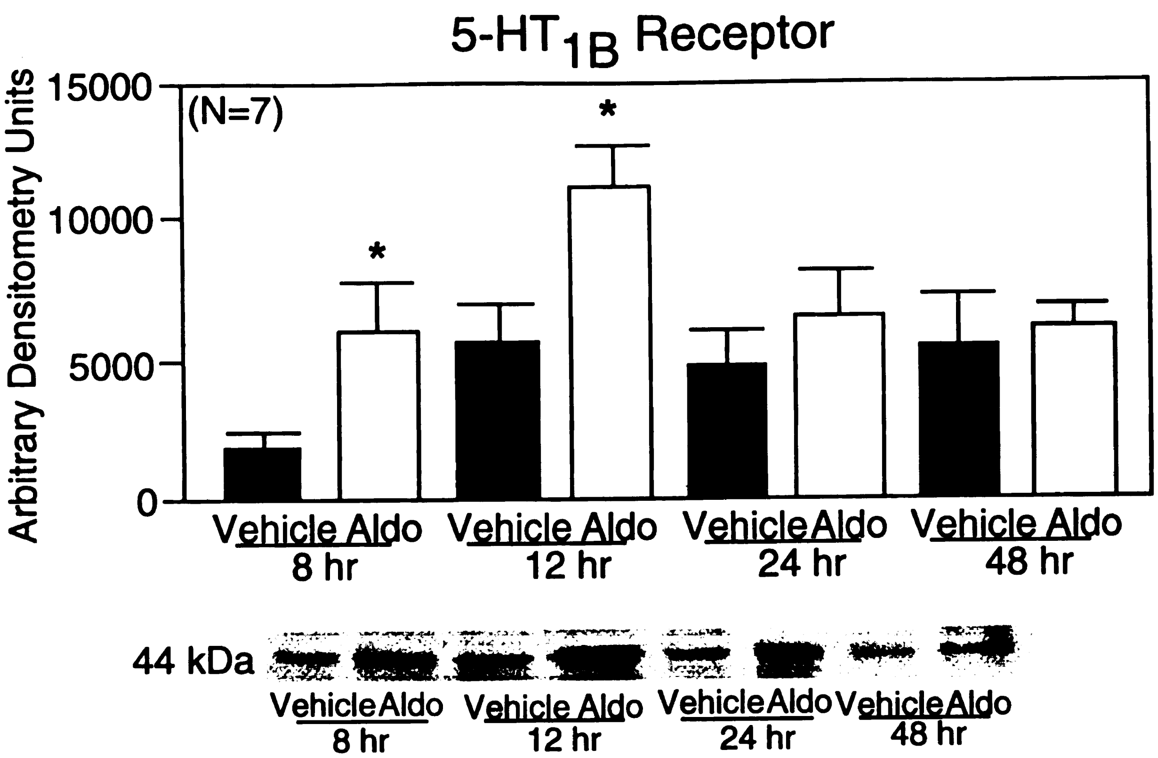
Aldosterone-incubation Time Course Studies

To test the effects of mineralocorticoid treatment in the absence of an increase in blood pressure, we performed aortic incubation studies using aldosterone. Endothelium-denuded thoracic aorta from male Sprague-Dawley rats with normal blood pressure was incubated under tissue culture conditions with aldosterone. Incubation with aldosterone (100 nM) for 8 and 12 hours resulted in a significant increase (approximately 2-fold above vehicle treated) in both 5-HT$_{1B}$ receptor (figure 20, top) and 5-HT$_{2B}$ receptor (figure 20, bottom) protein levels. Incubation for 24 and 48 hours with aldosterone (100 nM) did not result in a significant increase in either 5-HT$_{1B}$ or 5-HT$_{2B}$ receptor protein levels above vehicle. Unexpectedly, aldosterone-incubation at 24 and 48 hour time points did not result in an increase in 5-HT$_{1B}$ or 5-HT$_{2B}$ receptor proteins (figure 20, top and bottom). These data suggest that while aldosterone causes an acute increase in the level of the receptor proteins, a second cellular signal is necessary to maintain the increased levels of receptor proteins. A co-incubation pilot study with vasopressin and aldosterone (data not shown) demonstrated that treatment with vasopressin alone or in combination with aldosterone had no effect on 5-HT$_{2B}$ and 5-HT$_{1B}$ receptor protein levels. This suggests that vasopressin is not the maintenance signal for continued increase in the level of the 5-HT$_{2B}$ and 5-HT$_{1B}$ receptor proteins.

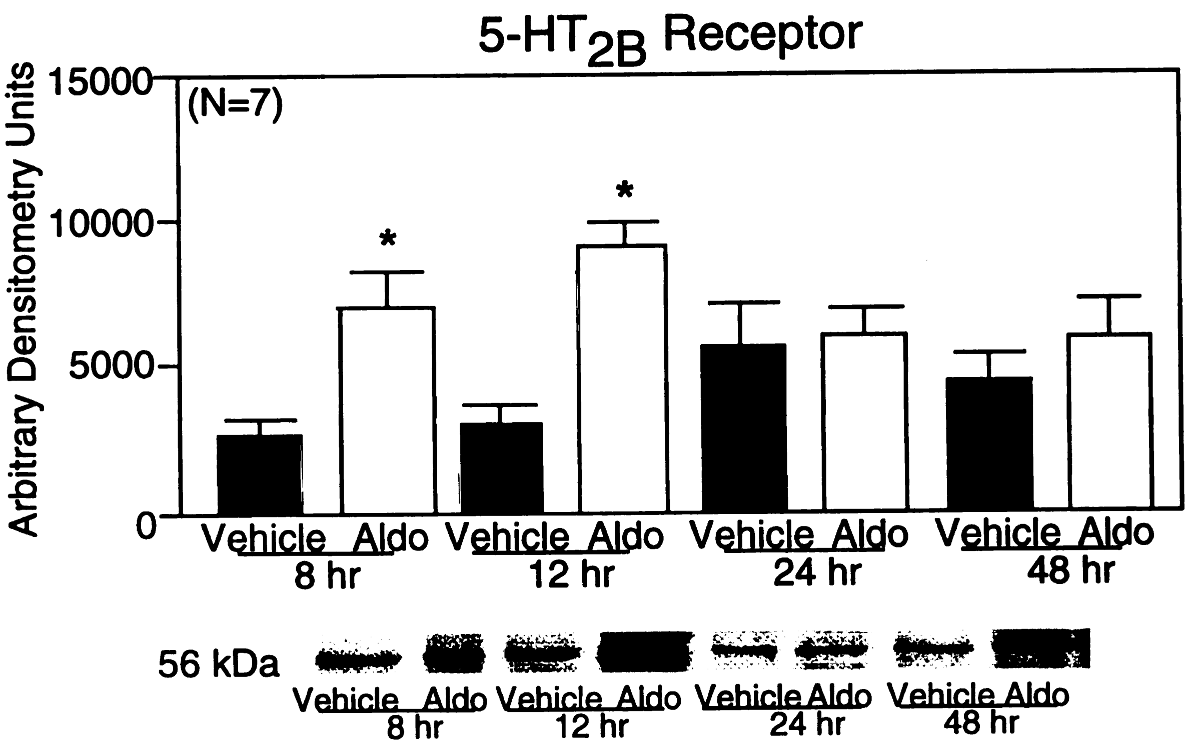
Aldosterone-incubation Concentration Response Curve Studies

Additionally, tissues were incubated for 12 hours with varying concentrations of aldosterone (1 nM – 100 nM). The concentrations of aldosterone which resulted in a statistically significant increase in 5-HT$_{1B}$ receptor protein levels above that of the vehicle were 30, 50 and 100 nM (figure 21, top). 5-HT$_{2B}$ receptor protein levels were statistically increased by the 10, 30, 50 and

Figure 20. Top: Measurement of 5-HT_{1B} receptor protein levels to determine the effects of Aldosterone (Aldo, 100 nM) incubation for variable lengths of time (8, 12, 24 and 48 hours) in thoracic aorta from Sprague-Dawley rats with normal blood pressure. Bottom: Measurement of 5-HT_{2B} receptor protein levels to determine the effects of Aldosterone (Aldo, 100 nM) incubation for variable lengths of time (8, 12, 24 and 48 hours) in thoracic aorta from Sprague-Dawley rats with normal blood pressure. Blots are representative of seven experiments. Units are reported as arbitrary densitometry units. Blots are representative of four experiments. Total protein loaded in each lane was 50 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the vehicle-incubated artery.

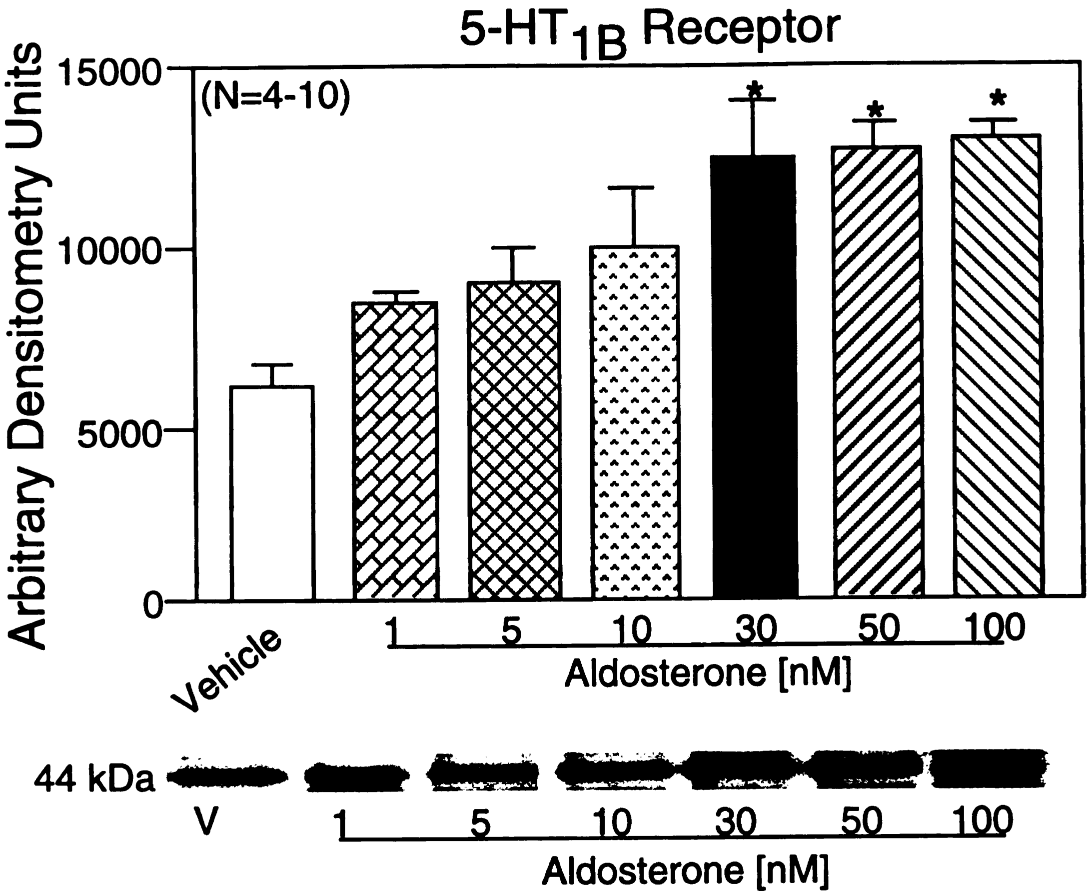


5-HT1B Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
(N=7)
*
*
Vehicle Aldo
8 hr
Vehicle Aldo
12 hr
Vehicle Aldo
24 hr
Vehicle Aldo
48 hr
44 kDa
Vehicle Aldo
8 hr
Vehicle Aldo
12 hr
Vehicle Aldo
24 hr
Vehicle Aldo
48 hr

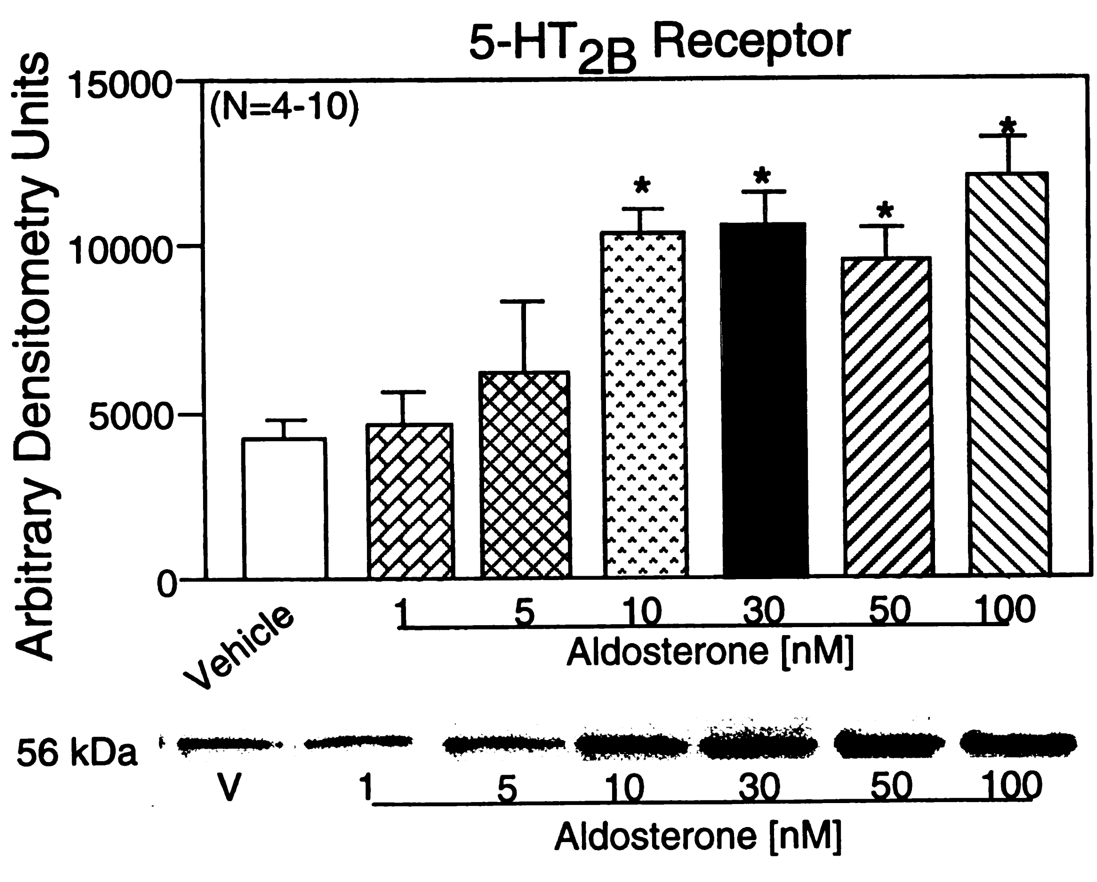


5-HT2B Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
(N=7)
*
*
Vehicle Aldo
8 hr
Vehicle Aldo
12 hr
Vehicle Aldo
24 hr
Vehicle Aldo
48 hr
56 kDa
Vehicle Aldo
8 hr
Vehicle Aldo
12 hr
Vehicle Aldo
24 hr
Vehicle Aldo
48 hr

Figure 21. Top: Measurement of 5-HT_{1B} receptor protein levels to determine the effects of Aldosterone (Aldo, 1 nM to 100 nM) incubation for 12 hours in thoracic aorta from Sprague-Dawley rats with normal blood pressure. Bottom: Measurement of 5-HT_{2B} receptor protein levels to determine the effects of Aldosterone (Aldo, 1 nM to 100 nM) incubation for 12 hours in thoracic aorta from Sprague-Dawley rats with normal blood pressure. V indicates vehicle treatment. Blots are representative of four to ten experiments. Units are reported as arbitrary densitometry units. Total protein loaded in each lane was 50 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the vehicle-incubated artery.



5-HT1B Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
(N=4-10)
Vehicle
1
5
10
30
50
100
Aldosterone [nM]
44 kDa
V
Aldosterone [nM]



5-HT2B Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
(N=4-10)
Vehicle
1
5
10
30
50
100
Aldosterone [nM]
56 kDa
V
Aldosterone [nM]

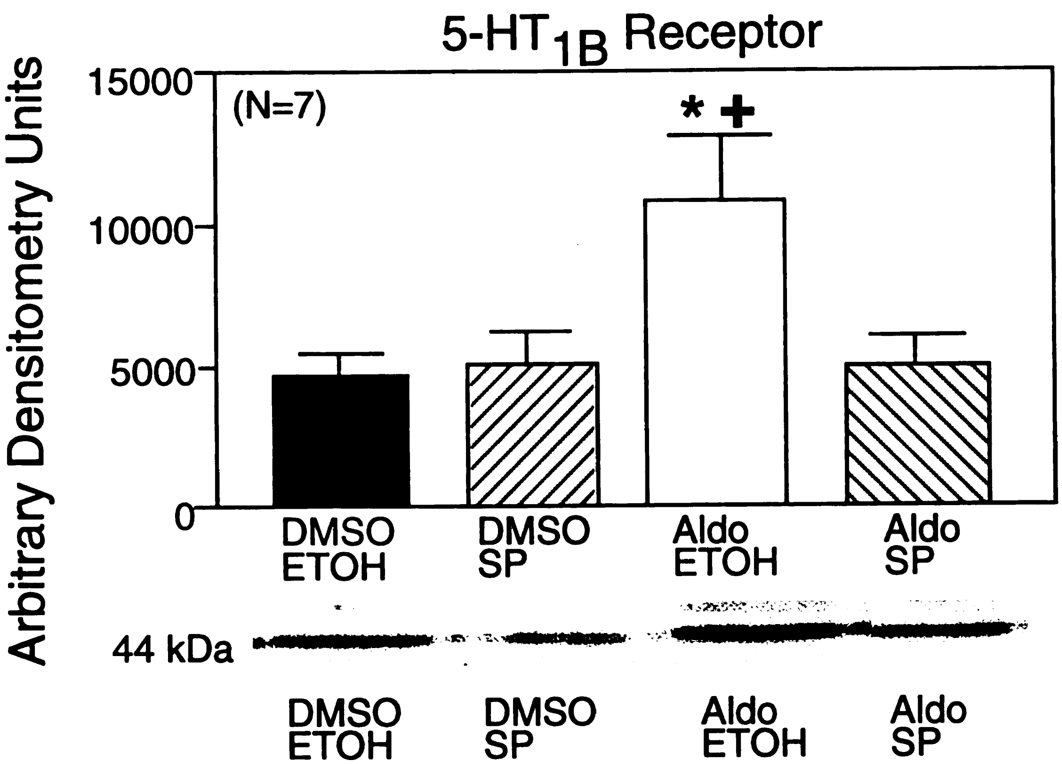
100 nM concentrations of aldosterone (figure 21, bottom).

Aldosterone- and DOCA-incubation Studies with Spironolactone

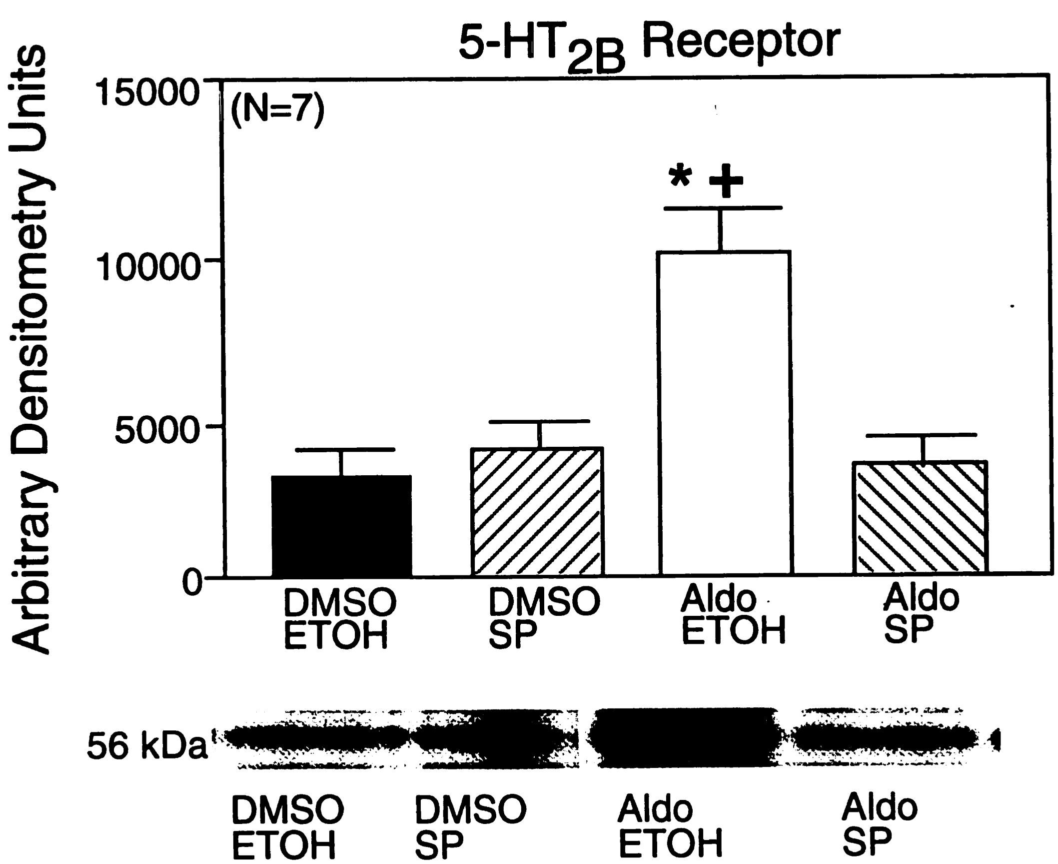
Furthermore, aldosterone-stimulated increase of 5-HT_{1B} and 5-HT_{2B} receptor protein levels was inhibited by the mineralocorticoid receptor antagonist spironolactone (10 μM)(figure 22 top and bottom, respectively). These data indicate that aldosterone acted *via* a mineralocorticoid receptor to cause the increase in the 5-HT_{1B} and 5-HT_{2B} receptor protein levels was inhibited by the mineralocorticoid receptor antagonist spironolactone. Taken together, these data support the hypothesis that aldosterone can increase the 5-HT_{1B} and 5-HT_{2B} receptor protein levels, independent of an increase in pressure.

To examine whether the increase in the levels of the 5-HT_{1B} and 5-HT_{2B} receptor proteins was selective to aldosterone we used deoxycorticosterone acetate (DOCA) as another mineralocorticoid receptor agonist. Thoracic aorta from male Sprague-Dawley rats was incubated with DOCA (100 nM) for twelve hours in the presence and absence of the mineralocorticoid receptor antagonist spironolactone (10 μM). DOCA incubation increased the level of the 5-HT_{2B} receptor protein approximately 2-fold (figure 23, top) and 1-fold (figure 23, bottom) for the 5-HT_{1B} and 5-HT_{2B} receptors, respectively. This increase in the level of receptor proteins was inhibited by the presence of the mineralocorticoid antagonist spironolactone. These data suggest that both mineralocorticoid receptor agonists, aldosterone and DOCA, increase the level of the 5-HT_{2B} and 5-HT_{1B} receptor proteins through the mineralocorticoid receptor.

Figure 22. Measurement of 5-HT_{1B} receptor protein levels (top) and 5-HT_{2B} receptor protein levels (bottom) to determine the effects of incubation of spironolactone (SP, 10 μM) on Aldosterone-induced (Aldo, 100 nM) increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor proteins in thoracic aorta from Sprague-Dawley rats with normal blood pressure. DMSO indicates dimethyl sulfoxide treatment. ETOH indicates ethanol treatment. Blots are representative of seven experiments. Units are reported as arbitrary densitometry units. Total protein loaded in each lane was 50 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the DMSO/ETOH-incubated artery. + represents statistically significant difference ($p<0.05$) between the response obtained in the Aldo/SP-incubated artery.

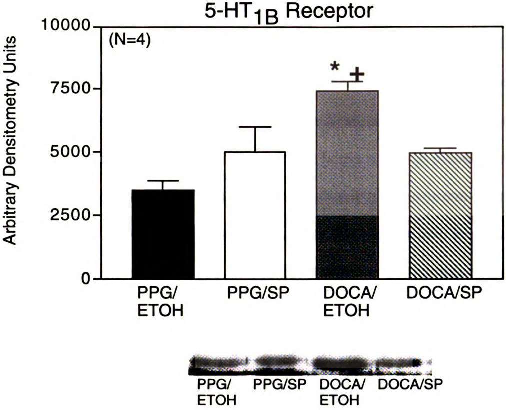


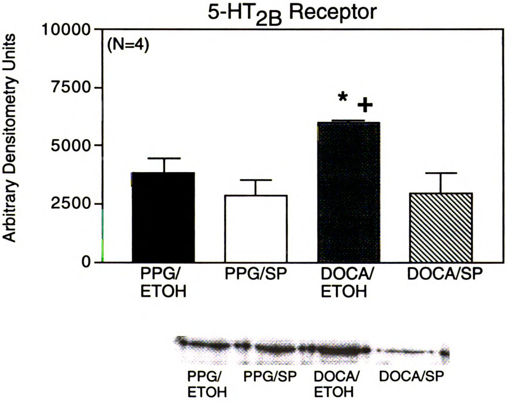
5-HT1B Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
(N=7)
* +
DMSO ETOH
DMSO SP
Aldo ETOH
Aldo SP
44 kDa
DMSO ETOH
DMSO SP
Aldo ETOH
Aldo SP



5-HT2B Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
(N=7)
* +
DMSO ETOH
DMSO SP
Aldo ETOH
Aldo SP
56 kDa
DMSO ETOH
DMSO SP
Aldo ETOH
Aldo SP

Figure 23. Measurement of 5-HT_{1B} receptor (top) and 5-HT_{2B} receptor (bottom) protein levels to determine the effects of incubation of spironolactone (SP, 10 μM) on deoxycorticosterone acetate-induced (DOCA, 100 nM) increases in the level of 5-HT_{1B} receptor and 5-HT_{2B} receptor proteins in thoracic aorta from Sprague-Dawley rats with normal blood pressure. PPG indicates propylene glycol treatment. ETOH indicates ethanol treatment. Blots are representative of four experiments. Units are reported as arbitrary densitometry units. Total protein loaded in each lane was 50 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the PPG/ETOH-incubated artery. + represents statistically significant difference ($p<0.05$) between the response obtained in the DOCA/SP-incubated artery.


5-HT1B Receptor
(N=4)
Arbitrary Densitometry Units
10000
7500
5000
2500
0
PPG/ ETOH
PPG/SP
DOCA/ ETOH
DOCA/SP
PPG/ ETOH
PPG/SP
DOCA/ ETOH
DOCA/SP


5-HT2B Receptor
(N=4)
Arbitrary Densitometry Units
10000
7500
5000
2500
0
PPG/ ETOH
PPG/SP
DOCA/ ETOH
DOCA/SP
PPG/ ETOH
PPG/SP
DOCA/ ETOH
DOCA/SP

Aldosterone-incubation and Contraction Studies

To determine if an acute increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor proteins was sufficient to cause an enhanced contractile response to 5-HT we incubated endothelium-denuded thoracic aorta from male Sprague-Dawley rats with aldosterone (100 nM) for 12 hours and then placed the tissues into an isolated tissue bath for contractile studies. The aldosterone-incubated tissues did not have an enhanced response to 5-HT (figure 24). Importantly, Western analysis of the tissues after removal from the bath demonstrated a significant increase in the levels of 5-HT_{2B} and 5-HT_{1B} receptor proteins (figure 25). These data suggest that an acute increase in the level of the receptor proteins is not sufficient to enable the hyperresponsiveness to 5-HT seen in arteries from hypertensive DOCA-salt rats. Alternatively, these data also suggest that other factors, such as the function of down stream signaling molecules, such as Rho-kinase (Weber and Webb, 2001) or the coupling of the receptor to the G-protein may also be chronically altered in the arteries from the hypertensive DOCA-salt rats by factors other than aldosterone.

DOCA-salt Time Course Studies

In order to determine the separate effects, *in vivo*, of mineralocorticoids, pressure and salt we performed a time course study. On day zero, male Sprague-Dawley rats were weighed and their blood pressure measured. All rats received an uninephrectomy and the rats in the DOCA-salt

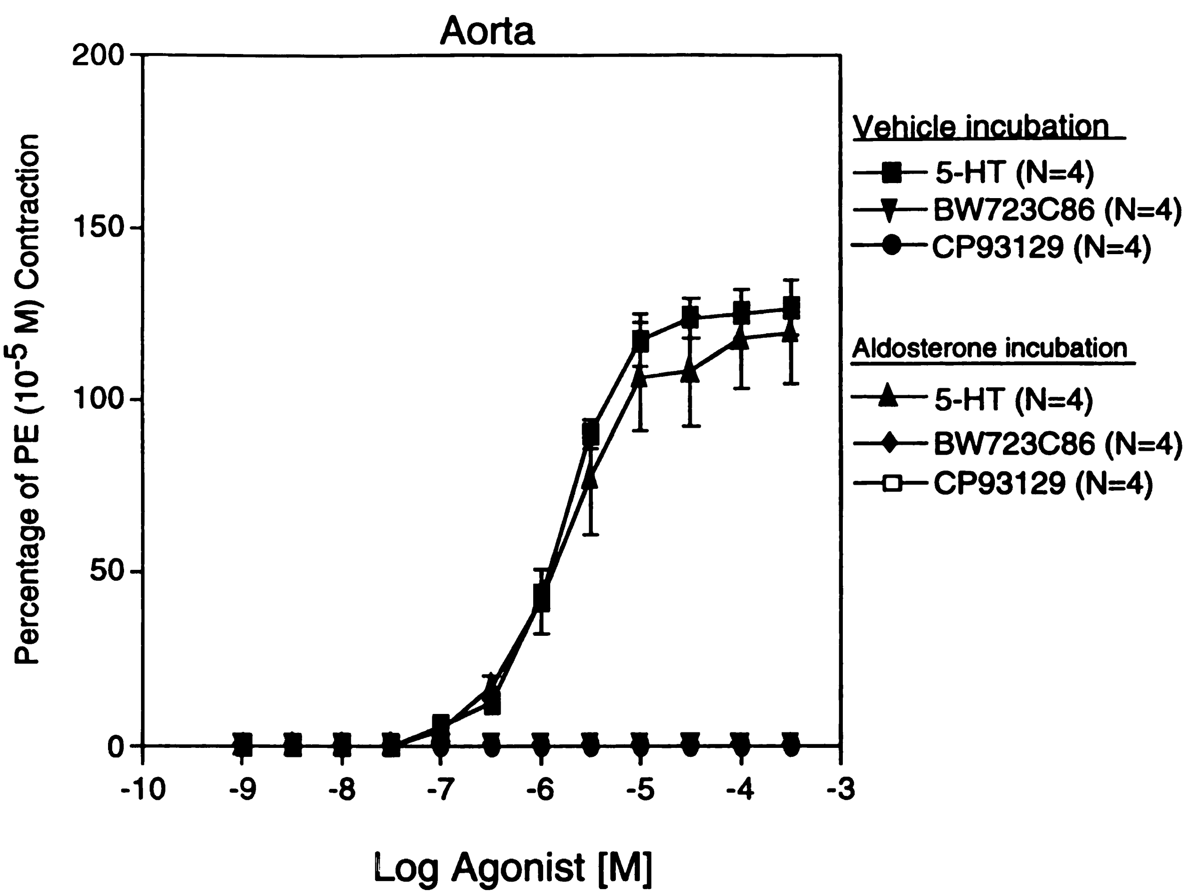
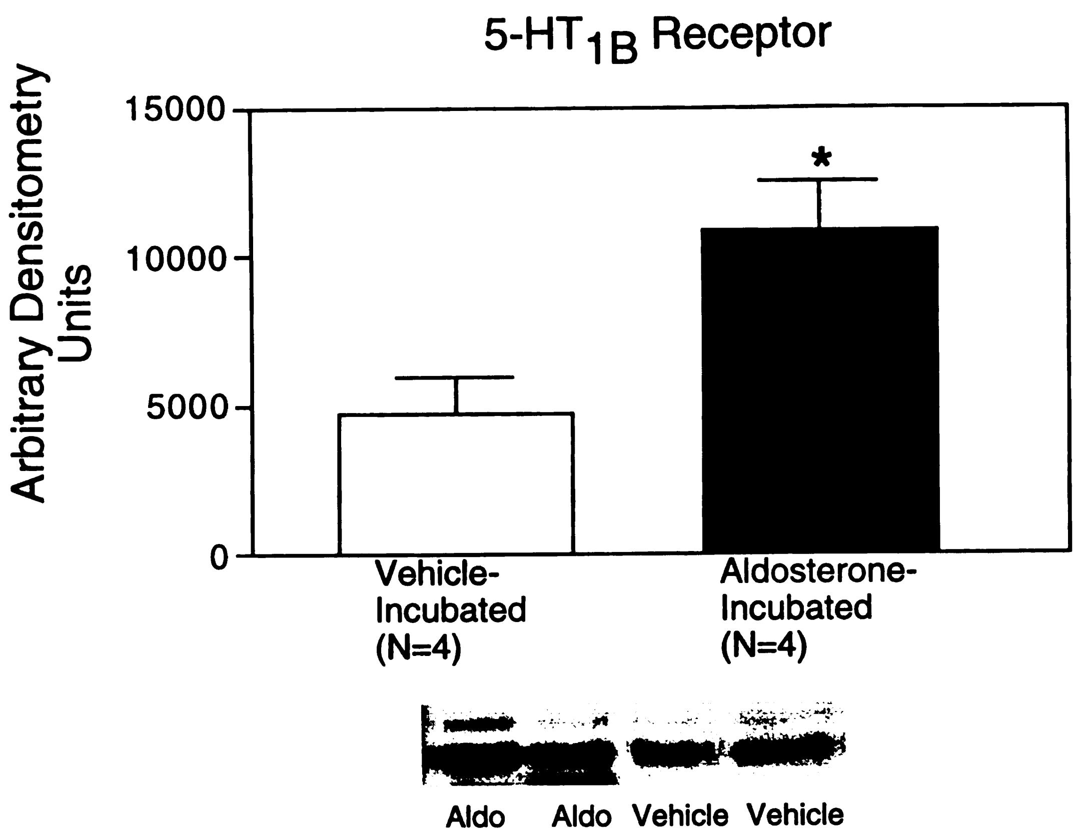
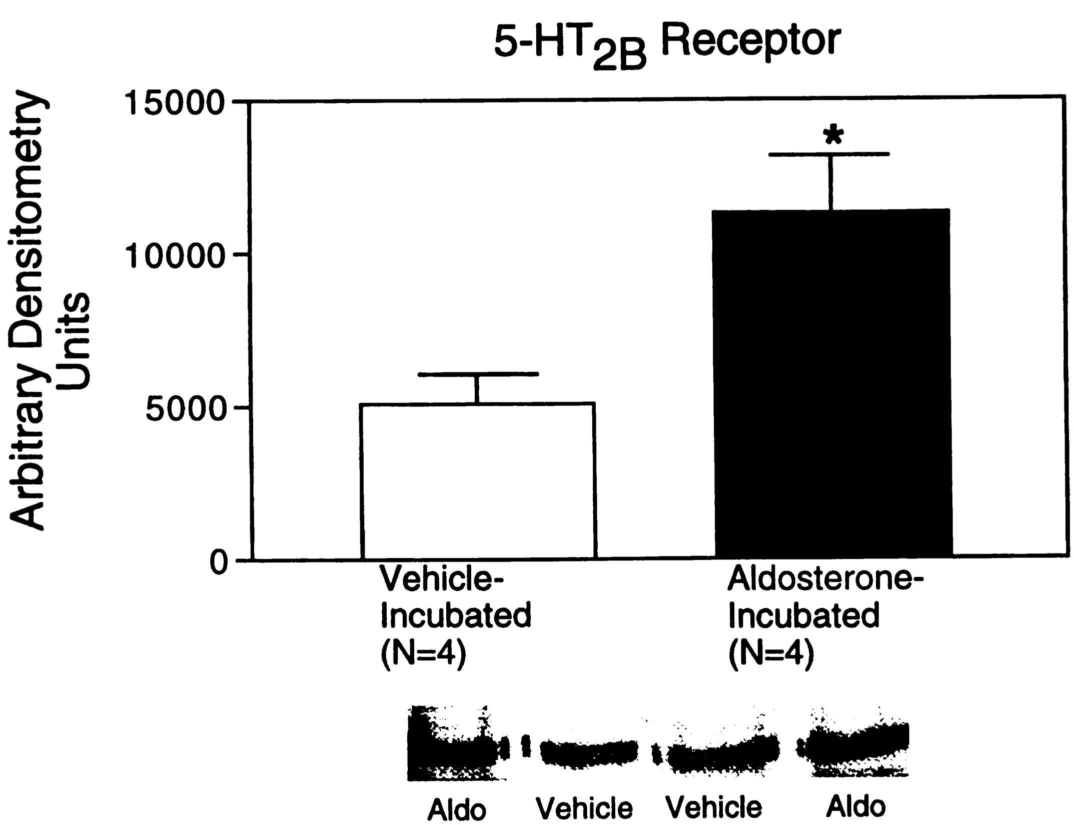


Figure 24. Effect of aldosterone (100 nM, 12 hr) incubation on 5-HT-, the 5-HT_{2B} receptor agonist BW723C86- and the 5-HT_{1B} receptor agonist CP93129-induced contraction in the endothelium-denuded rat thoracic aorta. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.

Figure 25. Top: Measurement of 5-HT_{1B} receptor protein levels in thoracic aorta from Sprague-Dawley rats incubated with aldosterone (100 nM; 12 hr) after contraction to 5-HT, the 5-HT_{2B} receptor agonist BW723C86 and the 5-HT_{1B} receptor agonist CP93129. Bottom: Measurement of 5-HT_{2B} receptor protein levels in thoracic aorta from Sprague-Dawley rats incubated with aldosterone (100 nM; 12 hr) after contraction to 5-HT, the 5-HT_{2B} receptor agonist BW723C86 and the 5-HT_{1B} receptor agonist CP93129. Blots are representative of four experiments. Units are reported as arbitrary densitometry units. Total protein loaded in each lane was 50 μg/μL. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) from the response obtained in the Vehicle-incubated artery.


5-HT_{1B} Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
*
Vehicle-Incubated (N=4)
Aldosterone-Incubated (N=4)
Aldo
Aldo
Vehicle
Vehicle


5-HT_{2B} Receptor
Arbitrary Densitometry Units
15000
10000
5000
0
*
Vehicle-Incubated (N=4)
Aldosterone-Incubated (N=4)
Aldo
Vehicle
Vehicle
Aldo

and DOCA-low salt groups received implants. Sham and DOCA-low salt rats were given normal tap water to drink. The DOCA-salt and high salt rats received high salt water (1% NaCl and 0.2% KCl) to drink. On days 1,3,5 and 7 after starting the protocol the rats were weighed and the systolic blood pressure was measured. Only the sham rats on day seven of the protocol showed a significant gain in body weight (figure 26, top right). None of the rats in the high salt, DOCA-salt and DOCA-low salt groups gained significant body weight during the protocol. By day three the DOCA-salt rats were consuming significantly more fluid than the sham, high salt and DOCA-low salt rats (figure 27, top right). The DOCA-salt rats continued to increase their fluid consumption through day seven. Additionally, the rats placed on the high salt water alone increased intake significantly by day five and remained elevated through day seven (figure 27, bottom left). The sham and DOCA-low salt rats did not vary significantly in their fluid consumption during the study.

Systolic Blood Pressure

The systolic blood pressure of the sham rats remained relatively constant over the seven days of treatment (figure 28). The DOCA-salt rats showed a significant increase in blood pressure by day three (average systolic blood pressure (SBP) 124 $\pm$ 7.6 mm Hg and 110 $\pm$ 2.6 mm Hg, DOCA-salt and sham, respectively) (figure 27). The systolic blood pressure of the DOCA-salt rats reached hypertensive levels by day five (table 2). High salt rats had increased blood pressures by day seven but did not reach hypertensive levels (table 2). By

Figure 26. Top left: Measurement of mass (g) of Sham rats on day s 1-7. Top right: Measurement of mass (g) of DOCA-salt rats on days 1-7. Bottom left: Measurement of mass (g) of DOCA-low salt rats on days 1-7. Bottom right: Measurement of mass (g) of High salt rats on days 1-7. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference (p<0.05) between the response obtained in the sham.

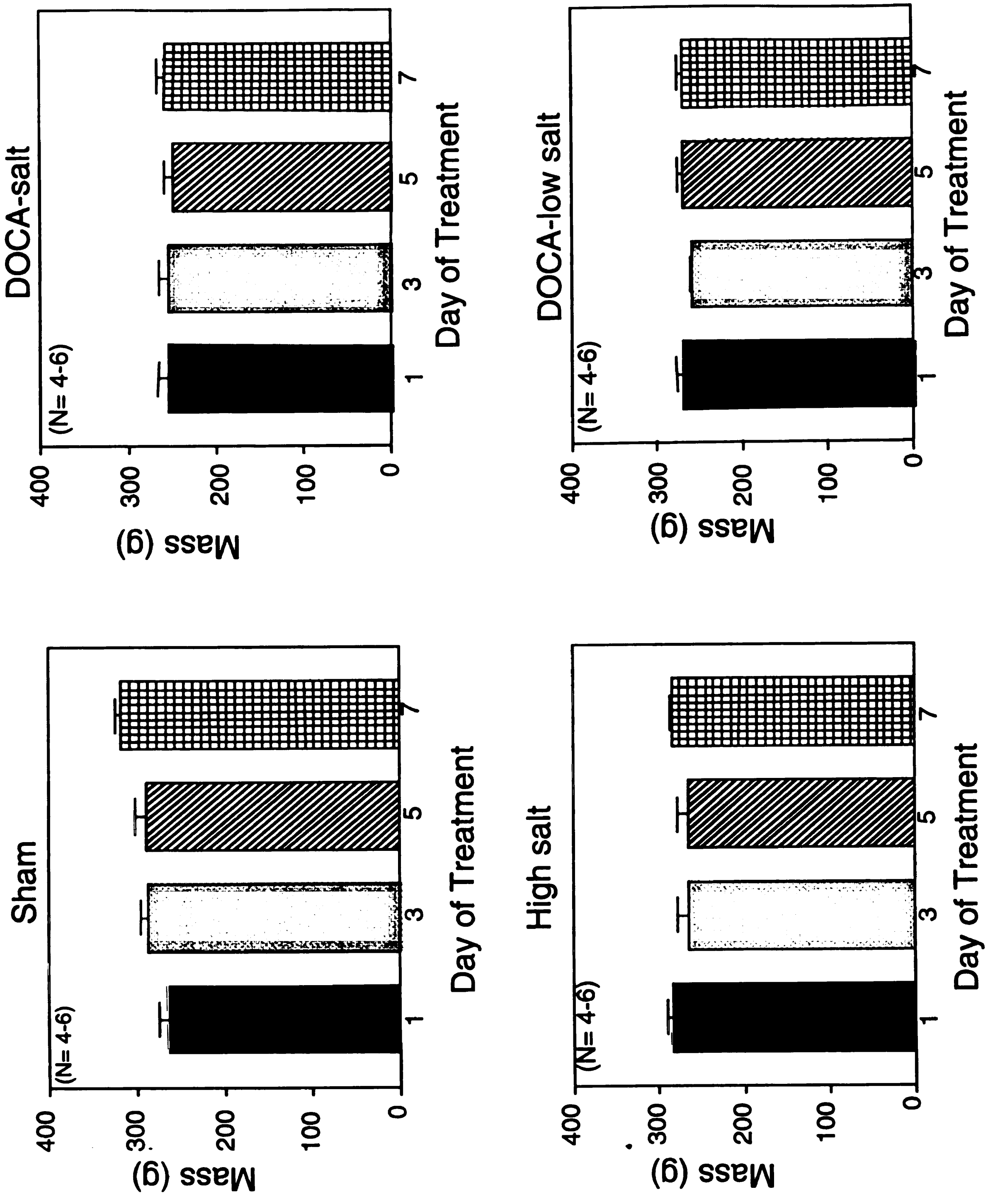
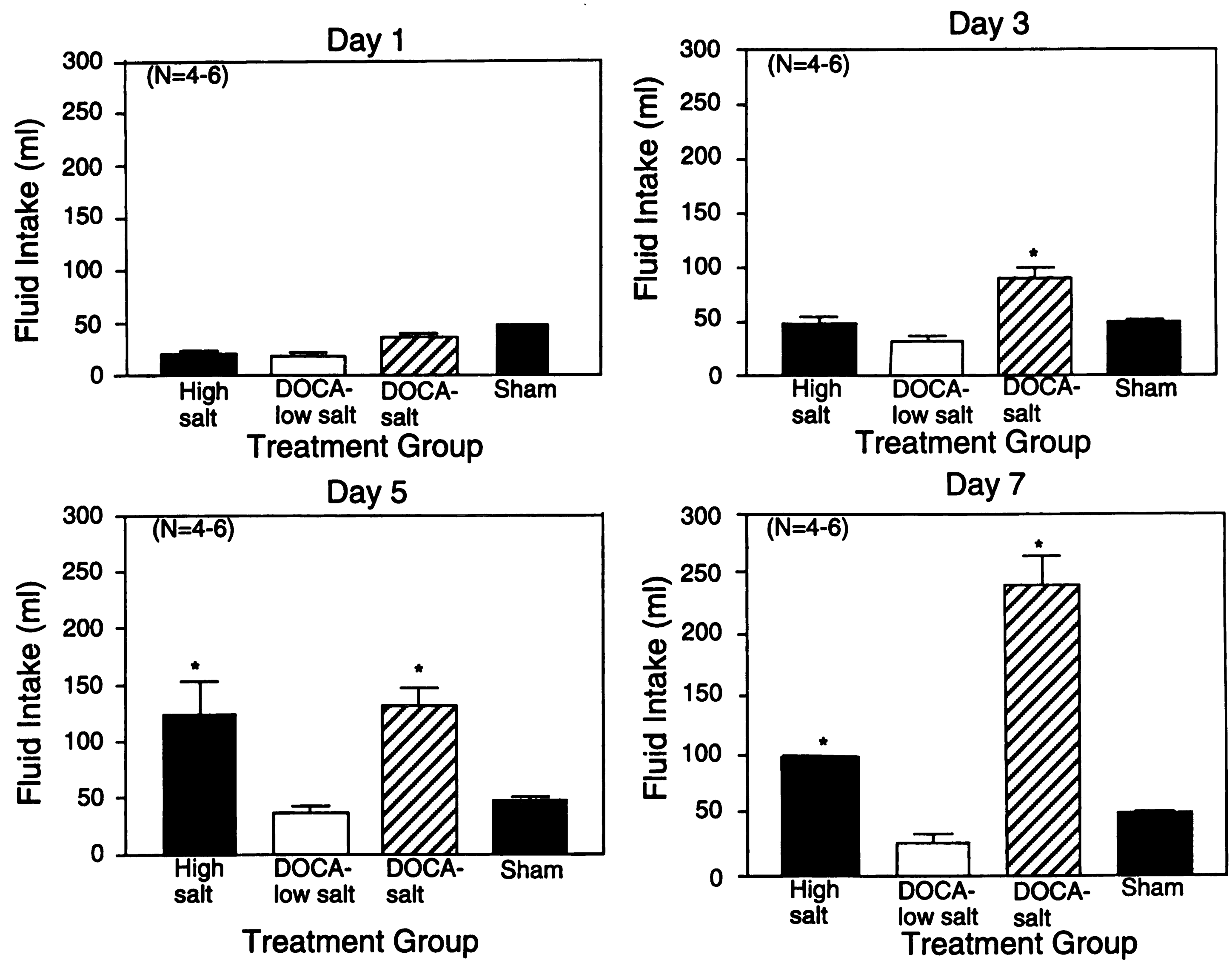

Sham
(N= 4-6)
Mass (g)
400
300
200
100
0
1
3
5
7
Day of Treatment
DOCA-salt
(N= 4-6)
Mass (g)
400
300
200
100
0
1
3
5
7
Day of Treatment
High salt
(N= 4-6)
Mass (g)
400
300
200
100
0
1
3
5
7
Day of Treatment
DOCA-low salt
(N= 4-6)
Mass (g)
400
300
200
100
0
1
3
5
7
Day of Treatment

Figure 27. Measurement of fluid consumption by day and treatment group. Sham and DOCA-low salt rats received normal tap water to drink. High salt and DOCA-salt rats received salt water containing 1 % NaCl and 0.2 % KCl to drink. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained from day one of treatment.



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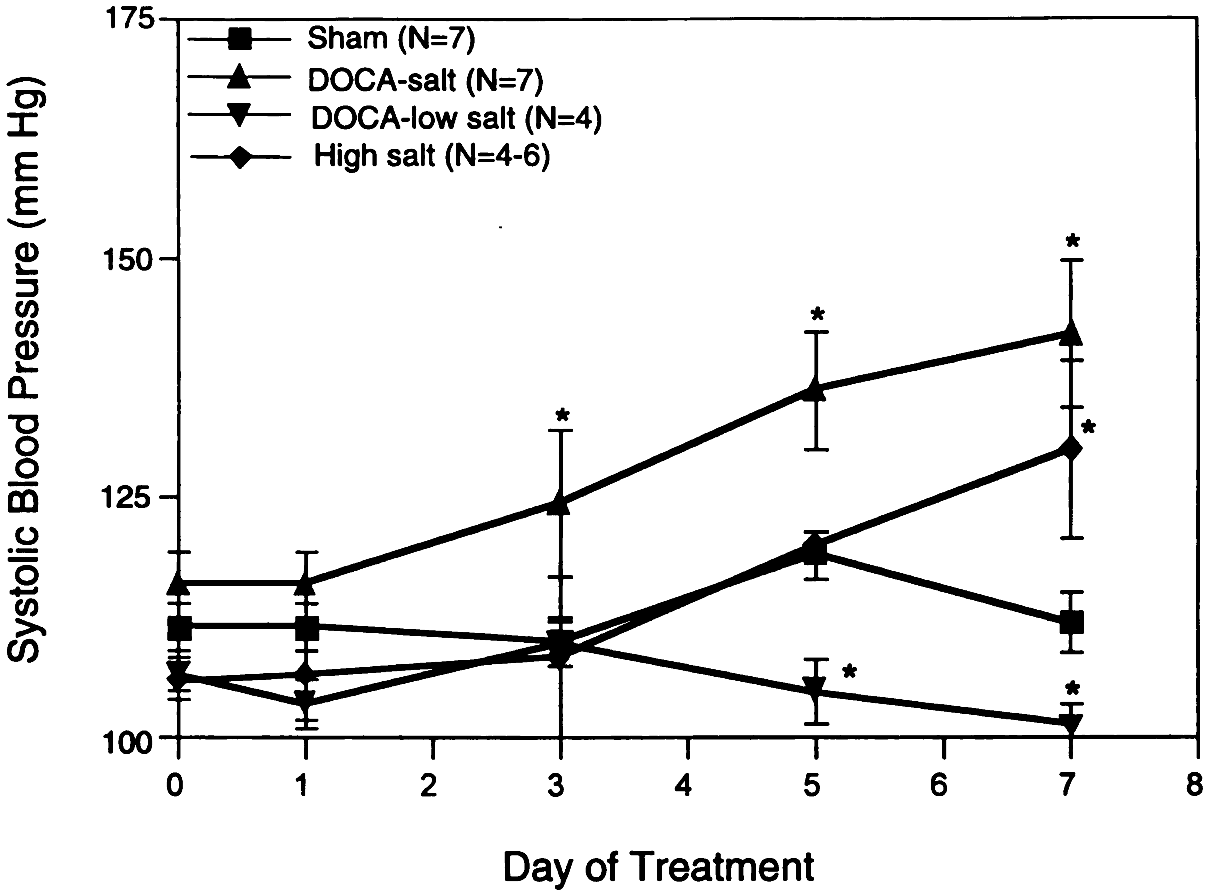

Figure 28. Measurement of systolic blood pressures. Data are reported in mm Hg. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained from the sham response on each day .

Table 2. Systolic blood pressures (SBP), EC_{50} values for BW723C86, CP93129 and 5-HT and the maximal contractile responses to BW723C86, CP93129 and 5-HT represented as a percentage of phenylephrine (10 μM); PE) for sham, high salt, DOCA-low salt and DOCA-salt rats. Values represent the means ± standard error of the mean for each group. The number of animals in each group is indicated in parentheses. * represents statistical difference from response obtained in the shams.

Treatment Group	SBP mm Hg	- log EC_{50} value [M] for 5-HT Response	Maximal 5-HT Response	Maximal BW723C86 Response	Maximal CP93129 Response
Day 1					
Sham (7)	112 ± 2.4	5.95 ± 0.03	106.7 ± 5.5	7.7 ± 7.7	0
DOCA-salt (7)	116 ± 3.4	5.87 ± 0.06	102.5 ± 8.4	52.5 ± 10.5	0
DOCA-low salt (4)	106 ± 2.5	6.00 ± 0.06	103.5 ± 0.5	16.8 ± 16.8	0
High salt (4)	107 ± 4.6	5.91 ± 0.03	104.3 ± 8.0	0	0
Day 3					
Sham (5)	110 ± 2.6	5.74 ± 0.05	93.2 ± 5.9	17.5 ± 11.0	0
DOCA-salt (5)	124 ± 7.6	6.03 ± 0.08	110.1 ± 7.8	73.1 ± 9.1	37.1 ± 14.3
DOCA-low salt (4)	110 ± 2.3	5.71 ± 0.08	141.2 ± 11.8	0	0
High salt (4)	108 ± 8.3	6.20 ± 0.03	128.4 ± 6.3	46.7 ± 15.0	20.0 ± 12.2
Day 5					
Sham (5)	119 ± 2.5	5.60 ± 0.07	106.2 ± 8.7	18.5 ± 11.6	0
DOCA-salt (5)	147 ± 14	5.98 ± 0.07	106.5 ± 7.7	54.3 ± 11.9	35.0 + 8.6
DOCA-low salt (4)	105 ± 3.4	5.88 ± 0.06	110.9 ± 1.8	0	0
High salt (4)	120 ± 1.4	6.06 ± 0.05	148.5 ± 11.4	46.3 ± 6.6	27.8 ± 7.4
Day 7					
Sham (5)	114 ± 2.3	5.70 ± 0.07	90.4 ± 6.5	0	0
DOCA-salt (5)	142 ± 8.4	6.41 ± 0.09	130.3 ± 19.5	57.4 ± 5.9	63.8 ± 7.8
DOCA-low salt (4)	101 ± 2.1	5.77 ± 0.08	110.1 ± 1.8	0	0
High salt (6)	130 ± 9.3	6.13 ± 0.03	119.5 ± 5.1	24.5 ± 9.4	19.6 ± 8.1

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day seven the DOCA-low salt rats had significantly lower blood pressure than their sham, DOCA-salt and high salt counterparts (average SBP 114 $\pm$ 2.3 mm Hg, 142 $\pm$ 8.4 mm Hg, 130 $\pm$ 9.3 mm Hg and 101 $\pm$ 2.1 mm Hg, sham, DOCA-salt, high salt and DOCA-low salt, respectively).

Contractile Studies on Day 1

Although there was no increase in systolic blood pressure on day one the arteries from the DOCA-salt rats contracted to the 5-HT_{2B} receptor agonist BW723C86 (maximal contraction 52.5 % $\pm$ 10.5 of phenylephrine (PE) 10^{-5} M contraction). The arteries from DOCA-low salt, high salt and sham rats did not respond to either BW723C86 or to the 5-HT_{1B} receptor agonist CP93129 (figure 29, top and middle). There was no enhanced contraction to 5-HT observed in any of the arteries from the treatment groups (figure 29, bottom). These data suggest that while the 5-HT_{2B} receptor is functional on day one, as indicated by contraction to BW723C86, this is not sufficient to result in an enhanced contraction to 5-HT. It is interesting to note that after twenty-four hours of exposure to elevated mineralocorticoid and salt levels the 5-HT_{2B} receptor becomes functionally linked to contraction. The change that enables the 5-HT_{2B} receptor to mediate contraction occurs in the absence of an increase in pressure.

Contractile Studies on Day 3

On day three of the time course the 5-HT_{2B} agonist BW723C86 elicits a contraction in the arteries from the DOCA-salt and high salt rats (figure 30, top). There is also a significant contraction observed to the 5-HT_{1B} agonist CP93129 in

Figure 29. Top: Effect of the 5-HT_{2B} receptor agonist BW723C86 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day one of treatment. Middle: Effect of the 5-HT_{1B} receptor agonist CP93129 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day one of treatment. Bottom: Effect of 5-HT in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day one of treatment. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery.

Day 1

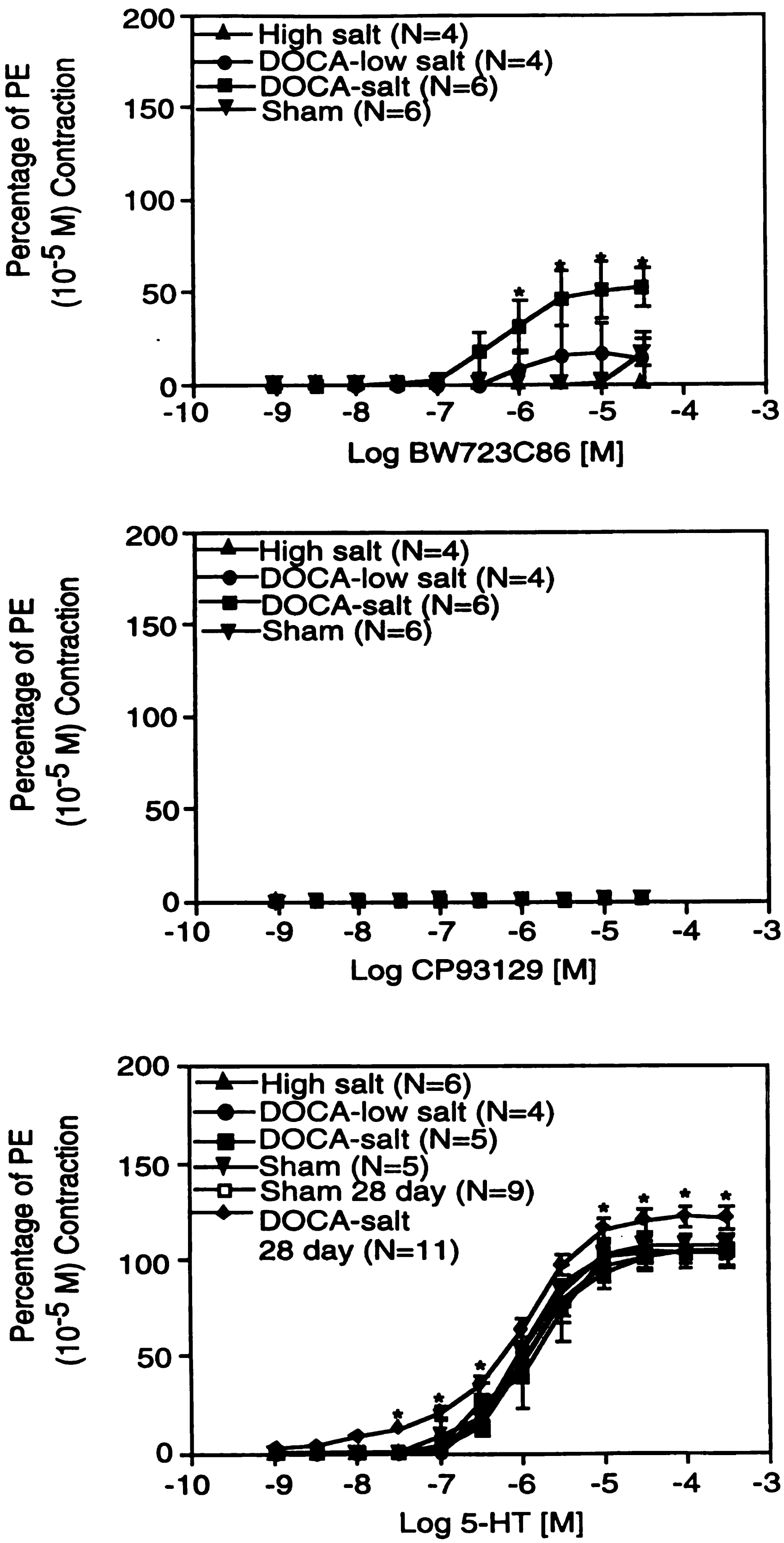
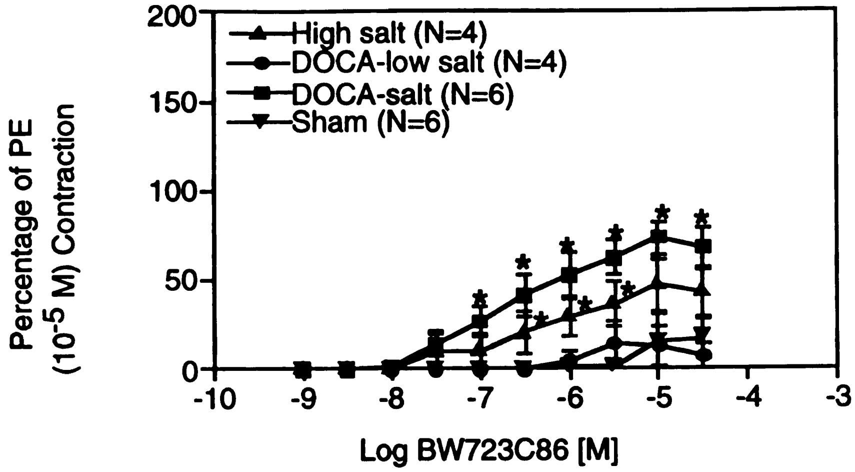
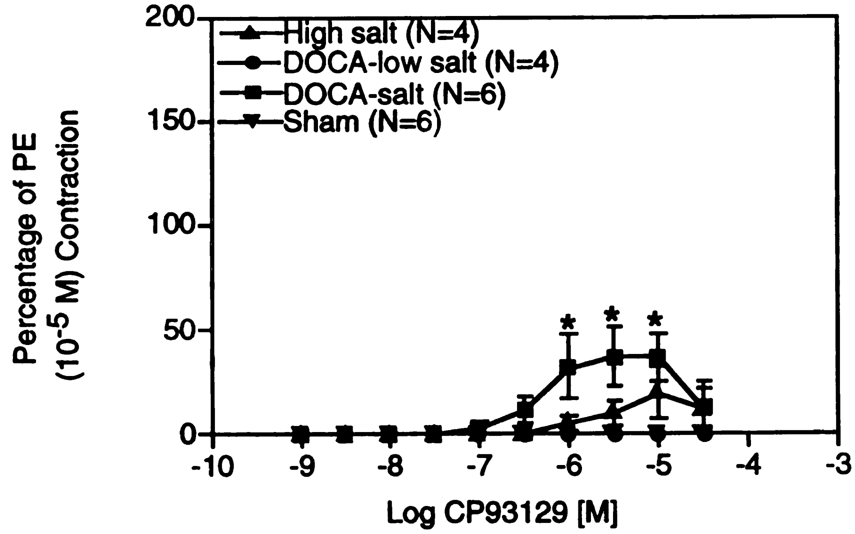

High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Percentage of PE
(10-5 M) Contraction
Log BW723C86 [M]
High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Percentage of PE
(10-5 M) Contraction
Log CP93129 [M]
High salt (N=6)
DOCA-low salt (N=4)
DOCA-salt (N=5)
Sham (N=5)
Sham 28 day (N=9)
DOCA-salt
28 day (N=11)
Percentage of PE
(10-5 M) Contraction
Log 5-HT [M]

Figure 30. Top: Effect of the 5-HT_{2B} receptor agonist BW723C86 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day three of treatment. Middle: Effect of the 5-HT_{1B} receptor agonist CP93129 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day three of treatment. Bottom: Effect of 5-HT in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day three of treatment. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery.

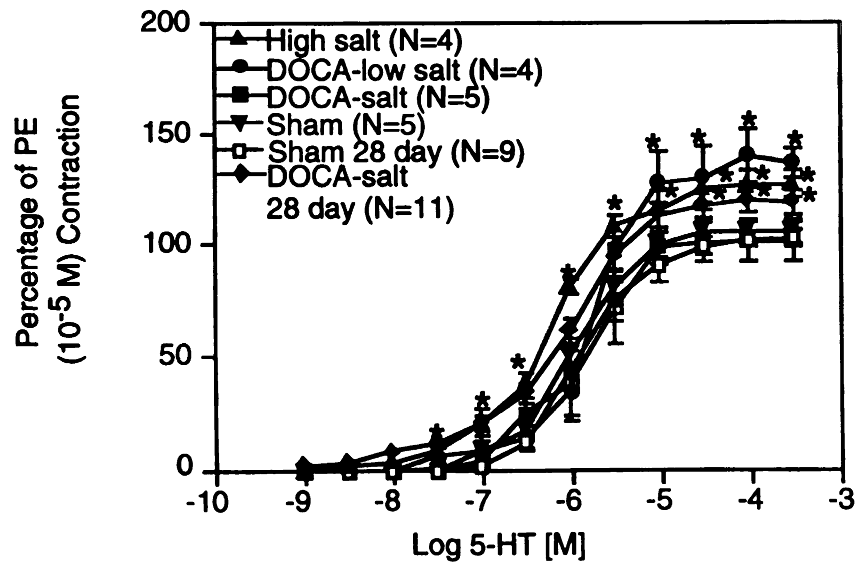
Day 3



High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Percentage of PE (10^{-5} M) Contraction
0
50
100
150
200
-10
-9
-8
-7
-6
-5
-4
-3
Log BW723C86 [M]



High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Percentage of PE (10^{-5} M) Contraction
0
50
100
150
200
-10
-9
-8
-7
-6
-5
-4
-3
Log CP93129 [M]



High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=5)
Sham (N=5)
Sham 28 day (N=9)
DOCA-salt 28 day (N=11)
Percentage of PE (10^{-5} M) Contraction
0
50
100
150
200
-10
-9
-8
-7
-6
-5
-4
-3
Log 5-HT [M]

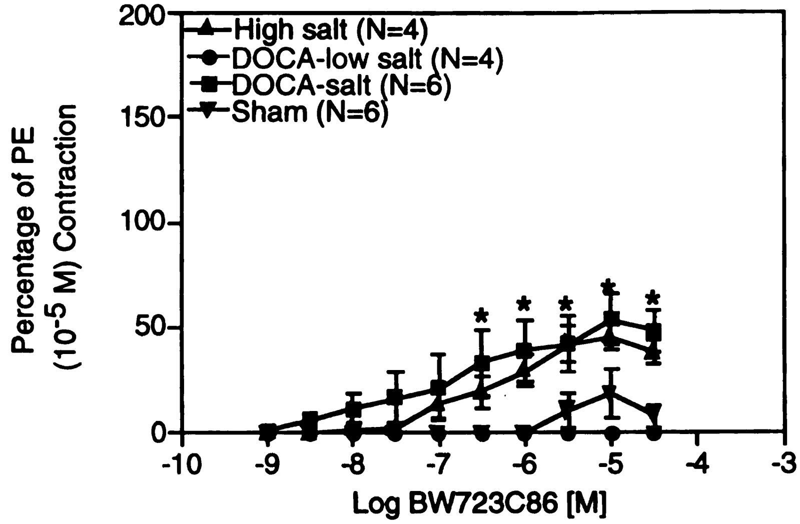
the arteries from the DOCA-salt rats (figure 30, middle). The arteries from the DOCA-low salt and sham rats do not show a significant contractile response to BW723C86 or CP93129 (figure 30, top and middle). Contraction to 5-HT is enhanced is arteries from high salt rats (figure 30, bottom). The arteries from the DOCA-salt rats showed no change in the maximal contraction to 5-HT but demonstrated enhanced 5-HT potency (-log EC_{50} [M] values 6.025 $\pm$ 0.08, 5.740 $\pm$ 0.05, 6.017 $\pm$ 0.03, DOCA-salt 3 day, Sham, DOCA-salt 28 day, respectively). Unexpectedly, the arteries from DOCA-low salt rats show an increased maximal response to 5-HT (maximal contraction 141.2 $\pm$ 11.8 % PE 10^{-5} M contraction). Since there was no response to the 5-HT_{1B} agonist CP93129 and very little response to the 5-HT_{2B} agonist BW723C86 this enhanced contraction to 5-HT is surprising. However, this enhancement of 5-HT-induced contraction in arteries from DOCA-low salt rats only occurs on day three.

Contractile Studies on Day 5

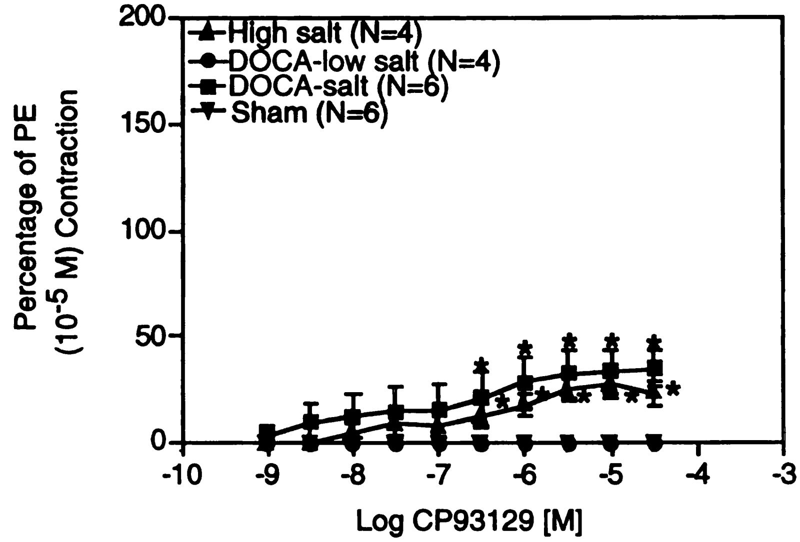
Experiments performed on day five of the time course demonstrate a similar profile of results. Arteries from the DOCA-salt and high salt rats contracted to both the 5-HT_{2B} agonist BW723C86 (maximal contraction 54.3 $\pm$ 11.9 % and 46.3 $\pm$ 6.6 % of PE 10^{-5} M contraction, DOCA-salt and high salt, respectively) and the 5-HT_{1B} agonist CP93129 (maximal contraction 35.0 $\pm$ 8.6% and 27.8 $\pm$ 7.4 % of PE 10^{-5} M contraction, DOCA-salt and high salt, respectively) (figure 31, top and middle). Arteries from high salt rats also displayed an increased maximal contraction (maximal contraction 148.5 $\pm$ 11.4 % and 106.2 $\pm$

Figure 31. Top: Effect of the 5-HT_{2B} receptor agonist BW723C86 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day five of treatment. Middle: Effect of the 5-HT_{1B} receptor agonist CP93129 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day five of treatment. Bottom: Effect of 5-HT in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day five of treatment. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery.

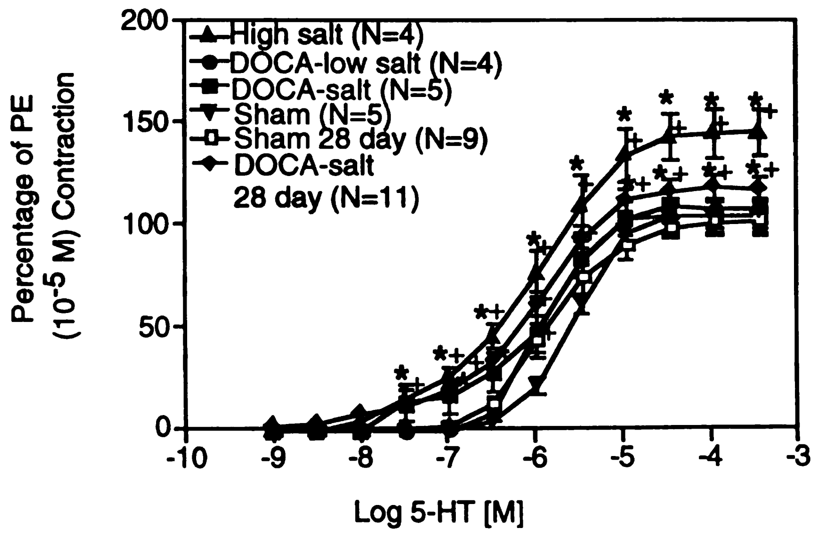
Day 5



High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Percentage of PE (10-5 M) Contraction
Log BW723C86 [M]



High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Percentage of PE (10-5 M) Contraction
Log CP93129 [M]



High salt (N=4)
DOCA-low salt (N=4)
DOCA-salt (N=5)
Sham (N=5)
Sham 28 day (N=9)
DOCA-salt
28 day (N=11)
Percentage of PE (10-5 M) Contraction
Log 5-HT [M]

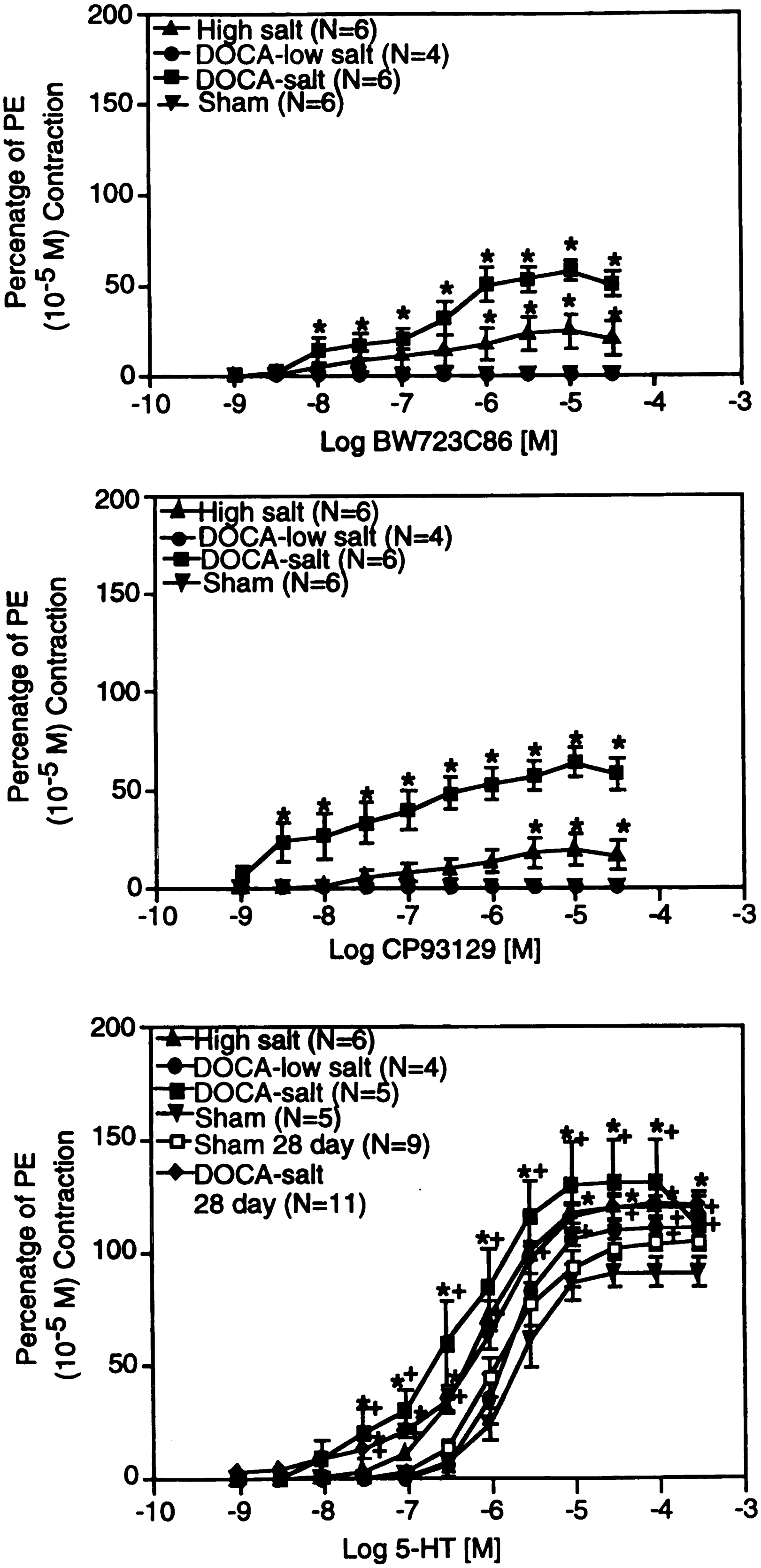
8.7 % of PE 10^{-5} M contraction, high salt and sham, respectively), a decrease in the threshold of activation of contraction and an increase in potency, and as compared to sham arteries (-log EC_{50} value [M] 6.06 $\pm$ 0.05 and 5.60 $\pm$ 0.07 high salt and sham, respectively) (figure 31, bottom). The arteries from DOCA-salt rats also show a decrease in the threshold of activation of contraction (figure 31, bottom).

Contractile Studies on Day 7

On day seven of the time course, arteries from DOCA-salt rats contracted to both BW723C86 (maximal contraction 57.4 $\pm$ 5.9 % of PE 10^{-5} M contraction) and CP93129 (maximal contraction 63.8 $\pm$ 7.8 % of PE 10^{-5} M contraction) (figure 32, top and middle). Arteries from high salt rats also contracted to both BW723C86 (maximal contraction 24.5 $\pm$ 9.4 % of PE 10^{-5} M contraction) and CP93129 (maximal contraction 19.6 $\pm$ 8.1 % of PE 10^{-5} M contraction) (figure 31, top and middle). Interestingly, arteries from high salt rats have been exposed to both increased levels of salt as well as an increased pressure. However, these arteries do not contract to the same magnitude of contraction in the presence of BW723C86 and CP93129 as those that are taken from DOCA-salt rats on day seven (table 2). These data suggest that the combination of increased levels of salt and mineralocorticoids as well as the increased pressure are all important regulators of the changes which enable the 5-HT_{2B} and 5-HT_{1B} receptors to participate in contraction. Additionally, arteries from DOCA-salt and high salt rats both show hyperresponsiveness to 5-HT (figure 32, bottom). The characteristic

Figure 32. Top: Effect of the 5-HT_{2B} receptor agonist BW723C86 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day seven of treatment. Middle: Effect of the 5-HT_{1B} receptor agonist CP93129 in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day seven of treatment. Bottom: Effect of 5-HT in endothelium-denuded thoracic aorta from high salt, DOCA-salt, DOCA-low salt and sham rats on day seven of treatment. Data are reported as a percentage of the initial phenylephrine (PE) 10^{-5} M contraction. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the sham artery.

Day 7


Percenatge of PE
(10^{-5} M) Contraction
High salt (N=6)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Log BW723C86 [M]
Percenatge of PE
(10^{-5} M) Contraction
High salt (N=6)
DOCA-low salt (N=4)
DOCA-salt (N=6)
Sham (N=6)
Log CP93129 [M]
Percenatge of PE
(10^{-5} M) Contraction
High salt (N=6)
DOCA-low salt (N=4)
DOCA-salt (N=5)
Sham (N=5)
Sham 28 day (N=9)
DOCA-salt
28 day (N=11)
Log 5-HT [M]

increased maximal contraction (maximal contraction 130.3 $\pm$ 19.5 % and 119.5 $\pm$ 5.1 % of PE 10^{-5} M contraction, DOCA-salt and high salt, respectively), the decreased threshold for contraction and the increased potency (- log EC_{50} value [M] 6.41 $\pm$ 0.09, 6.13 $\pm$ 0.03 and 5.70 $\pm$ 0.07, DOCA-salt, high salt and sham, respectively).

Protein Analysis Studies

There is no increase in 5-HT_{1B} receptor protein levels at any of the treatment groups at any time point (figure 33), although there is a trend towards increasing levels of protein in the aortic homogenates from the DOCA-salt rats. This was an unexpected finding as the arteries from both the DOCA-salt and high salt rats contract to the 5-HT_{1B} agonist CP93129 starting on day three of the time course. These data suggest that although the receptor protein levels were increased by 28 days of DOCA-salt treatment, increased receptor protein levels are not required to enable this receptor to participate in contraction. Furthermore, the 5-HT_{2B} receptor protein levels are significantly increased by day 3 of DOCA-salt treatment (figure 34). The 5-HT_{2B} receptor protein levels are not increased in any of the other treatment groups at any time point. Interestingly, the level of receptor protein is not significantly increased on day 1, even though there is a significant contraction to the 5-HT_{2B} receptor agonist BW723C86. There is also no significant increase in the 5-HT_{2B} receptor levels in the aortic homogenates from the high salt rats in which contraction to BW723C86 does

Figure 33. Top: Measurement of 5-HT_{1B} receptor protein levels in aortic homogenates from sham, DOCA-low salt, high salt and DOCA-salt rats on days 1, 3, 5,and 7. Bottom: Representative Western blot probed for the 5-HT_{1B} receptor protein. Total protein loaded in each lane was 50 μg/μL. Blots are representative of 4-6 experiments. Units are reported as arbitrary densitometry units. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the corresponding sham. Abbreviations: S= Sham; DS= DOCA-salt; DLS= DOCA-low salt; HS= High salt.

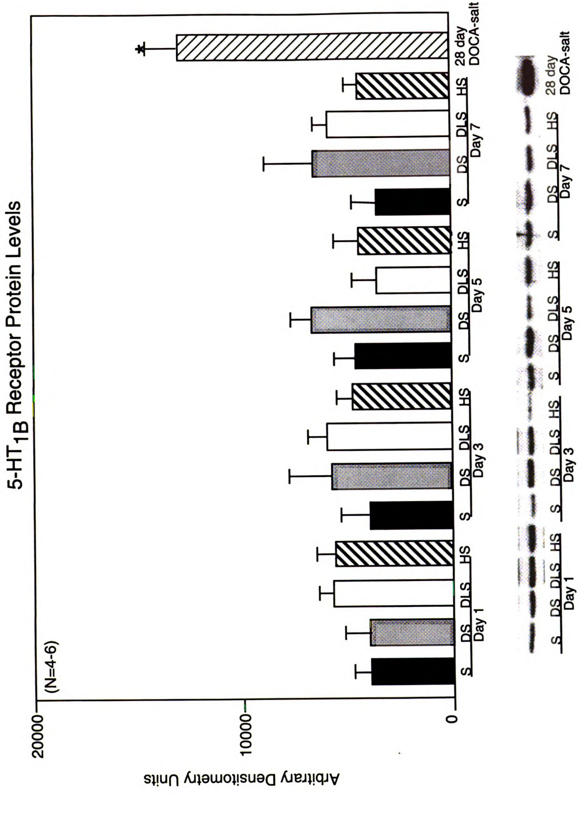
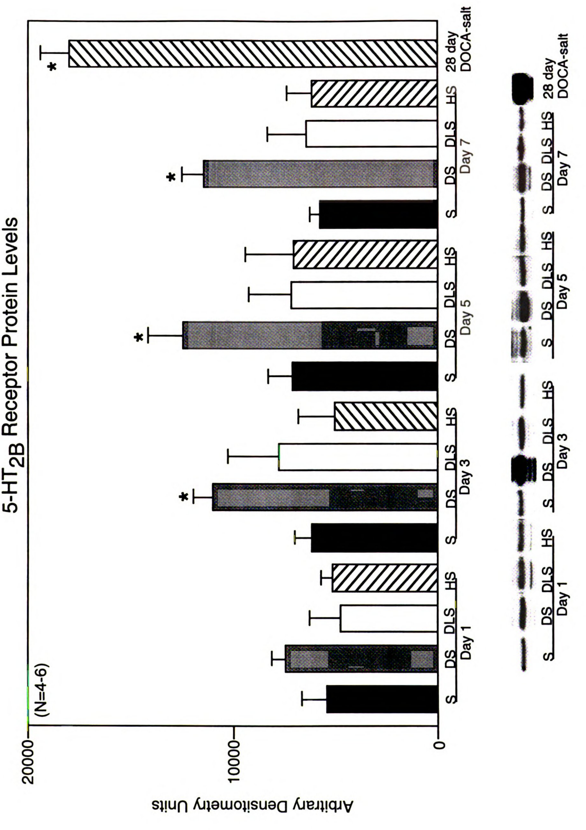

5-HT1B Receptor Protein Levels
(N=4-6)
Arbitrary Densitometry Units
20000
10000
0
S
DS
DLS
HS
Day 1
Day 3
Day 5
Day 7
28 day
DOCA-salt

Figure 34. Top: Measurement of 5-HT_{2B} receptor protein levels in aortic homogenates from sham, DOCA-low salt, high salt and DOCA-salt rats on days 1, 3, 5,and 7. Bottom: Representative Western blot probed for the 5-HT_{1B} receptor protein. Total protein loaded in each lane was 50 µg/µL. Blots are representative of 4-6 experiments. Units are reported as arbitrary densitometry units. Vertical bars represent the mean and the standard error of the mean for the number of experiments indicated in parentheses. * represents statistically significant difference ($p<0.05$) between the response obtained in the corresponding sham. Abbreviations: S= Sham; DS= DOCA-salt; DLS= DOCA-low salt; HS= High salt.


5-HT2B Receptor Protein Levels
(N=4-6)
Arbitrary Densitometry Units
20000
10000
0
*
S
DS
DLS
HS
Day 1
Day 3
Day 5
Day 7
28 day
DOCA-salt

occur. These data would suggest that increased levels of the 5-HT_{2B} receptor protein are not essential for contraction to occur. Additionally, these data suggest that an increase in pressure is also not required for the 5-HT_{2B} receptor to become functionally coupled to contraction. Collectively, these data support the hypothesis that mineralocorticoids can upregulate the 5-HT_{2B} and 5-HT_{1B} receptors, independent of an increase in blood pressure. These data also demonstrate that increased levels of salt and pressure are also important regulators of these receptors. Additionally, an increase in the level of receptor protein is not required to enable these receptors to mediate contraction. These data also suggest that changes in the signaling cascades and receptor/effector coupling are potentially involved in this enhanced contractility to 5-HT and agonists of the 5-HT_{2B} and 5-HT_{1B} receptors under conditions of DOCA-salt hypertension.

Hypothesis IV

Measurement of Free Plasma 5-HT Levels

To test the hypothesis that the level of free 5-HT circulating in the blood is increased in the DOCA-salt model of hypertension, we measured the levels of free 5-HT. The normal physiological levels of 5-HT are reported to be between 5 and 120 nM in both human models and rat models of hypertension (Marasini et al, 1985, Zhao *et al*, 1999, Biondo *et al*, 1986). The increase in free 5-HT may occur for several reasons. There may be a decrease in platelet uptake (Kamal *et al*, 1984) of 5-HT or pulmonary endothelial damage results in a decrease in 5-HT clearance. Platelets from hypertensive subjects tend to aggregate which would result in 5-HT release. This increase in aggregation may be attributable to an increase in calcium and a decrease in $NO^{\bullet}$ levels (Camilletti *et al*, 2001, Nityanand *et al*, 1993). There are also reports in the literature that the platelets from hypertensive subjects are defective in either storage and/or uptake mechanisms of 5-HT (Nityanand *et al*, 1990, Fetkovska *et al*, 1990). This decreased platelet 5-HT levels may be due to defects in the 5-HT transporter. There is presently no one mechanism that has been clearly elucidated to account for this increase seen in hypertension. However, even a small increase in free 5-HT could be sufficient to produce endogenous activation of the 5-HT_{2B} receptor in the vasculature of hypertensive subjects.

We have preliminary data which suggest a trend towards an increase in the levels of free 5-HT in the platelet poor plasma fraction from the DOCA-salt hypertensive rats (figure 35). There is a corresponding decrease in the level of 5-HT in the platelet rich plasma fraction from the DOCA-salt hypertensive rats (figure 35). These data are in agreement with previously published human studies which demonstrate an increase in the levels of 5-HT in the plasma. These data also suggest one possible mechanism for the endogenous activation of 5-HT_{2B} receptors reported in the DOCA-salt model of hypertension (Watts and Fink, 1999). Another possible mechanism for the endogenous activation of 5-HT_{2B} receptors in DOCA-salt hypertension is that the increase in the level of the receptor protein and change in the functional coupling of the 5-HT_{2B} receptors to mediate contraction is sufficient to result in activation of these receptors, which then participate in the maintenance of the increased blood pressure.

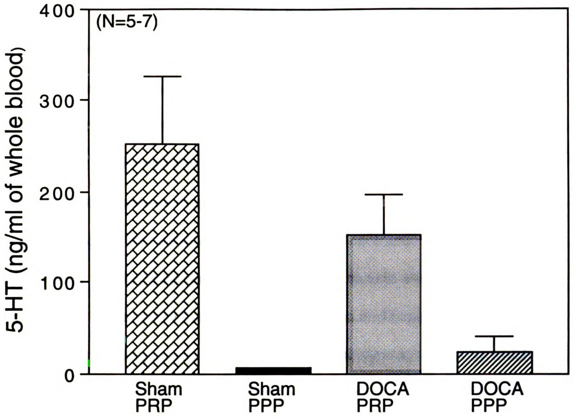


Figure 35. Level of 5-HT in the platelet poor and platelet rich fractions of plasma from normotensive sham and hypertensive DOCA-salt rats. Vertical bars represent the mean value and the standard error of the mean of the number of animals indicated in parenthesis. PPP represents platelet poor plasma. PRP represents platelet rich plasma.

Discussion

Since the isolation of 5-HT by Rapport and colleagues in 1948, a role for 5-HT in hypertension has been suggested. While the specific role which 5-HT plays in hypertension remains a controversial topic, there is no argument that 5-HT is both a vasoconstrictor and a mitogen. The ability of 5-HT to be involved in both of these processes may be important to 5-HT's role in hypertension. It has been established that enhanced agonist-induced contraction as well as hypertrophy and hyperplasia of vascular smooth muscle are characteristic of arteries from hypertensive subjects in both human and experimental models. Vascular remodeling can be seen as a compensatory mechanism by which the arteries attempt to cope with the increased pressure. Intriguingly, this remodeling may involve the dedifferentiation of vascular smooth muscle cells, migration of smooth muscle cells, replication and differentiation into a contractile phenotype. This situation may create an opportunity to observe receptors involved in growth and migration that may not be expressed under the normal contractile phenotype.

Furthermore, while there is controversy about the specific actions of 5-HT in many diseases, including depression, hypertension, pre-eclampsia, pulmonary hypertension, migraine and psychiatric disorders, the potential targeting of therapies to 5-HT receptors and transporters are currently being explored and utilized clinically. Therefore, understanding the 5-HT receptor subtypes and mechanisms which 5-HT utilizes in these pathological states is important to achieving maximal therapeutic benefits as well as avoiding unwanted and potentially harmful side-effects

This project was undertaken to characterize the 5-HT receptor subtypes utilized to mediate contractile hyperresponsiveness observed in arteries from hypertensive DOCA-salt rats and to investigate the mechanisms by which the expression and function of these receptors are changed under the conditions of DOCA-salt hypertension.

Characterization of 5-HT Receptors in Vascular Smooth Muscle

At the start of this project we set out to characterize which 5-HT receptors mediate 5-HT-induced contraction in vascular smooth muscle. Based on data from contractile experiments, we concluded that there was no contractile role for the 5-HT_{1D} and 5-HT_{1F} receptors in aorta from normotensive and hypertensive DOCA-salt rats. We were also unable to observe a contraction to the 5-HT_{1B} receptor agonists in aorta from normotensive rats. These findings for the 5-HT_{1D} and 5-HT_{1F} receptors are in agreement with findings by other investigators which demonstrate no role in smooth muscle contraction for the 5-HT_{1D} and 5-HT_{1F} receptors (Razzaque et al, 1999, Cohen and Schenck, 1999). Furthermore, in vascular smooth muscle cultured from aorta removed from rats with normal blood pressure, only the 5-HT_{2A} receptor was able to activate the ERK/MAPK pathway (Watts *et al*, 2001). Agonists of the 5-HT_{2B}, 5-HT_{1B} 5-HT_{1D} and 5-HT_{1F} receptors showed no increase in the level of ERK/MAPK activation. These data further support the idea that while the mRNA for these receptors are present, there does not appear to be an activatable receptor protein. These combined data support the conclusion that the 5-HT$_{1D}$, 5-HT$_{1F}$ and 5-HT$_{1B}$ receptors do not participate in 5-HT-induced contraction under conditions of normal blood pressure.

5-HT$_{1B}$ Receptors in Hypertension

Initially, we hypothesized that the 5-HT$_{1B}$ receptor mediated contraction under conditions of DOCA-salt hypertension. Our experiments using 5-HT, the 5-HT$_{1B}$ antagonist GR55562, the 5-HT$_{1B}$ receptor agonists RU24969, sumatriptan and the rodent selective 5-HT$_{1B}$ receptor agonist CP93129 support the conclusion that the 5-HT$_{1B}$ receptor does mediate at least a portion of the 5-HT- and CP93129-induced contraction under conditions of DOCA-salt hypertension. These findings are in agreement with those of MacLean and colleagues in pulmonary hypertension. (MacLean and Morecroft, 2001, MacLean *et al*, 1996, Keegan *et al*, 2001). These studies found that arteries taken from control rats with normal blood pressure in the pulmonary arteries did not respond to agonists of the 5-HT$_{1B}$ receptor. Additionally, the 5-HT$_{1B}$ receptor antagonist GR555562 had no effect on 5-HT-induced contraction in arteries from the control rats. However, 5-HT-induced contraction in arteries from the control rats was sensitive to inhibition by the 5-HT$_{2A/2C}$ receptor antagonist ketanserin. Interestingly, *in vivo* administration of the 5-HT$_{1B/1D}$ receptor antagonist GR127935 (3 mg/kg/day) attenuated the increased right ventricular pressure, right ventricular hypertrophy and pulmonary vascular remodeling in the hypertensive rats (Keegan *et al*, 2001). These data suggest that both the 5-HT_{2A} and 5-HT_{1B} receptors are involved in pulmonary hypertension. There have been no experiments which have examined the effects of *in vivo* administration of a 5-HT_{1B} antagonist on mean arterial blood pressure in the DOCA-salt model of hypertension. Additionally, in atherosclerotic coronary arteries from rabbits and rabbit carotid arteries which undergo collar placement, there is also a hypersensitivity to 5-HT and an increased contractile response to 5-HT_{1B} receptor agonists (Ishida *et al*; 2001, Geerts *et al*, 2000). Collectively, these data all suggest that under conditions of vascular diseases, such as hypertension and atherosclerosis, there is a hypersensitivity to 5-HT and a functional change in the 5-HT_{1B} receptor which contributes to the development and/or maintenance of the diseased state.

5-HT_{2B} Receptors in Hypertension

Data implicating the 5-HT_{2B} receptor in DOCA-salt hypertension has existed for several years. A change in the 5-HT receptor which primarily mediates contraction to 5-HT under the conditions of DOCA-salt hypertension has been suggested largely on the basis of functional data. These data include the use of the 5-$HT_{2A/2C}$ receptor antagonist ketanserin (Watts, 1998). The mesenteric arteries from the hypertensive DOCA-salt rats by day 7 no longer respond to the 5-$HT_{2A/2C}$ receptor antagonist ketanserin with a rightward shift in the 5-HT concentration response curve (Watts, 1998). These data support the idea of the 5-HT_{2B} receptor mediating 5-HT-induced contraction in arteries from hypertensive rats when one considers the low affinity of ketanserin for the 5-HT_{2B} receptor (table 1). The 5-HT_{2B} receptor is not sensitive to inhibition by ketanserin and any 5-HT-induced contraction mediated by this receptor would, therefore, not be inhibited. Further evidence for involvement of the 5-HT_{2B} receptor in hypertension comes from another study by Watts and colleagues in which they demonstrate that ketanserin did not shift the 5-HT-induced contraction in mesenteric arteries from 28 day hypertensive DOCA-salt rats (Watts *et al*, 1996). Additionally, the study by Watts and colleagues also demonstrated that in the rat stomach fundus the 5-HT_{2B} receptor is phenoxybenzamine-insensitive. The 5-HT_{2A} receptor is sensitive to alkylation by phenoxybenzamine as the 5-HT_{2A} receptor mediated contraction in the thoracic aorta from normotensive sham rats was inhibited in the presence of phenoxybenzamine (300 nM). The 5-HT-induced contraction in the arteries from the hypertensive DOCA-salt rats was relatively insensitive to the treatment with phenoxybenzamine (Watts *et el*, 1996).

The idea of the 5-HT_{2B} receptor involvement is also supported by the findings that the 5-HT_{2B} receptor antagonist LY272015 inhibited 5-HT-induced contraction in the aorta from the DOCA-salt hypertensive rats but not the sham rats (Watts, 1997). Further evidence for the involvement of the 5-HT_{2B} receptor comes from the use of the 5-HT_{2B} receptor agonist BW723C86. Watts and Harris showed that BW723C86 elicited a contraction in the mesenteric arteries from DOCA-salt rats but not in their corresponding sham counterparts (Watts and

Harris, 1999). These data suggest that the predominant receptor which mediates 5-HT-induced contraction in the mesenteric artery from DOCA-salt hypertensive rats is the 5-HT_{2B} receptor.

The physiological importance of this receptor is emphasized by the finding that administration of the 5-HT_{2B} receptor antagonist LY272015, *in vivo*, decreased the blood pressure of hypertensive DOCA-salt rats (Watts and Fink, 1999). However, chronic administration of LY272015 to determine whether 5-HT_{2B} receptors are required for development of the increased blood pressure as well as smooth muscle cell hypertrophy and hyperplasia has not yet been done in any model of hypertension. Additional support for the importance of 5-HT_{2B} receptors in the cardiovascular system comes from 5-HT_{2B} receptor knockout mice. 5-HT_{2B} receptor knockout mice show severe cardiovascular abnormalities (Choi *et al*, 1997, Nebigil *et al*, 2001b, Nebigil *et al*, 2000b). However, the use of 5-HT_{2B} receptor knockout mice to study the importance of this receptor in the development of hypertension has also not yet been done. Since 5-HT_{2B} receptors are required for morphogenesis, cell migration and cell cycle progression, one can speculate that 5-HT_{2B} receptors may be important to the vascular changes observed in arteries during the development of hypertension. However, immunohistochemical studies to examine location of the 5-HT_{2B} receptors in arteries from hypertensive rats have not been performed yet. Furthermore, localization of the 5-HT_{1B} and 5-HT_{2B} receptors within the smooth muscle cells, membrane or cytosol, has not been done. These localization studies would address the idea that 5-HT_{1B} and 5-HT_{2B} receptors may not be activated in smooth muscle cells from normotensive rats because they are stored in the cytosol and therefore not able to be activated by an agonist.

Additionally, no studies have been done, to date, which examine the involvement of the 5-HT_{2B} receptors in pulmonary hypertension although the 5-HT_{2B} receptor mRNA has been localized to pulmonary smooth muscle cells with immunohistochemistry (Choi and Maroteaux, 1996). Furthermore, the 5-HT_{2B} receptor has been implicated in the LNNA model of hypertension as well. (Russell and Watts, 1999). These findings suggest that the 5-HT_{2B} receptor may be involved in many models of hypertension and could be a potential therapeutic target for the treatment of established essential hypertension.

Investigation of 5-HT Receptor "Unmasking"

Currently, the mechanisms of receptor "unmasking" are not clearly understood. Additionally, there have been no studies which address the question of why the 5-HT_{1B} receptor requires "unmasking" in some but not all arteries. Furthermore, there are no studies to date which address the phenomenon of "unmasking" with regards to the 5-HT_{2B} receptor. However, the work by Smith and colleagues in the rabbit ear artery would suggest that both the 5-HT_{1B} and 5-HT_{2A} receptors may function as "silent receptors" (Smith *et al*, 1999). This may be due to a lack of coupling to second messenger systems under non-depolarized conditions. They suggest that the depolarization caused by elevated

K^+ may induce an influx of extracellular calcium which in turn enhances the coupling to the second messenger pathways which mediate vasoconstriction (Smith *et al*, 1999). It is not known if the depolarization-dependent "unmasking" is a phenomenon selective for 5-HT_{1B} and 5-HT_{2A} receptors or if other 5-HT receptors might also be "unmasked" by this mechanism.

Based on this information about "unmasking", we hypothesized that membrane depolarization alone would enable an artery from a normotensive sham rat to respond in the same manner to 5-HT and 5-HT_{1B} receptor agonists as an artery from a hypertensive DOCA-salt rat. However contrary to this hypothesis, we were unable to observe a contraction even in the presence of KCl (15 mM) to the rodent selective 5-HT_{1B} receptor agonist CP93129 in arteries from normotensive rats. Therefore, we conclude that these data do not support a role for silent 5-HT_{1B} receptors in the mesenteric arteries from normotensive rats. Additionally, we were also unable to observe a contraction to the 5-HT_{2B} receptor agonist BW723C86 and the 5-HT_{1B} receptor agonists RU24969 and sumatriptan in the rat thoracic aorta from normotensive rats in the presence of KCl (15 mM). The data presented herein demonstrate that acute depolarization alone was not sufficient to enhance arterial responses to agonists of the 5-HT_{1B} and 5-HT_{2B} receptors.

Interestingly, another artery from the rat, specifically the rat tail artery, has been described as requiring "unmasking" to observe a response to 5-HT_{1B} receptor agonists (Craig and Martin, 1993). The difference which enables the tail artery 5-HT_{1B} receptor to be "unmasked" and respond to CP93129 while the aorta and mesenteric artery do not is yet known. However, this difference may involve the ability of the 5-HT_{1B} receptor to activate mechanisms which lead to increases in intracellular calcium levels.

The role of calcium in 5-HT_{1B} receptor –mediated contraction appears to vary between species and vessel. In the rabbit ear artery 5-HT_{1B} receptor-induced contraction, following "unmasking" with preconstriction with the α_1 adrenergic receptor agonist phenylephrine, requires extracellular calcium and activation of L-type calcium channels (Movahedi and Purdy, 1997). However, in the rabbit saphenous vein contraction to 5-HT_{1B} receptor agonists is insensitive to the L-type calcium channel blocker nifedipine (Razzaque *et al*, 1995). Additionally, in vascular smooth muscle cells from the bovine basilar artery, 5-HT_{1B} receptor stimulation induces release of calcium from intracellular stores. In contrast, stimulation of the rabbit mesenteric artery and the rabbit renal artery fails to result in any release of intracellular calcium and appears to be dependent on L-type voltage gated calcium channels (Seager *et al*, 1994, Hill *et al*, 2000).

It is interesting to note that the 5-HT_{2B} receptor has been linked to activation of L-type calcium channels for contraction in the rat stomach fundus (Cox and Cohen, 1995). The signaling pathway used by the 5-HT_{1B} and 5-HT_{2B} receptors to activate the L-type calcium channels and intracellular calcium stores has not yet been elucidated. However, if these two receptors simultaneously couple to activation of different mechanisms which would increase intracellular calcium levels in vascular smooth muscle cells it might, in part, explain their combined contribution to the hyperresponsiveness to 5-HT observed in arteries from hypertensive DOCA-salt rats and the lack of effect in vessels from normotensive rats.

Increased Levels of 5-HT$_{1B}$ and 5-HT$_{2B}$ Receptor Proteins in DOCA-salt Hypertension

Based on the contractile data which suggested a functional change in the 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors, we speculated that an increase in the expression of these receptors in vascular smooth muscle which would provide an explanation for the physiological and pharmacological data which indicate a change in the complement of contractile serotonergic receptors in vascular smooth muscle under conditions of hypertension. These molecular data demonstrate an increase in the levels of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptor proteins under conditions of established DOCA-salt hypertension in the thoracic aorta as well as the mesenteric artery. These are the first studies which have demonstrated on increased level of 5-HT$_{2B}$ and 5-HT$_{1B}$ receptor proteins in a diseased state.

Regulation of 5-HT$_{1B}$ and 5-HT$_{2B}$ Receptors

With the knowledge of an increased level of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptor protein levels we next proposed to determine what factors might contribute to this increase in receptor protein levels. There are currently no studies which have investigated the regulation of either of these receptors at the transcriptional and translational levels. Analysis of the rodent 5-HT_{1B} promoter revealed that there are multiple mineralocorticoid response elements (MRE's) located in the promoter (Hamblin *et al*, 1992). Additional analysis also revealed that the rodent 5-HT_{2B} receptor promoter also contains two MRE's (Foguet *et al*, 1992). This is relevant to the regulation of these receptors in a disease state such as in the DOCA-salt model, where hypertension is induced by the use of mineralocorticoids, as well as other models of hypertension where elevated levels of aldosterone may influence expression of this receptor. As previously mentioned, aldosterone has been implicated in increasing the expression of the K-ras (Stockand *et al*, 1999). Aldosterone also decreases the mRNA expression of c-Myc, c-Jun, c-Fos by post-transcriptional mechanisms while increasing Fra-2 mRNA by a transcriptional mechanism in epithelial cells (Verry *et al*, 2000). This is interesting to note because it suggests that aldosterone may act directly at a promoter *via* the MRE as well as indirectly through its actions on transcription factors.

Additionally, aldosterone induces methylation of ras in renal epithelial cells (Al-Baldawi *et al*, 2000). This suggests that aldosterone not only changes the level of protein expression but also the state of activation of proteins involved in signaling cascades. While the signaling pathways used by the 5-HT_{1B} and 5-HT_{2B} receptors to cause contraction are currently unknown, both of these receptors have been shown to utilize the ERK/MAPK pathway, of which ras is a member, for mitogenesis. Whether aldosterone is acting directly at the MRE's or through its actions on other transcription factors to cause the upregulation of the 5-HT_{1B} and 5-HT_{2B} receptors *in vivo* is unclear as there is no increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor mRNA. However, the lack of an increase in mRNA is not conclusive as we did not measure the rate of mRNA degradation. The 5-HT_{1B} receptor promoter also contains eight basic helix loop helix (bHLH) sites, one glucocorticoid response element (GRE), several SP-1 sites as well as several AP-1 sites. The 5-HT_{2B} receptor promoter contains ten bHLH sites, SP-1 sites and multiple AP-1 sites. Transcription factors from the bHLH family include a wide variety of proteins such as the aryl hydrocarbon receptor nuclear transporter (ARNT) (Huffman *et al*, 2001), Myc (Miethe *et al*, 2001) and MyoD (Chen *et al*, 2001) among many others. Animals have been shown to contain members of 36 subfamilies of bHLH transcription factors, of the 44 subfamilies which have been defined (Ledent and Vervoot, 2001).

Interestingly, another transcription factor, the SP-1 transcription factor, is a target for $NO^•$ in vascular smooth muscle cells (Sellak *et al*, 2002). $NO^•$ was shown to inhibit the binding of SP-1 to the cGMP-dependent protein kinase (PKG) promoter (Sellak *et al*, 2002). Thus, in conditions such as hypertension where the increase in reactive oxygen species may affect the bioavailability of $NO^•$, transcriptional regulation of genes by $NO^•$ may be affected contributing to the changes observed in this disease. Collectively, this information suggests that characterization of these promoters and the transcriptional mechanisms of regulation of these receptors is an undertaking which will be both time and labor intensive.

It is also unknown at this point if aldosterone affects the members of the signaling cascades used by the 5-HT_{1B} and 5-HT_{2B} receptors to cause contraction. This is an intriguing possibility since the ability of the 5-HT_{1B} and 5-HT_{2B} receptors to mediate contraction does not appear to be dependent on the level of the receptor protein. We initially speculated that upregulation of 5-HT_{1B} and 5-HT_{2B} receptor proteins was, at least partially, responsible for the increased contractility to 5-HT seen in the arteries from hypertensive rats. Our data would argue that an increase in the level of receptor protein is not sufficient to allow these receptors to participate in contraction. Additionally, the data obtained in the DOCA time course studies *in vivo* demonstrate that contraction can occur in the absence of an increase in the receptor protein.

The mechanism by which the protein levels of the 5-HT_{1B} and 5-HT_{2B} receptors are increased may not to involve an increase in mRNA levels as these were unchanged in the Real Time PCR experiments from the rat thoracic aorta. However, a study by Watts and colleagues demonstrated an 2-fold increase in 5-HT_{2B} receptor mRNA in the mesenteric artery from hypertensive DOCA-salt rats (Watts *et al*, 1996). While the protein levels are increased in both the aorta and mesenteric artery (% increase from sham 218% and 171%, aorta and mesenteric artery, respectively), these data do not reflect the changes observed in the levels of 5-HT_{2B} receptor mRNA. Interestingly, in the mesenteric artery one observes a greater enhancement of the 5-HT-induced contraction than that observed in the aorta (figure 4). However, these data do not address the issues of mRNA degradation. Therefore, based on the data presented herein we can not completely rule out transcriptional regulation of the 5-HT_{1B} and 5-HT_{2B} receptors. Furthermore, these findings further support the idea that hyperresponsiveness to 5-HT involves the 5-HT_{2B} and 5-HT_{1B} receptors through mechanisms involving more than just an increase in the level of receptor protein. There appear to be changes in the signaling mechanisms and/or changes in the receptor-effector coupling mechanisms.

Speculation

These data suggest that in addition to the upregulation of 5-HT_{1B} and 5-HT_{2B} receptors, other changes must be occurring in the vascular smooth muscle to enable the contractile responses observed. Additional second messengers which may be involved in 5-HT_{2B} and 5-HT_{1B} receptor signaling as second messengers in vascular smooth muscle but have not yet been thoroughly examined include reactive oxygen species, the Rho-Rho-kinase pathway and MUPP1 (figure 34).

The use of reactive oxygen species as a signaling mechanism by 5-HT_{2B} and 5-HT_{1B} receptors has not yet been investigated. Interestingly, H_2O_2 mediates calcium-dependent contraction in canine basilar arteries by a mechanism which involves PKC and PI-3 kinase (Yang *et al*, 1999). The 5-HT_{2B} receptor is known to couple to PKC activation in the rat stomach fundus to cause contraction (Cox and Cohen, 1995). Therefore, the involvement of H_2O_2 in the contractile signaling pathways used by the 5-HT_{2B} receptor should be investigated. Furthermore, the 5-HT_{2B} receptor and the 5-HT_{2A} receptor use many of the same signaling pathways. 5-HT_{2A} receptors are known to use H_2O_2 and O_2^- (Lee et al, 2001, Greene et al, 2000). If activation of the 5-HT_{2B} or 5-HT_{1B} receptors leads to an increased generation of reactive oxygen species the results may be an increase in contraction as well as a decrease in the available $NO^{\bullet}$ with a reduction in vasorelaxation. Additionally, since the involvement of reactive oxygen species has been implicated in many models of hypertension (Zalba *et al*, 2001, Makino *et al*, 2002, Gao and Lee, 2001), this merits further investigation.

Another second messenger which should be examined further in regards to 5-HT–induced contractile signaling is the Rho-Rho-kinase pathway. The involvement of the Rho-Rho-kinase pathway in calcium sensitization induced by agonist stimulation (Hirata *et al*, 1992) has been suggested and provides another area which requires further study. While the mechanisms of calcium sensitization induced by agonist stimulation are not clearly understood this may be involved in the hyperresponsiveness to 5-HT observed in vessels from hypertensive rats. Interestingly, in mesenteric arteries from hypertensive DOCA-salt rats contracted with 5-HT, there is an enhanced relaxation to the Rho-kinase inhibitor Y-27632 (Weber and Webb, 2001). The results from this study suggest that the enhanced 5-HT-induced contraction may be due to involvement of the Rho-Rho-kinase pathway. Additionally, these data also suggest an increased level of Rho-Rho-kinase activity. Therefore, examining the ability of 5-HT_{2B} and 5-HT_{1B} receptors to couple to this pathway maybe of importance in disease states, such as hypertension, where there is an upregulation and change in the functional coupling of the 5-HT_{2B} and 5-HT_{1B} receptors.

Lastly, the PDZ-dependent signaling mechanisms used by the 5-HT_{2B} receptor also merits further study. To date, 5-HT_{2B} receptors have been shown to activate nitric oxide synthases *via* its group I PDZ motif located at the C-terminus (Manivet *et al*, 2000). This receptor-mediated stimulation of iNOS requires the 5-HT_{2B} receptor to be coupled to the $G\alpha_{13}$ (Manivet *et al*, 2000). The loss of a vasodilator, specifically $NO^{\bullet}$ from endothelial cells potentially, through a 5-HT_{2B} receptor, in arteries from hypertensive rats may contribute to the enhanced arterial tone. Interestingly, the MUPP1 protein, a PDZ binding protein which has 13 PDZ domains, interacts with the 5-HT_{2B} receptor (Bécamel *et al*, 2001). The presence of MUPP1, which may serve as a scaffolding protein, in the vasculature of hypertensive DOCA-salt rats has not been examined at this time. The MUPP1 protein appears to be involved in clustering of 5-HT_{2C} receptors at the cell membrane (Bécamel *et al*, 2001). This clustering of receptors may be required for dimerization of receptors as well as proper interaction with effector molecules. While no studies have yet been performed to examine if 5-HT_{2B} receptors form dimers, it has been established that 5-HT_{1B} receptors can form both hetero and homodimers. Dimerization affects the interaction of the receptor with G-proteins, agonists and members of the signaling pathways. Therefore, the involvement of MUPP1 and the ability of the 5-HT_{2B} receptor to form dimers should be studied.

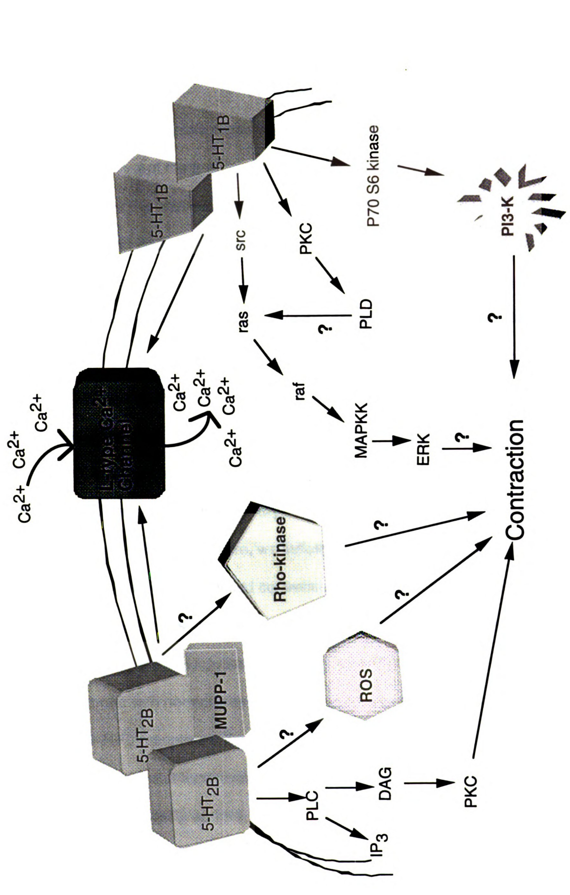
Furthermore, the class III Mint-1 protein, a PDZ domain containing scaffolding protein, has been linked to N-type Ca^{2+} channels (Maximov *et al*, 1999). The interaction of L-type Ca^{2+} channels and PDZ-domain containing proteins has not yet been investigated. However, it has been shown that both the 5-HT_{2B} and the 5-HT_{1B} receptors can couple to L-type calcium channels. The exact mechanisms of this interaction have not yet been elucidated. Additionally, in the family of membrane-associated guanylate kinases (MAGUKs), of which CASK is a member, PDZ-domains are found together with src homology (SH-3) domains (Li *et al*, 2002). Many adaptor molecules have SH-3 and SH-2 domains which facilitate protein-protein interactions. Proteins with PDZ-domains have been shown to interact with PLC and PKC (Bähner *et al*, 2002, Huber *et al*, 1998). Additionally, the interactions of scaffolding proteins with the 5-HT_{1B} receptor have also not yet been examined. However, this interaction between 5-HT receptors and their mechanisms of coupling to their signaling pathways may be an interesting avenue of future research.

Furthermore, changes in calcium channel density and/or function may also participate in the enhanced 5-HT-induced contraction and BW723C86- and CP93129-induced contractions observed in arteries from DOCA-salt hypertensive rats. In mesenteric arteries from hypertensive DOCA-salt rats L-type calcium channel expression was increased as compared to sham rats

(Molero *et al,* 2001). Changes in function of the calcium channels have also been suggested in several models of hypertension (Hermsmeyer, 1993, Matsuda *et al,* 1997, Cox and Lozinskaya, 1995). These changes in calcium channel density and function may contribute to the changes in the functional coupling of the 5-HT_{1B} and 5-HT_{2B} receptors. The combination of changes in calcium channel function and/or number combined with changes in the signaling pathways, such as Rho-Rho-kinase and calcium sensitization induced by agonist stimulation, may be important to the hyperresponsiveness to 5-HT observed in hypertension.

Physiologically, the changes in the levels of the 5-HT_{2B} and 5-HT_{1B} receptors and their functional coupling may contribute to either the development and/ or maintenance of hypertension. The ability of the 5-HT_{2B} receptor antagonist LY272015 to lower the blood pressure of DOCA-salt hypertensive rats suggests endogenous activation of this receptor to maintain the increased blood pressure (Watts and Fink, 1999). While no 5-HT_{1B} receptor antagonists have been studied *in vivo* in the DOCA-salt model of hypertension, they have been used to study pulmonary hypertension. 5-HT has been implicated as a causative agent in pulmonary hypertension (Egermayer *et al,* 1999, Kereveur *et al,* 2000, MacLean *et al,* 2000, Miyata *et al,* 2000). The administration of 5-HT_{1B} receptor antagonists attenuate the pulmonary hypertension (Miyata *et al,* 2000). The common finding between these two models of hypertension, pulmonary and DOCA-salt-induced, is an increase in the level of free 5-HT in the plasma. These findings suggest that increases in plasma free 5-HT may be a marker for future 5-HT based treatments. These studies suggest that 5-HT_{1B} and 5-HT_{2B} receptors are involved in mediating the arterial hyperresponsiveness to 5-HT observed in hypertension. This involvement appears to be due to changes independent of the level of receptor proteins as the functional response of contraction can be separated from an increase in the level of 5-HT_{2B} and 5-HT_{1B} receptor proteins. Currently, there is no precedent for receptors to be present but not activatable under conditions of normal blood pressure but which are involved as mediators of contraction and the development of a pathological condition such as hypertension.

Figure 36. Speculations on changes in signaling pathways which may be involved in 5-HT-induced contraction *via* the 5-HT_{2B} and 5-HT_{1B} receptors in arteries from DOCA-salt hypertensive rats. Abbreviations: ERK= Extracellular signal regulated kinase; PKC= Protein Kinase C; MUPP1= multi-PDZ domain protein 1; PLC= Phospholipase C; DAG= Diacylglycerol; IP3= Inosital triphosphate; ROS= Reactive oxygen species; MAPKK= Mitogen activated protein kinase kinase; PLD= Phospholipase D; PI3-K= Phosphoinosital 3 kinase.


5-HT1B
5-HT1B
src
PKC
P70 S6 kinase
PI3-K
ras
PLD
?
?
raf
MAPKK
ERK
?
Contraction
L-type Ca2+ Channel
Ca2+
Rho-kinase
?
?
?
ROS
?
5-HT2B
MUPP-1
5-HT2B
PLC
DAG
PKC
IP3

Conclusion

The main goals of this thesis were to investigate the mechanisms of the increase in the level of the 5-HT_{2B} and 5-HT_{1B} receptor proteins in DOCA-salt hypertension and the physiological significance of these changes. The 5-HT_{2B} receptor has been implicated as a possible mediator of the maintained increase in pressure observed in DOCA-salt hypertension. Additionally, hyperresponsiveness to 5-HT observed in arteries from hypertensive rats is another a physiological phenomenon which we investigated to determine if the 5-HT_{2B} and 5-HT_{1B} receptors were involved in the mechanisms of hyperresponsiveness.

5-HT is a known mitogen and vasoconstrictor. With the use of 5-HT receptors and transporters as drug targets, it is necessary to understand how 5-HT mediates its actions. Therefore, we initially characterized the 5-HT receptors which are involved in 5-HT-induced contraction under conditions of both normal blood pressure as well as hypertension. From the studies presented herein we conclude that only the 5-HT_{2A} receptor mediates 5-HT-induced contraction in arteries from rats with normal blood pressure. We also conclude that the 5-HT_{2B}, 5-HT_{1B} and 5-HT_{2A} receptors are involved in 5-HT-induced contraction under conditions of DOCA-salt hypertension. We were unable to detect any involvement of the 5-HT_{1D} and 5-HT_{1F} receptors under either conditions. We also conclude that the 5-HT_{1B} and 5-HT_{2B} receptors in the thoracic aorta of normotensive rats can not be "unmasked" to participate in the 5-HT-induced contraction.

We initially speculated that increases in the level of 5-HT_{1B} and 5-HT_{2B} receptor proteins were, at least partially, responsible for the increased contractility to 5-HT seen in the arteries from hypertensive rats. However, the results of our aldosterone incubation studies and the DOCA-salt time course studies do not support this idea. These data support the argument that an increase in the level of 5-HT_{1B} and 5-HT_{2B} receptor proteins are not necessary to observe an increased contractile response to agonists of these receptors. However, there is an increased contractile response to the 5-HT_{2B} receptor agonist BW723C86 prior to an increase in blood pressure in the DOCA and salt treated rats. Therefore, while the 5-HT_{2B} and 5-HT_{1B} receptor protein levels are increased after four weeks of DOCA-salt treatment, the precise role of these receptors in the development of hypertension and possibly vascular remodeling are yet to be elucidated.

The studies presented here also support the idea that mineralocorticoids in conjunction with increases in pressure and salt may be important mediators of the changes to both the level of 5-HT_{2B} and 5-HT_{1B} receptor protein levels and the signaling mechanisms utilized by these receptors. Aldosterone incubation, *in vitro,* produced an increase in the level of the 5-HT_{1B} and 5-HT_{2B} receptor protein levels. The *in vivo* time course experiments, however, did not show any increase in the level of the 5-HT_{1B} receptor protein levels. Additionally, only the DOCA-salt rats showed an increase in the level of the 5-HT_{2B} receptor protein. These data suggest that *in vivo* regulation mechanisms of these receptors maybe more complicated than *in vitro* experiment suggested as elevated levels of mineralocorticoids alone *in vivo* was not sufficient to increase the levels either the 5-HT_{1B} or the 5-HT_{2B} receptor proteins. These data suggest that *in vivo* mineralocorticoids may not be acting directly at the transcriptional level. However, as we have not measured mRNA degradation or done an RNAse protection assay, we can not rule out the possibility of transcriptional regulation.

The functional changes resulting from a change from a 5-HT_{2A} receptor to the addition of the 5-HT_{2B} and 5-HT_{1B} receptors have significant physiological ramification. 5-HT has a 300 fold higher affinity for the 5-HT_{2B} receptor than for the 5-HT_{2A} receptor. When comparing the 5-HT_{2B}, 5-HT_{1B} and 5-HT_{2A} receptors, 5-HT has the greatest affinity for the 5-HT_{2B} receptor followed by the 5-HT_{1B} receptor (table 1). This increased sensitivity to 5-HT in hypertension coupled to the possibility of an increase in free 5-HT, as observed in the HPLC experiments, and increased level of receptor proteins creates a situation where 5-HT may be acting endogenously to mediate an increase in total peripheral resistance. This increased 5-HT receptor activation may contribute either to the development or maintenance of hypertension. The finding that the 5-HT_{2B} receptor antagonist LY272015 can act as an antihypertensive agent in weeks three and four of DOCA-salt hypertension suggests that this may be an important receptor to consider in the treatment of established hypertension. While 5-HT_{1B} receptor antagonists have not been tested in the DOCA-salt model of hypertension, they are effective in the treatment of pulmonary hypertension. If the 5-HT_{1B} receptor antagonists prove effective in lowering blood pressure of the DOCA-salt hypertensive rats, this could create another potential target for therapy. This project is also significant in that the determination of serotonergic receptors which mediate vascular contraction will allow for targeting of drug therapy for migraine with fewer systemic side effects. One of the major side effects reported for migraine treatment with Imitrex®, also known as sumatriptan, is chest pain associated with vasoconstriction of 5-HT_{1B} receptors (Ottervanger *et al*, 1998). With the increasing interest in utilizing 5-HT receptors as targets for treatment of neurological disorders, such as depression, determining the serotonergic receptors which mediate contraction to 5-HT in the vasculature of normotensive and hypertensive subjects is imperative. Understanding of the regulation of 5-HT receptors, their signaling pathways and functions in disease states may contribute to advances in these fields of therapeutic research.

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