OCCIPITAL ARTERY FUNCTION DURING THE DEVELOPMENT OF
2-KIDNEY, 1-CLIP HYPERTENSION IN THE RAT

by

STEPHEN P. CHELKO

(Under the Direction of Thomas P. Robertson)

ABSTRACT

Renovascular hypertension (RVH) is the most common form of secondary hypertension. Decreased renal perfusion activates the renin-angiotensin-aldosterone system, increases vascular resistance as a result of angiotensin II (AII)-dependent constriction of resistance arteries, reduces the sensitivity of the baroreflex system, and increases aldosterone- and arginine vasopressin (AVP)-induced sodium and water retention. Although these aspects of RVH have been known for decades, further research is required to elucidate the precise mechanisms responsible for the development of RVH. Recently, it has been reported that blood-borne factors can gain access to the nodose ganglion exclusively by the occipital artery (OA). Since the nodose ganglion plays an important role in blood pressure homeostasis, this thesis is centered around the hypothesis that the OA may also play a similarly vital role in blood pressure regulation in health and disease.

This dissertation details 1) the first characterization of the contractile responses of OA of the rat, 2) the development of a novel vascular clip designed to provide significant improvements to the most commonly-used model of RVH (2-kidney, 1-clip, 2K1C), and 3) the effects of RVH on the function of rat OA.
The results of these studies are consistent with AII, AVP and 5-hydroxytryptamine (5-HT) induced OA vasoconstriction being mediated by AII$_1$, AVP$_1$ and 5-HT$_2$ type receptors, respectively. Additionally, the design elements of the novel vascular clip prevented dislodgment after implantation, and produced reliable increases in blood pressure and plasma renin concentrations in 2K1C rats. In 2K1C rats, RVH-associated alterations in OA contractile function were both agonist- and segment-specific. Collectively, these studies represent the first steps to elucidating the role of the OA in blood pressure regulation in a reliable model of RVH.

INDEX WORDS: Renovascular hypertension; 2-kidney, 1-clip; Occipital artery; Renin-angiotensin-aldosterone system; Vascular reactivity
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DEDICATION

Dedicated to

my mother, Carla Pitts Chelko,
for her faith in me, encouragement, and unconditional love;

and my partner, Danny Lenox Fay,
for his emotional support, unquestioned trust, and continual love
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CHAPTER 1
INTRODUCTION

High blood pressure is a major cause of morbidity and mortality affecting more than one-third of adults in the United States every year. Renovascular hypertension (RVH) is the most common cause of secondary hypertension, resulting from a decrease in renal blood flow and activation of the renin-angiotensin-aldosterone system (RAAS). RVH is associated with increases in blood volume, via sodium and water retention, and circulating vasoconstrictors which decrease resistance artery diameter, thereby raising vascular resistance and arterial blood pressure. There are numerous causes of RVH such as renal arterial stenosis, renal parenchymal disease, fibromuscular dysplasia, hyperglycemia and hyperlipidemia, and alcohol or drug abuse. Given the prevalence of this disease, it is vital that experimental models accurately and reliably reflect the pathogenesis of RVH.

One of the most commonly-used models to study RVH is the two-kidney, one-clip (2K1C) technique. First described by Goldblatt et al. in 1934, the original 2K1C technique involved application of variable size silver clips to partially reduce the diameter of renal arteries in dogs and monkeys. (39, 40) Decreased kidney perfusion activates granular cells, located in the juxtaglomerular apparatus of renal afferent arterioles, causing renin release. Renin cleaves angiotensinogen into angiotensin I, which is subsequently converted into the potent vasoconstrictor angiotensin II (AII) by angiotensin-converting enzyme (ACE). Circulating AII causes hypertension by: 1) eliciting smooth muscle constriction in resistance arterioles, thereby
raising systemic vascular resistance, and 2) renal absorption of sodium and water via arginine vasopressin (AVP) and aldosterone released from the pituitary and adrenal glands, respectively, thereby raising blood volume.

In addition to increasing AII synthesis, decreased renal perfusion activates renal baroreceptors (BR). decreases renal-afferent nerve activity, which in turn, increases renal-efferent sympathetic nerve (ESN) activity, known as the renorenal reflex. (68, 70) Furthermore, activation of ESN, innervating granular cells of the juxtaglomerular apparatus, causes renin secretion via norepinephrine release and activation of β1-adrenergic receptors.(69, 70) Although BR are present in visceral organs, they are most abundant in the aortic arch and carotid bodies. BR respond to changes in blood flow and arterial stretch, and are responsible for maintaining blood pressure homeostasis. Blood pressure shifts from a normotensive to a hypertensive state as a result of: 1) prolonged exposure of systemic arteries to vasoconstricting hormones, and 2) the reduction in BR sensitivity due to chronic stimulation of renal-afferent nerves by the sympathetic system. When stimulated, as in 2K1C hypertension, BR relay the change in blood pressure to sensory afferent cell bodies of the vagus and glossopharyngeal nerves, such as the nodose (NG) and petrosal ganglion (PG), respectively.

The function of the vagal and glossopharyngeal cell bodies is vital for the maintenance and regulation of blood pressure and blood flow and thus the vasculature that supplies them is also important. The occipital artery (OA) is one of three arterial branches arising from the common carotid artery (CCA), along with the internal carotid (ICA) and external carotid (ECA) arteries. The ICA and OA supply blood to the NG and PG. However, recent research has demonstrated that low molecular weight markers can gain direct access to the cells bodies of the NG and PG via the OA, but not via the ICA.(71) This is consistent with the concept that the OA
may play an important role in the regulation of NG and PG function and, in turn, blood pressure homeostasis. Indeed, the OA has recently become an area of interest in the development of 2K1C hypertension. Lacolley et al. demonstrated that 2K1C rats with bilateral OA ligations had significantly higher mean arterial pressures compared to 2K1C rats with sham OA ligations or normotensive rats with either bilateral or sham OA ligations.(71)

Consequences of hypertension, such as myocardial infarctions, left ventricle hypertrophy, stroke, congestive heart failure, and/or renal failure are extremely detrimental to the body. The 2K1C model is clinically relevant as it mimics unilateral renal arterial stenosis and has provided valuable insights into the pathogenesis of RAAS-associated hypertension. Since vascular reactivity plays a central role in the control of blood pressure, several studies have detailed vascular changes in blood vessels from hypertensive animals, including aorta,(88, 98, 118, 127) common carotid,(99, 127) and mesenteric(82, 91, 108, 118) arteries. However, there have been no in vitro studies regarding the contractile properties of the OA isolated from rats. Determining the contractile properties of the OA will help elucidate the role of this potentially important blood vessel in the development of hypertension. The overall goal of this project is to provide novel insights into the contractile properties of the OA isolated from control, sham and 2K1C hypertensive rats.

The goal of this project will be met by achieving the following specific aims:

**AIM 1** To determine the contractile effects of AII, arginine vasopressin (AVP), and 5-hydroxytryptamine (5-HT) on OA isolated from male Sprague-Dawley rats using small vessel myography.
AIM 2 To elicit reliable hypertension via unilateral renal hypoperfusion utilizing an improved 2K1C methodology in rats.

AIM 3 To determine the contractile properties of the OA isolated from sham and 2K1C rats, 9 days following renal artery clipping.
CHAPTER 2
LITERATURE REVIEW

SECTION I: RENOVASCULAR HYPERTENSION

Prevalence of Disease

Hypertension increases a patient’s risk of developing heart disease and stroke, the first and third leading cause of death in the United States. (85, 122) It is estimated that every forty seconds someone in the United States will have a stroke and 13% of these individuals will experience a second stroke within five years. (85) Heart disease and stroke are more prevalent among men than women, (97) and this risk is exacerbated in African-Americans who have a higher incidence of developing hypertension than Caucasians. (122) The World Health Organization estimates that over 17 million people in the world die from cardiovascular diseases every year. The American Heart Association’s Statistical Committee and the American Stroke’s Statistical Subcommittee estimates the United States spent $503.2 billion on cardiovascular diseases in 2010. (81)

Although primary (essential) hypertension is idiopathic and thus harder to diagnose and treat, RVH is the most common cause of secondary hypertension. (121) RVH is a form of hypertension secondary to stenotic or obstructive lesions in renal arteries. (9) Renal artery stenosis decreases renal blood flow and afferent-arteriole pressure thereby activating the RAAS. (48) Individuals with a history of hypertension (diastolic > 100mmHg) are 50 - 60% more likely to develop
atherosclerotic renal arterial stenosis (ARAS) which affects nearly 90% of RVH cases. ARAS is a chronic arterial inflammatory response due to fatty acid (cholesterol and lipid) accumulation in renal arteries which impedes blood flow. Renocardiovascular complications typically remain asymptomatic until stenosis causes a 75% reduction in renal arteriole blood flow. Abnormally high fats, sugars and sodium in the circulation, (hyperlipidemia, hyperglycemia and hypernatremia, respectively), sedentary lifestyle, drug and alcohol abuse, diabetes mellitus, obesity and atherosclerosis have all been shown to activate the RAAS which increases cardiac output and fluid retention, increasing blood volume.

**Renal and Cardiovascular Consequences**

Decreased renal perfusion increases systemic blood pressure via activation of the RAAS and sympathetic nervous system (SNS). Activation of the RAAS is the primary cause of the increase in blood pressure, which is then amplified by further pathophysiological changes, such as increased oxidative stress and circulating endothelin-1, vascular remodeling and parenchymal disease. Renal ischemia and the formation of microvascular atheromatotic plaques cause renal parenchymal disease, which in turn results in a further deterioration in renal function.

As previously mentioned, obesity and diabetes are associated with an increased incidence of chronic kidney disease and end-stage organ failure. Diabetic individuals are hyperglycemic, which is the result of either a lack of insulin production by the pancreas (type-1 diabetes) or the presence of insulin resistance (type-2 diabetes). Hyperglycemia is associated with increases in vascular resistance, blood volume and blood pressure, further weight gain, decreased glucose
tolerance, insulin and leptin resistance, activation of the RAAS and SNS and endothelial
dysfunction.\textsuperscript{(31, 67, 80, 86)}

Hyperglycemia increases intracellular protonated nicotinamide adenine dinucleotide
(NADH) levels, which results in more NADH, an electron donor, being pushed into the electron
transport chain. This increase causes a change in the membrane potential until a critical threshold
is reached whereupon Complex III is inhibited, which in turn increases mitochondrial production
of reactive oxygen species (ROS), such as superoxide.\textsuperscript{(131)} Although hyperglycemia is not
always a contributing factor in renal diseases, increased ROS production \textit{per se} is associated
with diabetic nephropathy and chronic kidney disease and may play a critical role in the
development of RVH.

The vascular endothelium plays a vital role in the local regulation of blood flow.
Endothelial dysfunction is associated with increased ROS production, elevated circulating
endothelin-1 (ET-1), decreased nitric oxide (NO) production, and alterations in vascular function
and structure.\textsuperscript{(80)} ET-1 can increase or decrease vascular tone via activation of vascular ET\textsubscript{A}
receptors or ET\textsubscript{B} receptors, respectively.\textsuperscript{(31, 134)} Increased ET-1 production, coupled with
endothelial dysfunction, often results in an overall increase in vascular tone due to the decrease
in ET-1 stimulated NO release.\textsuperscript{(111)}

Accumulation of cholesterol and lipids in the circulation leads to plaque formation and
pervasive cardiovascular problems, such as thrombosis and intimal cell proliferation.\textsuperscript{(14, 43, 64,
98, 110, 114)} Intimal cell proliferation is a major consequence of hypertension and is
characterized by mitogenic growth of vascular smooth muscle cells from the intima to media,
thus reducing blood flow by reducing lumen diameter.\textsuperscript{(88)} Additionally, ACE mRNA, low-
density lipoproteins and advanced glycation end-products have been found in common carotid
atherosclerotic plaques of obese and diabetic individuals with renal disease, which exacerbate the effects of plaque formation. These plaques not only contribute to vascular dysfunction but can alter blood flow, blood pressure and BR activity (see \textit{Neuronal and Hormonal Regulation} section).

Another major consequence of RVH is left ventricle hypertrophy (LVH). In order for the heart to effectively supply blood to the body in the face of increased blood pressure, the left ventricle must contract with a greater force. This increase in load on the cardiac myocytes leads to hypertrophy of these cells, in turn leading to LVH, which is often observed in patients with hypertension, aortic or coronary stenosis, obesity and diabetes.\textsuperscript{(1, 15)} As such, LVH is the result of hypertrophic cardiomyopathy in which the cells of the myocardium (cardiomyocytes) undergo abnormal enlargement. Increased blood pressure causes the left ventricle to forcefully expel a larger stroke volume via increased ventricle contractions. However, this promotes sarcomere synthesis which, in turn, results in increased cardiomyocyte size due to increases in the number of myosin-actin cross-bridges. Initially, these effects cause thickening of the ventricular wall, thereby increasing the level of contractile force that can be achieved, but over time the hypertrophic myopathy leads to heart failure. Additionally, systemic and/or local AII has been implicated in myocardial fibrosis, another contributor to the development of LVH.\textsuperscript{(8)} Hardening of the heart valves due to inappropriate fibroblast proliferation and increased synthesis of type I collagen was demonstrated by Weber \textit{et al.} in the hypertrophied left ventricle of renovascular hypertensive rats.\textsuperscript{(129)} ACE inhibitors and AII type-1 receptor (AII\textsubscript{1}R) blockers have shown to reverse myocardial fibrosis.\textsuperscript{(41)} A 2003 meta-analysis showed that angiotensin receptor blockers and ACE inhibitors were the first and third most efficacious in reducing left ventricular mass in patients with essential hypertension.\textsuperscript{(65)}
In the adrenal gland circulating AII influences the synthesis and release of aldosterone into the bloodstream. In order for bodily organs to function properly, the extracellular environment must have an appropriate concentration of ions to regulate blood volume and cell membrane potential. In addition, an adequate supply of metabolic substrates, such as glucose and oxygen, is required for cells to produce ATP. For example, ATP is essential for the production of cAMP which activates Protein Kinase A and stimulates mitochondrial uptake of cholesterol in the adrenal medulla for steroidogenesis.(107) The adrenal medulla is comprised of two distinct regions, namely the cortex and medulla. The adrenal cortex is comprised of three layers of cells, the zona glomerulosa which produces aldosterone and the zona fasciculata and zona reticularis which produce cortisol and corticosterone.(107) Surrounding the adrenal vasculature are chromaffin cells, in which preganglionic stimulation causes epinephrine and norepinephrine release into the adrenal venous sinuses. Although adrenocorticotropic hormone (ACTH) can bind to ACTH receptors on zona glomerulosa cells and stimulate aldosterone synthesis and secretion, AII is the primary regulator of aldosterone synthesis in these cells. Angiotensin II binding activates phospholipase C (PLC) which in turn cleaves phosphatidylinositol 4,5 bisphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol triphosphate (IP$_3$). Intracellular calcium (Ca$^{2+}$) levels rise through IP$_3$-dependent release of Ca$^{2+}$ from intracellular stores via activation of IP$_3$ receptors on the endoplasmic reticulum. Calcium activates calmodulin-dependent protein kinase whereas DAG activates Protein Kinase C (PKC). These two enzymes stimulate mitochondrial uptake of cholesterol where it is converted to aldosterone and is secreted into the adrenal venous sinus via cAMP-dependent mechanisms.(107) Aldosterone promotes sodium and water retention and excretion of potassium in renal collecting duct cells through stimulation of the H$^+$/ATPase
pump. The retention of sodium and water increases blood volume which contributes to the increase in blood pressure.\( (42, 52, 60) \)

Increased plasma concentrations of AII and aldosterone can eventually lead to glomerulosclerosis, which refers to the thickening and hardening of the glomerular basement membrane.\( (72) \). The glomeruli of the kidneys are comprised of arterioles and capillary beds that are under constant high pressure (glomerular hydrostatic capillary pressure of 55mmHg compared to skeletal muscle capillary pressure of 25mmHg) that facilitates the filtration of urea and other metabolites from the blood. Therefore, decreases in afferent renal blood flow or dilation of efferent-arterioles will result in a decline in the glomerular filtration rate (GFR). Direct microinjection of AII in isolated perfused kidneys reduces GFR by constricting afferent-arterioles, in turn decreasing proximal blood flow and reducing glomerular hydrostatic capillary pressure. The perfused kidneys had progressive and sustained decreases in renal blood flow via afferent constriction and increased filtration fraction via efferent constriction, and proteinuria.\( (72) \) The net effect of AII-dependent vasoconstriction on afferent- and efferent-arterioles determines GFR.\( (27) \) For example, efferent vascular resistance far exceeds afferent vascular resistance due to the significantly smaller efferent-arteriole diameter. Additionally, afferent-arterioles are distinct from efferent-arterioles in that they can undergo vasodilation through AII-mediated release of prostaglandins and nitric oxide. Therefore, in the case of a stenotic kidney, GFR is determined by AII-dependent constriction of efferent-arterioles.\( (27) \)

Proteinuria is another major consequence of RVH and is the result of podocyte atrophy in the glomerular basement membrane. Prolonged efferent-arteriole constriction leads to podocyte atrophy and necrosis, which eliminates the tight filtration slits in the glomerular basement membrane and results in proteinuria.\( (10, 14, 18, 32, 54, 105, 112) \)
Therapeutic Intervention

RAAS inhibitors, dietary restrictions of sodium and fat, and herbal regimens have all been used to treat RVH. Antihypertensive treatments, such as ACE inhibitors, and AII, α/β-adrenergic, and aldosterone receptor antagonists have been shown to be effective in RVH. (16, 18, 50, 60, 130) Although administration of RAAS inhibitors alone has been shown to alleviate RVH, daily *Hibiscus sabdariffa* herbal supplements added to a captopril regime, decrease blood pressure and showed a return to natriuretic homeostasis. (50) ACE inhibitors and AII antagonists have been reported to slow the rate of renal dysfunction (13) and increases renal blood flow and GFR in RVH. (51, 89) Mineralcorticoid receptor antagonists are effective in decreasing renal vascular injury, mainly podocyte inflammation and glomerulosclerosis. (110) Moreover, a combination of AII and mineralcorticoid antagonists increase nephron and podocin mRNA levels in patients with nephrotic syndrome, thus leading to a recovery in glomerular basement membrane structure in the kidney. (93)

Although, RAAS inhibitors and diet restrictions are the most common interventions in RVH, these treatments can be ineffective and costly and invasive procedures may be required. Bilateral renal artery stenting can reduce blood pressure and proteinuria in patients with RVH. (6) However, if stenting fails to restore renal arterial blood flow, a renal arteriole bypass may be performed, which has been reported to reverse RVH. (62, 75)

In addition to atherosclerotic renal arteriole stenosis, atherosclerosis of the CCA and coronary arteries may occur in hypertensive patients. Occlusion of the CCA by atherosclerosis considerably reduces blood flow to the brain and is a contributing risk factor for ischemic stroke. When the CCA is occluded, the only surgical possibilities are carotid endarterectomy, plaque
buildup removal, or a subclavian- and/or saphenous-carotid artery bypass in order to restore blood flow.(19, 21, 23, 37, 99, 126)

SECTION II: HORMONAL AND NEURONAL REGULATION

*The Renin-Angiotensin-Aldosterone System*

Renin cleaves angiotensinogen into angiotensin I (AI) that is, in turn, cleaved by ACE to form AII. Angiotensin II-dependent vasoconstriction, via activation of $G_{q}$-protein coupled receptors ($G_{q}$PCR), is mediated by the PLC-IP$_{3}$-DAG pathway. Release of stored Ca$^{2+}$ via activation of IP$_{3}$-dependent calcium channels on the sarcoplasmic reticulum (SR) primarily occurs in conduit arteries. In contrast, increased cytosolic Ca$^{2+}$ concentrations in resistance arteries often depends on Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels.(78) In either vessel type, increases in cytosolic Ca$^{2+}$ concentrations leads to the binding of four Ca$^{2+}$ ions to calmodulin, forming a Ca$^{2+}$-calmodulin complex, which activates myosin light chain kinase (MLCK). Subsequently, MLCK phosphorylates myosin light chains allowing myosin to bind to actin, resulting in smooth muscle constriction.

DAG can increase intracellular Ca$^{2+}$ by activating transient receptor potential channels (TRPC) in resistance arteries, causing Na$^{+}$ influx and Cl$^{-}$ efflux, thereby initiating membrane depolarization and activation of voltage-dependent Ca$^{2+}$ channels.(116) As previously mentioned, conduit vessels typically utilize IP$_{3}$-dependent release of stored Ca$^{2+}$. However, increased cytosolic concentrations of Ca$^{2+}$ can also activate Ca$^{2+}$-activated chloride channels, further depolarizing the cell and augmenting Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels.(78)
In both, small and large arteries, the presence of receptor-operated calcium channels also contribute to elevations in cytosolic Ca\textsuperscript{2+}. For example, AII-dependent activation of G\textsubscript{q}PCR increases Ca\textsuperscript{2+} influx via DAG-activated receptor-operated calcium channels.(78) (78)

The aforementioned pathways contribute to the initiation phase of smooth muscle constriction, however PKC and RhoA-GTPase aid in the maintenance phase. The latter phase, called Ca\textsuperscript{2+} sensitization, maintains the force of contraction in the face of steady or declining cytosolic Ca\textsuperscript{2+} concentrations. Protein kinase C and RhoA-GTPase activate their respective targets, CPI-17 and Rho kinase, which inhibit myosin light chain phosphotase-mediated dephosphorylation of MLC.(63, 78) (63, 78) This results in a net increase in myosin head phosphorylation leading to sustained constriction.

While AII binding on smooth muscle cells results in vascular constriction, binding of AII on renal tubule cells causes an increase in sodium reabsorption. The latter effect is also mediated by increased DAG formation via the aforementioned pathway. The rise in plasma membrane-bound DAG, along with increases in intracellular Ca\textsuperscript{2+}, activates PKC, which stimulates sodium-hydrogen exchanger 3 in proximal tubules and the thick ascending limbs of the loop of Henle to increase sodium reabsorption, thereby increasing blood volume and blood pressure.(11)

While decreased blood flow in afferent-arteries releases renin, macula densa cells can also indirectly influence renin secretion from granular cells by sensing a fall in plasma sodium concentrations. Macula densa cells respond to the decline in plasma osmolality by stimulating NO production from (117) neighboring endothelial cells (eNOS) or neuronal cells (nNOS). Increased bioavailability of NO catalyzes the formation of prostaglandins that bind to and activate G\textsubscript{sa}PCR which in turn activates adenylate cyclase and cAMP production, which in turn elicits renin secretion.(117)
As previously mentioned, secretion of renin can restore plasma solute concentrations by promoting aldosterone-mediated sodium retention. Angiotensin II promotes aldosterone synthesis and release from zona glomerulosa cells of the adrenal medulla into the renal venous sinuses. Aldosterone binds to and activates mineralcorticoid receptors on renal distal tubules to increase apical $\text{Na}^+/\text{K}^+$-ATPase pumps, thereby increasing sodium and water retention.(42, 52, 60) However, when sodium concentrations rise in the distal convoluted tubule, macula densa cells absorb sodium via epithelial $\text{Na}^+/$Cl$^-$ cotransporters. Through mechanisms which are still a topic of debate, macula densa cells transmit the rise in sodium through neighboring extraglomerular mesangial cells to granular cells in order to release renin. This process, called the tubuloglomerular feedback mechanism, increases arteriolar resistance and reduces renal blood flow to counter the rise in sodium.(92, 106) Additionally, renin release, in response to decreased renal perfusion, is regulated by the baroreflex system.

The Baroreflex System

The baroreceptor reflex system is comprised of mechanoreceptors that relay afferent sensory information to the nucleus tractus solitarii (NTS) regarding the status of blood volume and arterial pressure. The NTS is responsible for controlling cardiac output and systemic vascular resistance in order to maintain arterial pressure and organ perfusion.(33, 44, 104) Located in the medulla oblongata, the NTS regulates cardiac output and vascular resistance through the rostral ventrolateral medulla and nucleus ambiguous, which control sympathetic and parasympathetic input to target organs, respectively. (33, 44, 104) For example, sympathetic release of norepinephrine activates $\beta_1$-adrenergic receptors to increase heart rate and contraction,
whereas parasympathetic fibers innervating the heart release acetylcholine that activates muscarinic receptors, which results in a decrease in heart rate and conduction velocity. In addition, increased norepinephrine release from sympathetic nerves activates $\alpha_1$-adrenergic receptors on smooth muscle cells causing these cells to contract. In contrast, sympathetic-mediated release of epinephrine from the adrenal medulla activates $\beta_2$-adrenergic receptors and initiates smooth muscle relaxation (dilation).(107)

Neurons of the autonomic nervous system (ANS) are densely populated with BR. Although BR are more abundant on the aortic arch and carotid bodies, they are found throughout visceral organs, venous and arterial vessels, and peripheral and central neurons of the ANS. (33, 44, 104) These uniquely-tuned baroreceptors monitor changes in blood volume and arterial stretch and respond by depolarizing or hyperpolarizing afferent neurons in order to increase or decrease action potential frequency, respectively. Ultimately, these action potentials are sent to the NTS via afferent sensory neurons in order to regulate blood pressure homeostasis.

Action potentials originating from aortic arch BR are transmitted from the aortic nerve to the vagus nerve, whereas carotid body action potentials are transmitted to the glossopharyngeal nerves via the carotid sinus nerve. However, both the vagus and glossopharyngeal central nerve terminals are located in the NTS where regulation of sympathetic and parasympathetic activity is controlled. (33, 44, 79, 104) Therefore, arterial dilation or a fall in blood volume decreases the frequency of BR action potentials sent to the NTS, resulting in inhibition of nucleus ambiguous neurons (decreased parasympathetic activity) and excitation of rostral ventrolateral medulla neurons (increased sympathetic activity). In contrast, arterial distension (stretch) or increased blood volume raises the frequency of BR action potentials sent to the NTS in order to decrease
sympathetic activity and increase parasympathetic activity, thus reducing cardiac output and vascular resistance to return blood pressure to normal. (44)

In the case of 2K1C hypertension, renal-afferent BR innervating renal afferent arteries sense the fall in renal perfusion (e.g. decline in blood flow/pressure), therefore diminishing the frequency of action potentials sent to the NTS via the dorsal root ganglia. The NTS decreases glutamate release onto caudal ventrolateral medulla terminals which in turn disinhibits GABA release onto terminals of the rostral ventrolateral medulla, increasing sympathetic activity. (107, 112) Alleviation of the inhibitory effects of GABA allows for sympathetic activation of adrenal chromaffin cells, resulting in epinephrine release and initiation of the flight-or-fight response (e.g. increased heart rate, blood flow to skeletal muscles and dilation of coronary blood vessels.) (24, 113) Additionally, arginine vasopressin is released in response to decreased kidney perfusion. The decline in renal blood flow decreases the frequency of BR action potentials sent to the paraventricular nucleus of the hypothalamus and results in further arginine vasopressin release from the posterior pituitary gland. (107)

Although both systems of the ANS innervate the kidney, only sympathetic neurons terminate with chromaffin cells in the adrenal medulla. Acetylcholine release from preganglionic neurons of the sympathetic system activates nicotinic receptors that results in an increase in secretion of epinephrine from granule cells via raising intracellular Ca$^{2+}$. Epinephrine is a nonselective adrenergic receptor agonist. (115) Although epinephrine has a higher affinity for β-adrenergic receptors, α-adrenergic receptor activation dominates with increasing concentrations of epinephrine in the circulation.

The continuous stress of elevated arterial pressure changes BR sensitivity in maintaining and recognizing normotension by resetting to a newly developed level of hypertension. The
mechanism behind BR adaptation and sensitivity is dependent on action potential firing and sustained arterial pressure. Acute hypertension causes an increase in BR action potential firing to the NTS in order to maximize blood pressure reductions. However, if hypertension is maintained BR action potential firing decreases until the original firing rate is reached (within 24 to 48hrs.). Therefore, in the face of sustained elevated blood pressure, BR effectively "re-set" to this higher pressure and no longer initiate reductions in cardiac output and vascular resistance.(107)

**Models of Angiotensin II-Dependent Hypertension**

Currently, inducing AII-dependent hypertension is achieved via angiotensin II-infusion or renal arterial clipping.(27, 29) AII-infusion, via intravenous or subcutaneous injections, is relatively easy, mimics AII-dependent systemic hypertension, and facilitates the study of baroreflex function. However, this technique has little clinical relevance as it involves supraphysiological concentrations of AII, and suppresses pressure-natriuresis in both kidneys. (128) Additionally, AII-infusions in rodents causes marked reductions in plasma renin activity, which is the exact opposite of renin activity in patients.(66, 101)

In contrast to AII-infusion techniques, renal arterial clipping, via 2-kidney, 2-clip (2K2C) or 2K1C models, resembles clinical cases of renal arterial stenosis and facilitates studies utilizing pharmacological compounds upstream of AII in the RAAS, such as ACE and renin inhibitors. Renal clipping is a RAAS-dependent model of hypertension, in which the animals have increased aldosterone- and AVP-mediated sodium and water retention, respectively. (27, 29) The 2K1C model of RVH allows for the contralateral kidney to achieve renal excretion via pressure diuresis, whereas 2K2C animals are unable to adequately achieve renal excretion.(27)
Both the 2K1C and 2K2C models involve AII-dependent constriction and increased vascular resistance. In addition to studies on pressure- versus volume-overload hypertension, both kidneys in 2K2C experience increased renin production, whereas the contralateral kidney in the 2K1C model shows a decline in renin production. However, although widely used as a means of inducing RVH, insufficiencies with respect to renal-clipping plague the 2K1C model. For example, renal clip dislodgment and inadequate reductions in renal arterial diameter are costly in terms of supplies, investigator time, and mortality and morbidity, have been detailed extensively. Moreover, these deficiencies lead to unacceptably low success rates in terms of the desired outcome, namely hypertension. Therefore, additional work and research is needed to reliably elicit hypertension in the 2K1C model. Considering the relatively low success rate of 2K1C, it is likely that flaws in the design elements of the clip are culpable. Currently, 1) the malleability of traditionally used silver U-shaped clips, and 2) perseverance of clip placement around the renal artery following implantation are two variables which have shown to be problematic. Therefore, we propose the use of a far superior metal, such as titanium, and a design element that would provide perseverance of clip placement following implantation will significantly benefit the field of 2K1C models and studies on the pathogenesis of RVH.
SECTION III: NEURONAL CELL BODIES AND THEIR VASCULATURE

*The Nodose and Petrosal Ganglia and Carotid Bodies*

Bipolar vagal-afferent cell bodies of the nodose ganglia (NG) receive sensory signals, such as blood pressure, cardiac output and intestinal distention, via vagal-afferent peripheral axons of the cardiopulmonary system, gastrointestinal tract and visceral organs and transmit these signals via vagal-afferent central axons terminating in the nucleus tractus solatarii (NTS). (22, 53, 124, 135) Changes in afferent action potential frequencies are received by the NTS, which regulates baroreflex activity through the autonomic nervous system. (135) The NTS then relays information back through motor efferent neurons in order to control visceral organ and cardiopulmonary functions such as smooth muscle contraction, bronchial dilation, intestinal unloading and cardiac output. In the rat, cell bodies of the glossopharyngeal and vagus nerves form the petrosal ganglia (PG) and NG, respectively.

During embryogenesis cranial neural crest cells form the cell bodies of the PG while epidermal placodes give rise to the cell bodies of the NG. (76, 135) Cell bodies of the PG and NG project axons through the tympano-occipital fissure, an aperture of the skull near the occipital and internal carotid arteries, (135) which enter the lateral medulla and terminate in the ipsilateral NTS. (22, 45, 57, 58, 132) For example, experiments have demonstrated a marked reduction in labeled AIIR in the ipsilateral NTS in rats with an ipsilateral vagal-transection (between the NG and central axons projecting to the NTS). (3) While, cell bodies of the NG provide sensory-afferent innervations to the visceral organs, the esophagus and trachea, and cardiopulmonary plexuses, (76, 135) cell bodies of the aortic depressor nerve also reside in the NG. (30)
However, sensory-afferent and motor-efferent neurons of the PG are confined to muscles, glands and arteries of the face and neck. Specifically, PG motor-efferent neurons innervate the stylopharyngeal and digastricus muscles in order to dilate and elevate the pharynx to allow food passage during swallowing and provide neck and mandible movement, respectively.(135) In addition, the PG sensory-afferent neurons project to facio-cranial muscles and organs, such as the pharyngeal and laryngeal organs, salivatory glands in the mouth, taste receptors in the tongue and to carotid body chemoreceptors via the carotid sinus nerve.(135) Chemoreceptors of the carotid bodies, located near the common carotid bifurcation, transmit signals such as O₂ and CO₂ levels and pH via the carotid sinus nerve which terminates in the NTS via the glossopharyngeal nerve.(96)

Cell bodies of the NG synthesize neuropeptides (NP) for central or peripheral transportation to the NTS or target organs, respectively. Other neurotransmitters (NT) are synthesized and stored at nerve terminals awaiting an action potential and Ca²⁺ influx to initiate release and/or further transcription.(135) Therefore, sensory signals can occur in the form of neurosensory, chemosensory, or both, and can involve two or more neuroactive signals (such as NT, NP, Ca²⁺-binding proteins, or substance P) to form a complex neurochemical signal.(135) Zhuo et al. reported the wide variety of receptor populations in NG cell bodies and their terminals, such as glutamate, GABA, somatostatin, substance P, arginine vasopressin (AVP), serotonin (5-HT) and AII receptors.(135) In addition, Czyzyk-Krzeska et al. demonstrated that NG cell bodies and their central and peripheral terminals also contain substance P and somatostatin mRNA, which may act as neuromodulators by altering cell polarization.(25) These neuromodulators act to maintain the membrane potential of NG cell body axon hillock, thus regulating action potential firing via acting as de- or hyperpolarizing agents.(119) Two other
important modulators of neuronal function, substance P and somatostatin, have been reported to increase 6-fold at proximal nerve termini when the vagus nerve is ligated.(83, 84) Additionally, NO has been reported to be a modulator of glutamate release in the NTS, and NG cell bodies and central vagal-afferent terminals express neuronal nitric oxide synthase (nNOS) mRNA.(74) Moreover, in rats with unilateral vagal-double ligations, Fong et al. reported that NADPH-diaphorase staining, a marker of NOS, increased and significantly accumulated at sites adjacent to proximal and distal ligatures, with an absence of NADPH-staining in between. These findings suggest the bidirectional transport of nNOS to sites during conditions such as neuronal damage or occlusion. However, in rats with a distal vagal transection and a proximal ligation, distal NADPH-diaphorase labeling was abolished.(36)

Along with nNOS, glutamate is used as a NT to communicate with the NTS, where NG transection showed a decrease in glutamate (87) and nNOS radiolabeling in the ipsilateral NTS.(73) Together, these findings demonstrate the complex nature of NG-associated neurotransmission. Conditions such as hypoxia, stroke and/or hemorrhage can affect the NG-NTS axis. For example, Aydin et al. reported a decline in NG neuron densities in rabbits with induced subarachnoid hemorrhages (SAH). Additionally, ischemic NG neuronal degeneration was more pronounced in SAH rabbits compared to sham SAH rabbits.(4) It should also be noted that PG and NG neuronal cell death can result in hypertension.(5) Therefore, the integrity of NG cell bodies and their nerve terminals is crucial for the maintenance of blood pressure.

As previously mentioned, carotid body chemoreceptors sense changes in circulating O\(_2\) levels and increase action potential firing during hypoxia.(96) Decreases in O\(_2\) partial pressure causes type-1 (chief) cell depolarization,(34) thus opening voltage-gated Ca\(^{2+}\) channels, increasing intracellular Ca\(^{2+}\) concentrations and releasing NT to the carotid sinus nerve via
vesicular exocytosis.(34, 96) These type-1 cells have abundant mitochondria and highly developed endoplasmic reticulum, where hypoxia-induced NT release has demonstrated increased synthesis of hypoxia inducible factor-1, ET-1, and vascular endothelial growth factor.(94) These signals are then transmitted via glossopharyngeal nerves to the NTS in order to regulate respiratory and cardiovascular responses, such as increased expiratory and tidal volume, bronchial dilation, increased venous return and cardiac output in acute hypoxia.(35) In a rodent model of chronic hypoxia there is a sustained sensitization of carotid body chemoreceptors,(100) due to increased expression of Na\(^+\)-channels in the type-1 cell membranes, further contributing to cellular depolarization.(17) Furthermore, NG vascular ischemia and hypoxia have marked affects on the baroreflex response and hormonal regulation. Recently, it has become apparent that baroreflex activity during the development of 2K1C hypertension is affected by circulating factors delivered to the NG by the occipital artery (OA). Specifically, 2K1C rats with bilateral OA ligations had a markedly increased mean arterial pressure (MAP) compared to rats with sham ligations or normotensive rats with either bilateral or sham OA ligations.(71) Therefore, the OA may play a crucial role in blood pressure maintenance during the development of RVH.

*The Occipital Artery*

The OA is located directly behind the digastric muscles of the neck and supplies blood to the occipital lobe, muscles of the sterno-mastoid region and cell bodies of the PG and NG.(71) The OA arises from the common carotid artery, which also bifurcates into the internal (ICA) and external carotid (ECA) arteries. However, it is essential to note that blood flow to the NG and PG is via the ICA and OA, and not the ECA (*Figure 1.*) (71)
Neuromodulators synthesized by, or transported to, NG cell bodies affect NG membrane potential and thus regulate action potential firing to the NTS. Therefore, if circulating factors, gain access to the cell bodies of the NG, via the OA and ICA, they could potentially influence the resting potential of the NG axon hillock, therefore contributing to the regulation of baroreflex activity.

Commonly, ganglia are considered to possess a tight blood-ganglion barrier (BGB) that prevents circulating factors from gaining direct access to the neuronal cell bodies.(102) However, recent reports have demonstrated that circulating low molecular weight molecules can gain access to the cell bodies of the NG via the OA and in turn affect baroreflex responses.(71) Specifically, OA injections of 5-HT (MW = 212) caused immediate reductions in heart rate and blood pressure in rats, whereas these findings were absent in rats with ICA injections of 5-HT.(71) Furthermore, the observed bradycardia and hypotension upon OA injections of 5-HT were reduced in rats with prior vagal transections (between the NG and the ascending vagus nerve to the NTS).(71) Jacobs and Comroe et al. reported that CCA injections of 5-HT in cats induced hypotension and bradycardia, however this was abolished when the OA was ligated prior to injection.(55) These findings, which will be discussed below, demonstrate the unique difference between the permeability of the OA and ICA microvasculature with the NG and the ability of the NG to act as a potential sensor of blood-borne factors.

Although blood supply to the NG is provided by the ICA and OA, it has recently been reported that there are marked differences in the permeability of the BGB at the OA-NG complex and the ICA-NG complex.(71) Specifically, the small molecular weight tracer, Basic Blue 9 (BB9, MW = 374), entered NG and PG cell bodies, 30 mins after injection into the
jugular vein of rats with open OA. In contrast, there was no BB9 staining of ipsilateral NG cell bodies in rats with OA ligations.(71)

As previously mentioned, cell bodies of the NG have a wide array of receptors, such as glutamate, GABA, somatostatin, AVP, AII and 5-HT.(135) Of these, it is known that AII, AVP, and 5-hydroxytryptamine (serotonin, 5-HT) can be up- or down-regulated by organ damage and culture, reactive oxygen species, cardiovascular diseases, neuronal damage, blood pressure oscillations and/or hypertension.(82, 108, 118, 125) Specifically, AIIR are down-regulated in ischemic kidneys and up-regulated in hypertrophic cardiomyopathy and heart failure.(26, 29)(26, 29) In rodent models of Parkinson’s disease, peripheral sympathectomy is performed by neurotoxic administration of 6-hydroxydopamine (6-OHDA). Neurodegeneration of dopaminergic and adrenogenergic neurons is achieved through 6-OHDA endocytosis via dopamine and norepinephrine reuptake into nerve terminals.(108) The exact pathway is still unclear, however dopaminergic and adrenogenergic neurodegeneration is considered to be the result of 6-OHDA-induced production of reactive oxygen species.(108) These rats were reported to have uncontrolled and spontaneous blood pressure oscillations and increased AVP-induced vasoconstriction on isolated mesenteric arteries compared with controls.(108) Whether this is due, in part, to vascular hypersensitivity or increased AVPR deposition on smooth muscle cells is unknown, however it is clear that blood pressure oscillations and neuronal damage influence the contractile properties of AVP-induced contractions in resistance arteries.

Concentrations of circulating 5-HT are elevated in rodent models of hypertension and 5-HT elicits a greater contractile response in arteries isolated from these animals.(82, 118) Utilizing Western blot analysis, Banes et al. demonstrated a 2-fold increase in 5-HT type-1 and type-2 receptors in denuded aortas from hypertensive deoxycorticosterone-salt (DOCA) rats
compared with controls. (7) Interestingly, deoxycorticosterone possesses mineralcorticoid activity, such as aldosterone, where aldosterone is well documented to be elevated in rodent models of RVH. (27) As such, in a rat tissue culture model, aortas incubated with aldosterone showed a concentration-dependent upregulation of 5-HT type-1 and type-2 receptors, whereas these findings were blocked in aortas incubated with the mineralcorticoid antagonist, spironolactone. (7) These findings may demonstrate increased 5-HT receptor synthesis via a mineralcorticoid receptor activation-dependent mechanism.

Angiotensin II type-1 receptors have shown to be transported bidirectionally to central nerve terminals of the NTS and to peripheral terminals of the vagus nerve after nodose gangliectomy. (2, 28) In contrast, Gao et al. demonstrated that AVP type-1 receptors are not transported after nodose gangliectomy. However, AVP type-1 receptors are present on cell bodies due to the fact that administration of AVP to NG cell bodies elicits immediate cell depolarization and blood pressure reductions. (38) Therefore, it is possible that AVP, AII and 5-HT may be important regulators of NG cell bodies and, in turn, baroreflex activity.

SECTION IV: THE RATIONALE

Novel Research in Occipital Artery Function

Emerging lines of evidence indicate that blood-borne factors, such as AVP, 5-HT and AII, may access the cell bodies of the NG exclusively via the OA. (71) Therefore, the contractile status of the OA may play a vital role in the homeostatic regulation of blood pressure in both normotensive and hypertensive states. However, no current reports exist concerning the
contractile properties on the OA isolated from the rat and our first objective was to determine the contractile responses of rat OA to AII, AVP, and 5-HT from control, sham- and 2K1C-operated rats using small vessel myography.

**Procedures Used to Determine the Contractile Properties of the Isolated Occipital Artery**

Through the pioneering work of Mulvany *et al.* and the increasingly innovative developments by Danish Myo Technology, the small vessel myograph can now afford the researcher studies on the contractile properties of blood vessels (~80 - 400um i.d.). (91) Although, this technique is more advantageous than perfusion myography (where the vessel is pressurized) in terms of the large amount of arteries that can be studied at one time, it has some limitations. For example, the myogenic response of arteries is highly dependent on transmural pressure and communication with the sympathetic nervous system, (78, 79) In the small vessel myograph, the vessels are mounted on two 40 micron stainless steel wires that allow the vessel to be placed under tension. However, this tension is in one plane (i.e., horizontal) and does not exactly replicate the stress that the artery wall undergoes *in vivo*, namely pressure from the blood in the lumen. Both wire and perfusion myography techniques allow for the study of endothelium-dependent or –independent responses, by performing experiments in endothelium-intact and endothelium-denuded vessels.(20, 56)

Furthermore, the small vessel myograph can be used to provide morphological data concerning artery diameter and thickness of the intima, media and adventitia.(103) Small vessel myography utilizes the law of Laplace (Wall Tension = Pressure x Radius/2 x Wall Thickness) in order to determine the resting wall tension and the appropriate pressure corresponding to the
dimensions of arterial diameter and thickness. (78, 79, 103) The myograph has two stainless steel jaws; one of which can be moved horizontally by a micrometer while the other is stationary and is attached to a force transducer. Vessels are mounted on two 40µM-diameter stainless steel wires, with one wire being attached to each of the two jaws. Vessels are then stretched to reach an equivalent transmural pressure that produces the maximum contractile response, as originally described by Mulvany et al., (91). Contractile responses can then be measured via the force transducer, which relays these responses to an interface that converts the millivolt signal to force (in mN). This conversion is achieved by pre-calibrating the force transducer using a known mass applied to the jaw attached to the transducer.

**Occipital Artery Isolation**

A high-powered dissecting microscope and micro-fine instruments are used in order to remove the digastricus muscles of the neck, therefore exposing the ventrally located CCA bifurcation. Due to the abundant vascular and neuronal networks in this area, care is taken when removing the glossopharyngeal nerve (GVN) so as to not pull, cut or snag the OA. A cut is then made at the caudal end of the GVN and then once again, proximal to the PG cell bodies, as the GVN moves rostrally. Sequentially, an axial-distal and –proximal cut is made before and after the CCA bifurcation, respectively. Extreme care is taken to cut between the ICA and OA while moving dorsally along the two arteries. Following removal of the ICA, an axial-proximal cut is made between the OA and ECA. Lastly, a rostral-dorsal cut of the OA nearest the aperture of the tympano-occipital fissure is made and the OA is removed and placed in ice cold physiological saline solution (PSS), containing, (in mM): NaCl 118, NaHCO₃ 24, KCl 4, glucose 5.6, MgSO₄
1, NaH$_2$PO$_4$ 0.435, CaCl$_2$ 1.8. Occipital arteries will be isolated and bisected such that proximal (proximal segment) or distal (distal segment) segments can be mounted separately on small vessel myographs for the measurement of isometric tension. (Model 500A, Danish Myo Technology, Denmark).

After equilibrating for 30 mins in PSS gassed with 12% O$_2$, 5% CO$_2$, and 83% N$_2$ (pH 7.4, 37°C), OA are stretched using the methods described by Mulvany et al. for systemic arteries. (91) The maximum responses of OA segments to a depolarizing stimulus are then established by exposing them to 80 mM K$^+$ (KPSS; isotonic replacement of Na$^+$ by K$^+$; 3 x 2 min exposures, 15-20 mins apart), as described previously. (109)

SECTION V: EXPERIMENTAL DESIGN

This project is divided into three aims:

**AIM 1** studies will determine the contractile effects of AII, AVP and 5-HT receptor agonists on the OA isolated from rats using small vessel myography. Additionally, AII, AVP and 5-HT receptor antagonists will be used to further aid the identification of specific receptor subtypes for these agonists (Objective 1).

**AIM 2** studies will focus on designing a novel methodology for inducing reliable and consistent levels of hypertension at 9 days of renal artery clipping in the 2K1C model of renovascular hypertension (Objective 2).

**AIM 3** studies will determine the contractile effects of AII, AVP and 5-HT on OA isolated from sham-operated and 2K1C rats nine days after clip implantation (Objective 3).
The Hypotheses

_Hypothesis 1:_ OA will constrict when exposed to AII, AVP or 5-HT, via activation of AVP₁, AII₁, and 5-HT₂ receptors.

_Hypothesis 2:_ A novel solid titanium renal clip with a design based upon a radial non-concentric groove with the addition of a drilled hole located at the outer edge will consistently produce reliable levels of hypertension in rats. 2K1C rats will have increased mean arterial pressure (MAP) and peak systolic blood pressure (PSBP) 9 days post-surgery, when compared to sham-operated controls. Physiological parameters such as plasma renin activity, body, and kidney and heart weights will be adversely affected in 2K1C rats compared to sham-operated controls.

_Hypothesis 3:_ OA isolated from 2K1C rats will have greater maximum responses and be more sensitive to AII, AVP, or 5-HT when compared to the responses of OA isolated from sham-operated rats.

The Objectives

**Objective 1:** To provide the first characterization of the contractile responses of rat occipital arteries to three physiologically relevant agonists, namely AII, AVP, and 5-HT.

**Objective 2:** To determine the effects of novel renal clips with gap widths of 0.23, 0.25 and 0.27mm on blood pressure, plasma renin activity, non-clipped and clipped (or sham-clipped) kidney weights, cardiac hypertrophy, and body weight in rats.

**Objective 3:** To determine whether the contractile properties of rat occipital arteries is altered at 9 days following renal artery clipping in the 2K1C model in rats.
SECTION VI: REFERENCES


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Figure 1. Schematic drawing of the occipital and carotid arteries and the cell bodies of the nodose and petrosal ganglion. n: nerve; a: artery.
CHAPTER 3

CONTRACTILE EFFECTS OF ANGIOTENSIN II, ARGinine VASOPRESSIN AND 5-HYDROxyTRYPTAMINE ON ISOLATED OCCIPITAL ARTERIES OF THE RAT

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ABSTRACT

Circulating factors delivered to the nodose ganglion by the occipital artery (OA) may affect vagal afferent activity by direct actions on the cell bodies. The aim of this study was to characterize the contractile responses of OA to angiotensin II (AII), arginine vasopressin (AVP), and 5-hydroxytryptamine (5-HT). Rat OA (250-400 µm diameter) were isolated and bisected into proximal (proximal segment) and distal (distal segment) segments and mounted separately on myographs. Maximum responses to AII and the AII₁ receptor agonist, Val⁵-AII (100pM-300nM for both) were similar in each segment type, and were abolished by the AII₁ receptor antagonist, losartan (1 µM). Maximum responses to AVP and the AVP₁ receptor agonist, oxytocin (100pM-1 µM for both), were significantly higher in distal than proximal segments, and were abolished by the AVP₁ receptor antagonist, V2255 (10nM). Distal segments were significantly more sensitive to either 5-HT or the 5-HT₂ receptor agonist, α-CH₃-5-HT (1nM-100 µM for both), than proximal segments though the maximum responses to 5-HT were similar between segment types. Responses to 5-HT or α-CH₃-5-HT were blocked by the 5-HT₂ receptor antagonist, ketanserin (100nM). Agonists for the AII₂ (CPG-42112A), AVP₂ (V1005), 5-HT₁B/₁D (sumatriptan) and 5-HT₁A (8-OH-DPAT) receptors did not elicit contractile responses in OA segments. The results of this study are consistent with the concept that the contractile properties of rat OA may vary depending on the agonist studied and the specific segment of the OA under investigation. Further studies are warranted to determine whether the function of this potentially important artery alters during the development of hypertension.
INTRODUCTION

The occipital arteries (OA) arise from the external carotid arteries (ECA), proximal to the bifurcation of the common carotid arteries (CCA) into the internal carotid arteries (ICA) and ECA. The OA supply blood to the occipital lobe and external cranium, muscles of the sterno-mastoid region and cell bodies of the petrosal ganglia (PG) and nodose ganglia (NG).(11) Whereas blood supply to the PG and NG is provided by both the ICA and OA, recent research has demonstrated that there are differences in the permeability of the blood-ganglion barrier at the OA-NG complex compared to the ICA-NG complex.(11) Specifically, Lacolley et al. demonstrated that the small molecular weight tracer, Basic Blue 9 (BB9, MW = 374), entered NG cell bodies 30 minutes after injection into the jugular vein of rats with patent OA. In contrast, there was no BB9 staining of NG cell bodies in rats with ipsilateral OA ligations.(11) Therefore, the lack of a tight blood-ganglion barrier at the OA-NG complex may allow for circulating factors, such as angiotensin II (AII), arginine vasopressin (AVP) and 5-hydroxytryptamine (5-HT) to gain access to, and affect the function of, NG cell bodies via the OA. These data are consistent with the concept that the OA may play a vital role in the regulation of NG function and, in turn, blood pressure homeostasis.

The cell bodies of the NG possess a wide array of receptor types such as glutamate, gamma-aminobutyric acid, AII, AVP and 5-HT.(27) Of these, it is known that AII, AVP, and 5-HT receptors can be up- or down-regulated by organ culture, reactive oxygen species, neuronal damage, and/or hypertension.(13, 17, 20, 22) Additionally, concentrations of circulating AVP and AII are raised in hypertension and have been reported to regulate baroafferent activity. (5, 6, 16, 21, 23-25) Lacolley et al. demonstrated that microinjections of 5-HT directly into the OA of
rats elicits a rapid decrease in heart rate and mean arterial pressure, whereas injections of 5-HT in the ICA does not.\(^{(11)}\) Furthermore, Jacobs \textit{et al.} demonstrated that bradycardia and hypotension ensued upon CCA injections of 5-HT in cats with patent OA, but not in cats in which OA were ligated.\(^{(8)}\) Since no studies currently exist that detail the contractile properties of OA, the aim of this initial study was to determine the contractile effects of three physiologically relevant agonists (AII, AVP and 5-HT) on OA isolated from male Sprague-Dawley rats.

**MATERIALS AND METHODS**

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all protocols were approved by The University of Georgia Institutional Animal Care and Use Committee.

\textit{Occipital Artery Isolation and Small Vessel Myography}

Male Sprague-Dawley rats (350 - 380g) were euthanized by decapitation and the heads immediately placed in ice cold physiological saline solution (PSS) containing (in mM): NaCl 118, NaHCO\(_3\) 24, KCl 4, glucose 5.6, MgSO\(_4\) 1, NaH\(_2\)PO\(_4\) 0.435, CaCl\(_2\) 1.8. On the stage of a high-powered dissecting microscope, OA (250 – 400 \(\mu\)m internal diameter; 3.5 – 4.5 mm long) were isolated and bisected into proximal (proximal segment) and distal (distal segment) segments and mounted separately (\textbf{Figure 1}) on small vessel myographs (Model 500A, Danish
Myo Technology, Denmark). After equilibrating for 30 mins in PSS gassed with 12% O\(_2\), 5% CO\(_2\), and 83% N\(_2\) (pH 7.4, 37°C), OA were stretched using the methods described by Mulvany et al. for systemic arteries.\(^{(14)}\) The maximum responses of OA segments to a depolarizing stimulus were then established by exposing them to 80 mM K\(^+\) (KPSS; isotonic replacement of Na\(^+\) by K\(^+\); 3 x 2 min exposures, 15-20 minutes apart), as described previously for other vessel types.\(^{(18)}\)

**Experimental Protocols**

Concentration response curves for AII, AVP, 5-HT and agonists for specific receptor subtypes, were established in OA segments in the absence or presence of receptor antagonists. In experiments where antagonists were used, the antagonists were added to the bathing solution 15 minutes prior to the first concentration of agonist.

**Agonists and Antagonists**

[Val\(^5\)]-Angiotensin II acetate salt hydrate (AII\(_1\) receptor agonist), CPG-42112A (AII\(_2\) receptor agonist), losartan potassium (AII\(_1\) receptor antagonist), [AVP\(^8\)]-Vasopressin acetate salt (AVP receptor agonist), [deamino-Cys\(^1\), D-Arg\(^8\)]-Vasopressin acetate salt hydrate (AVP\(_2\) receptor agonist, V1005), [β-Mercapto-β,β-cyclopentamethylenepropionyl\(^1\), O-me-Tyr\(^2\), Arg\(^8\)]-Vasopressin (AVP\(_1\) receptor antagonist, V2255), 5-hydroxytryptamine hydrochloride, (R)-(+) -8-Hydroxy-DPAT hydrobromide (5-HT\(_{1a}\) receptor agonist, 8-OH-DPAT), α-CH\(_3\)-5-hydroxytryptamine maleate salt (5-HT\(_2\) receptor agonist, α-CH\(_3\)-5-HT), NAN-190 hydrobromide
(5-HT\textsubscript{1a} receptor antagonist), sumatriptan succinate (5-HT\textsubscript{1b/1d} receptor agonist), and ketanserin tartrate salt (5-HT\textsubscript{2} receptor antagonist) were purchased from Sigma Chemical Co. (St. Louis, MO); Angiotensin II was purchased from the American Peptide Co. (Sunnyvale, CA); and oxytocin (AVP\textsubscript{1} receptor agonist) was purchased from Bachem (Torrance, CA).

**Statistical Analysis**

Contractile responses were expressed as a percentage of the maximal contractile response to KPSS (% T\textsubscript{K}) for each vessel. Data are presented as mean ± S.E.M. Maximal contractile responses (E\textsubscript{max}) were analyzed by 2-way analysis of variance (ANOVA) with post hoc analysis using the Bonferroni correction for multiple comparisons procedure. Differences between vasoconstrictor potency (EC\textsubscript{50}) were determined by Student's modified t-test. A value of P < 0.05 was deemed to be significant.

**RESULTS**

**Occipital Artery Responses to Angiotensin II**

AII (100pM – 300nM) elicited contractions in proximal and distal segments of OA in a concentration-dependent manner (Figure 2). Maximal contractile responses to AII were not significantly different between distal and proximal segments of OA (distal segment E\textsubscript{max}: 14.1 ± 2.4% T\textsubscript{K}; and proximal segment E\textsubscript{max}: 19.6 ± 3.4% T\textsubscript{K}, n = 9 for both; Table 1). Similar results were found for the AII\textsubscript{1} receptor agonist, Val\textsuperscript{5}-AII (100pM – 300nM; Figure 3; distal segment
$E_{\text{max}}$: 13.5 ± 2.6% $T_K$; and proximal segment $E_{\text{max}}$: 14.0 ± 2.1% $T_K$, $n = 9$ for both, Table 1).

Distal segments of OA were significantly more sensitive to AII ($EC_{50}$: 3.1 ± 1.5 nM, $n = 9$) than proximal segments ($EC_{50}$: 8.2 ± 1.4 nM, $n = 9$, Table 1), though there was no significant difference in OA sensitivity to Val$^5$-AII between segments. Addition of 1µM losartan (AII$_1$ receptor antagonist) completely inhibited the contractile effects of AII and Val$^5$-AII in both proximal and distal segments of OA (Table 1). The AII$_2$ receptor agonist, CPG-42112A (100pM – 100nM) did not elicit any contractile responses in proximal and distal OA segments, and did not elicit dilation in OA segments pre-constricted to ~40% $T_K$ with prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; data not shown).

**Occipital Artery Responses to Arginine Vasopressin**

Cumulative addition of AVP (100pM – 1µM) to the bathing solution elicited contractions in both proximal and distal OA segments (Figure 4). However, distal segments constricted to a significantly higher degree than proximal segments when exposed to AVP (distal segment $E_{\text{max}}$: 74.2 ± 6.8% $T_K$, $n = 11$; and proximal segment $E_{\text{max}}$: 29.7 ± 8.5% $T_K$, $n = 10$; Table 2). This was also the case for the AVP$_1$ receptor agonist, oxytocin (100pM – 1µM; distal segment $E_{\text{max}}$: 66.6 ± 10.7% $T_K$; and proximal segment $E_{\text{max}}$: 26.7 ± 3.8% $T_K$, $n = 10$ for both; Figure 5). There was no difference in OA sensitivity to AVP between segments, however distal segments were significantly more sensitive to oxytocin ($EC_{50}$: 24.2 ± 1.4 nM, $n = 10$) than proximal segments ($EC_{50}$: 36.7 ± 1.3 nM, $n = 10$; Table 2). Addition of 10nM V2255 (AVP$_1$ receptor antagonist) completely inhibited the contractile effects of AVP and oxytocin in both OA segments (Table 2).
The AVP$_2$ receptor agonist, V1005 (100pM to 100nM), did not elicit any change in tone (constriction and/or dilation) in either segment of the OA (data not shown).

**Occipital Artery Responses to 5-Hydroxytryptamine**

The proximal and distal segments of the OA contracted robustly when 5-HT (1nM - 100µM) was added cumulatively to the bathing solution (Figure 6). Maximal contractile responses to 5-HT were not significantly different between OA segment types (distal segment $E_{\text{max}}$: 130.7 ± 5.7% $T_K$; proximal segment $E_{\text{max}}$: 129.2 ± 10.5% $T_K$, $n = 11$ for both, Table 3). However, when the 5-HT$_2$ receptor agonist, α-CH$_3$-5-HT (1nM - 100µM), was added to the bathing solution, distal segments constricted to a significantly higher degree than proximal segments (distal segment $E_{\text{max}}$: 108.1 ± 3.7% $T_K$, $n = 21$; proximal segment $E_{\text{max}}$: 82.0 ± 3.9% $T_K$, $n = 16$, Figure 7). Distal segments were significantly more sensitive than proximal segments to 5-HT and α-CH$_3$-5-HT (Table 3). Pre-incubation of distal or proximal OA segments with 100nM ketanserin (5-HT$_2$ receptor antagonist) completely inhibited the contractile responses to 5-HT and α-CH$_3$-5-HT (Table 3).

The 5-HT$_{1a}$ receptor agonist, 8-OH-DPAT (1nM - 10µM), elicited small, yet significant contractile responses in all OA segments at the highest concentration used, and distal segments had significantly higher maximal responses than proximal segments (distal segment $E_{\text{max}}$: 8.6 ± 1.3% $T_K$; proximal segment $E_{\text{max}}$: 5.7 ± 1.4% $T_K$, $n = 15$ for both, Figure 8). However, pre-incubation with ketanserin (100nM) completely abolished the contractile responses to 8-OH-DPAT in both segment types (Table 3). The 5-HT$_{1b/1d}$ receptor agonist, sumatriptan (1nM to
10µM), did not elicit any change in tone in either distal or proximal OA segments (data not shown).

**DISCUSSION**

The activities of vagal afferents can be affected by direct activation of cell-surface receptors on the cell bodies located within the NG (1, 7, 27). Blood-borne factors can gain direct access to the cell bodies of the NG exclusively via the OA. (see 10, 11) Accordingly, it is possible that blood-borne factors within the OA blood supply to the NG may play a vital role in regulating vagal afferent-mediated control of physiological functions such as blood pressure homeostasis. For example, plasma concentrations of AVP and AII are increased in hypertension (5, 6, 16, 21, 23-25) and may affect baroafferent activity via actions on NG cells. Since no reports currently exist detailing the contractile properties of this potentially important blood vessel, our aim was to provide an initial characterization of the contractile responses of OA isolated from rats to three physiologically relevant agonists, namely AII, AVP and 5-HT.

The OA isolated from the rats used in this study were approximately 4mm in length, and since vessels of 2mm in length are suitable for small vessel myography, (14) our initial rationale for bisecting the OA was to increase the number of vessels that could be studied from each rat. However, this approach led to the serendipitous finding that the contractile properties of OA segments appear to change significantly from the external carotid artery to the NG, depending on the agonist under investigation. Specifically, we determined that distal OA segments contract to a significantly greater degree than proximal segments when exposed to AVP, oxytocin, and the
5-HT\textsubscript{2} receptor agonist, \(\alpha\)-CH\textsubscript{3}-5-HT. Moreover, distal segments of the OA were also more sensitive, with regard to EC\textsubscript{50} values, to AII, oxytocin, 5-HT and \(\alpha\)-CH\textsubscript{3}-5-HT.

The sensitivity of OA segments to AII in the present study were similar to those reported for other vessel types (e.g., rat and mouse aorta). (2, 26) The inhibition of AII and the AII\textsubscript{1} receptor agonist, Val\textsuperscript{5}-AII, -induced contractions by the AII\textsubscript{1} receptor antagonist, losartan, and the lack of effect of the AII\textsubscript{2} receptor agonist, CPG-42112A, are consistent with AII-induced contractions of the OA being mediated via activation of AII\textsubscript{1} receptors. It is apparent that activation of AII\textsubscript{2} receptors is associated with vasodilation via the production of endothelium-derived relaxing factors.(15) However, addition of CPG-42112A to OA pre-constricted with prostaglandin F\textsubscript{2\alpha} had no effect on OA tone (data not shown).

Distal segments from OA constricted to a significantly higher degree than proximal segments when exposed to AVP or the AVP\textsubscript{1} receptor agonist, oxytocin. Although distal segments were significantly more sensitive to oxytocin than proximal segments, there was no difference in OA sensitivity to AVP in either segment type. However, EC\textsubscript{50} values for AVP were significantly lower than EC\textsubscript{50} values for oxytocin, and were similar to those reported for rat aorta, and mesenteric and tail arteries.(2, 4) Pre-incubation with the AVP\textsubscript{1} receptor antagonist, V2255, completely inhibited the contractile effects of AVP and oxytocin. Similar to AII\textsubscript{2} receptors, AVP\textsubscript{2} receptor activation primarily induces vasodilation via stimulation of vascular endothelial cells.(9) However, the AVP\textsubscript{2} receptor agonist, V1005, elicited no significant change in either OA segment type either from baseline or in the presence of prostaglandin F\textsubscript{2\alpha}-induced pre-constriction (data not shown). Hence, these data are consistent with an elevation in plasma concentrations of AII or AVP eliciting OA constriction via AII\textsubscript{1} or AVP\textsubscript{1} receptor activation, respectively.
It is well established that the vasoactive effects of 5-HT are predominantly mediated via activation of 5-HT$_1$ and 5-HT$_2$ receptors.\(^{(19)}\) In the present study, both distal and proximal OA segments constricted robustly when exposed to either 5-HT or the 5-HT$_2$ receptor agonist. While distal segments were significantly more sensitive to either 5-HT or α-CH$_3$-5-HT than proximal segments, contractile responses in both segment types were completely inhibited by the 5-HT$_2$ receptor antagonist, ketanserin. In contrast to 5-HT$_2$ stimulation, the 5-HT$_{1a}$ receptor agonist, 8-OH-DPAT, elicited only weak contractions in OA segments, and even then only at the highest concentration used (10µM). The lack of constrictor effects of nanomolar concentrations of 8-OH-DPAT, and the blockade of the small contractile effect by ketanserin, are consistent with reported effects of 8-OH-DPAT in rat coronary \(^{(12)}\) and caudal \(^{(3)}\) arteries, and with the possibility that 8-OH-DPAT may activate 5-HT$_2$ receptor at higher concentrations. Since the 5-HT$_{1b/1d}$ receptor agonist, sumatriptan, did not have any effect on tone in OA, the present study are consistent with 5-HT$_2$ receptors being the predominant 5-HT receptor subtype in OA of the rat, which is a common finding for a variety rat blood vessel types.\(^{(12, 13, 17, 20)}\)

In summary, the present study is the first to characterize the contractile responses of OA isolated from the rat to AII, AVP and 5-HT, which appear to be mediated by AII$_1$, AVP$_1$ and 5-HT$_2$ receptors, respectively. The serendipitous finding that the contractile properties of the OA may depend on the precise anatomical location from which segments are taken, and also upon the specific agonist under investigation, highlights the importance of these considerations when studying segments of small blood vessels \textit{in vitro}, even when the entire vessel is only 4mm in length. Further studies are needed to determine whether the function of this potentially important blood vessel changes in disease states, such as hypertension, and whether such changes play a
role in alterations in baroreceptor function. It is hoped that this initial characterization of the OA will provide a basis for such studies.
REFERENCES


Table 1. Contractile effects of AII and Val₅-AII on the proximal and distal segments of the occipital artery.

<table>
<thead>
<tr>
<th>Segment</th>
<th>AII</th>
<th>Val₅-AII</th>
<th>AII + losartan</th>
<th>Val₅-AII + losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ (%$T_{\text{K}}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>19.6 ± 3.4, n = 9</td>
<td>14.0 ± 2.1, n = 9</td>
<td>0.0 ± 0.6,† n = 9</td>
<td>0.1 ± 0.2,† n = 9</td>
</tr>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>8.2 ± 1.4</td>
<td>4.7 ± 1.3</td>
<td>NA</td>
</tr>
<tr>
<td>Distal</td>
<td>14.1 ± 2.4, n = 9</td>
<td>13.5 ± 2.6, n = 9</td>
<td>0.3 ± 0.5,† n = 9</td>
<td>0.0 ± 0.4,† n = 9</td>
</tr>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>3.1 ± 1.5*</td>
<td>4.2 ± 1.4</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M, n = 10 for all segments. Significance set at $P < 0.05$; *significant difference between segment type; †significant difference between agonists in specific OA segment. NA = not applicable.
Table 2. Contractile effects of AVP and oxytocin on the proximal and distal segments of the occipital artery.

<table>
<thead>
<tr>
<th>Segment</th>
<th>AVP</th>
<th>oxytocin</th>
<th>AVP + V2255</th>
<th>oxytocin + V2255</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ (%$T_K$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>$29.7 \pm 8.5, n = 10$</td>
<td>$26.7 \pm 3.8, n = 10$</td>
<td>$0.5 \pm 0.5, \dagger, n = 7$</td>
<td>$0.1 \pm 0.1, \dagger, n = 6$</td>
</tr>
<tr>
<td></td>
<td>$EC_{50}$ (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>$5.2 \pm 1.6, \dagger$</td>
<td>$36.7 \pm 1.3$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>$E_{\text{max}}$ (%$T_K$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>$74.2 \pm 6.8, * n = 11$</td>
<td>$66.6 \pm 10.7, * n = 10$</td>
<td>$0.1 \pm 0.4, \dagger, n = 7$</td>
<td>$0.2 \pm 0.5, \dagger, n = 6$</td>
</tr>
<tr>
<td></td>
<td>$EC_{50}$ (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>$3.7 \pm 1.2, \dagger$</td>
<td>$24.2 \pm 1.4, *$</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. Significance set at $P < 0.05$; *significant difference between segment type; †significant difference between agonists in specific OA segment. NA = not applicable.
Table 3. Contractile effects of 5-HT, α-CH₃-5-HT, 8-OH-DPAT and Sumatriptan on the proximal and distal segments of the occipital artery.

<table>
<thead>
<tr>
<th>Segment</th>
<th>5-HT</th>
<th>α-CH₃-5-HT</th>
<th>8-OH-DPAT</th>
<th>5-HT + ketanserin</th>
<th>α-CH₃-5-HT + ketanserin</th>
<th>8-OH-DPAT + ketanserin</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Eₘₐₓ (%T₀)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td></td>
<td>129.2 ± 10.5, n = 11</td>
<td>82.0 ± 3.9, n = 16</td>
<td>5.7 ± 1.4,† n = 15</td>
<td>0.9 ± 0.2,† n = 7</td>
<td>1.5 ± 0.7,† n = 8</td>
</tr>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>3,303 ± 115</td>
<td>2,024 ± 110†</td>
<td>3,702 ± 177*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Distal</td>
<td>Eₘₐₓ (%T₀)</td>
<td>130.7 ± 5.7, n = 11</td>
<td>108.1 ± 3.7, n = 21</td>
<td>8.6 ± 1.3,⁎† n = 15</td>
<td>4.3 ± 0.7,† n = 7</td>
<td>4.0 ± 1.5,† n = 8</td>
</tr>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>1,503 ± 110*</td>
<td>1,096 ± 110*†</td>
<td>8,622 ± 875†</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. Significance set at P < 0.05; *significant difference between segment type; †significant difference when 5-HT receptor subtype agonist is compared to 5-HT. NA = not applicable.
Figure 1. Isolation of occipital artery segments. Occipital arteries were isolated and bisected into proximal (proximal segment) and distal (distal segment) segments and mounted separately on small vessel myographs for the measurement of isometric tension. n: nerve; a: artery.
Figure 2. Typical small vessel myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to AII. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to AII (Panel C).
Figure 3. Typical small vessel myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to Val⁵-II. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to Val⁵-II (Panel C).
Figure 4. Typical small vessel myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to AVP. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to AVP (Panel C), *P<0.05.
Figure 5. Typical small myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to Oxytocin. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to Oxytocin (Panel C), *P<0.05.
Figure 6. Typical small myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to 5-HT. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to 5-HT (Panel C), *P<0.05.
Figure 7. Typical small myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to α-CH₃-5-HT. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to α-CH₃-5-HT (Panel C), *P<0.05.
Figure 8. Typical small myograph traces of the proximal (Panel A) and distal (Panel B) segments of the OA to 8-OH-DPAT. Mean ± SEM responses of proximal (■) and distal (▲) segments of the OA to 8-OH-DPAT (Panel C), *P<0.05.
CHAPTER 4

A NOVEL VASCULAR CLIP DESIGN FOR THE RELIABLE INDUCTION OF 2-KIDNEY, 1-CLIP HYPERTENSION IN THE RAT

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2S. Chelko, C. Schmiedt, T. Lewis, S. Lewis, T. Robertson. Accepted by Journal of Applied Physiology. Reprinted here with permission of publisher
ABSTRACT

The 2-kidney, 1-clip (2K1C) model has provided many insights into the pathogenesis of renovascular hypertension. However, studies using the 2K1C model often report low success rates of hypertension, with typical success rates of just 40-60%. We hypothesized that these low success rates are due to fundamental design flaws in the clips traditionally used in 2K1C models. Specifically, the gap widths of traditional silver clips may not be maintained during investigator handling and these clips may also be easily dislodged from the renal artery following placement. Therefore, we designed and tested a novel vascular clip possessing design features to maintain both gap width and position around the renal artery. In this initial study, application of these new clips to the left renal artery produced reliable and consistent levels of hypertension in rats. Nine day application of clips with gap widths of 0.27, 0.25, and 0.23 mm elicited higher mean arterial blood pressures of 112 ± 4, 121 ± 6, and 135 ± 7 mmHg, respectively (n = 8 for each group) than those of sham-operated controls (95 ± 2 mmHg, n = 8). Moreover, 8 out of 8 rats in each of the 0.23 and 0.25 mm 2K1C groups were hypertensive whereas 7 out of 8 rats in the 0.27 mm 2K1C group were hypertensive. Plasma renin concentrations were also increased in all 2K1C groups as compared to sham-operated controls. In summary, this novel clip design may help eliminate the large degree of unreliability commonly encountered with the 2K1C model.
INTRODUCTION

Hypertension is a major cause of morbidity and mortality affecting more than one-third of adults in the United States. Renovascular hypertension is a common form of secondary hypertension that results from a decrease in renal blood flow due to renal artery stenosis and subsequent activation of the renin-angiotensin-aldosterone system (RAAS). One of the most commonly-used models to study renovascular hypertension is the two-kidney, one-clip (2K1C) model. First described by Goldblatt and colleagues, the original 2K1C technique involved application of variable size silver clips to reduce the diameter of renal arteries in dogs and monkeys. Although the 2K1C model has provided significant insights into the pathogenesis of renovascular hypertension, few modifications have been made to the original clip design and there are noteworthy inadequacies in this model. Most notably, there is a large failure rate with respect to the number of animals that develop hypertension in the 2K1C model. For example, success rates of 44-80%, in rats, and a 45% success rate in mice, have been reported, which is costly in terms of animals, supplies and investigator time. Consequently, although the 2K1C model is associated with many changes similar to those found in patients with renovascular hypertension, its use by researchers may be limited by the unreliability of the clip procedure.

It is likely that the clip design is a major reason for the relatively low success rate of eliciting hypertension in the 2K1C model. Commonly, the clips are made from silver, which is malleable and easily fashioned into a U-shaped clip. However, the very ductile nature of silver may contribute to the unreliability of the clip because it increases the likelihood of investigator-induced changes in gap width while smoothing (deburring) or during clip application, thereby
resulting in inconsistent reduction in renal artery diameter. Recently, Lorenz et al. reported promising results in producing reliable 2K1C hypertension in mice by use of a modified polyurethane cuff (internal diameter, i.d., 0.27mm).(17) However, this modified cuff is also subject to investigator-induced changes due to the ductile nature of polyurethane, which was reflected by the reported variability in cuff size (0.25 – 0.29mm), and thus renal artery diameter, upon post-mortem examination.(17)

Another confounding factor in current 2K1C models is the potential for inflammation or an increased immunological response as a result of the material from which the cuff or clip is made. Silver has been shown to cause substantial perivascular inflammation, tissue granulation, and intimal proliferation,(7) especially if silver oxides are formed on the surface during sterilization and handling. This perivascular reaction may further alter renal artery lumen diameter, especially in chronic applications. Kraft et al. (2000) demonstrated that silver and stainless-steel implants elicited moderate to severe increases in leukocyte extravasation and venular dilation, respectively, when silver and stainless-steel implants were applied to the dorsal skin-fold in hamsters. Moreover, severe inflammation and edema occurred in 5 out of 6 silver implant preparations 3 days after implantation. In contrast, titanium implants did not cause significant changes in leukocyte leakage or venular dilation.(13) Use of polymers, such as polyurethane-coated silicone implants, has been associated with increased histological staining of vascular endothelial growth factor, transforming growth factor-β, and inflammatory cells at the site of implantation compared with textured-silicone implants in rats.(24)

Another confounding factor is that existing clips do not include design elements to prevent displacement from the renal artery. In the aforementioned polyurethane 2K1C cuff method by Lorenz et al., a silk ligature was tied around the cuff after implantation to secure its
placement around the renal artery. However, the application of the ligature was not sufficient to prevent all cuffs from becoming dislodged, and the fact that the ligature must be tied around the ductile polyurethane cuff likely contributed to the variability in final cuff diameter. (17) The use of a ‘circumferential constriction’ 2K1C method by Lorenz et al. is a novel approach to the 2K1C model, yet due to the method needed to secure the cuff it may be extremely difficult to minimize variations between individual investigators or between research laboratories.

In an attempt to address these problems, we designed a novel titanium vascular clip for use in the 2K1C model. The new clip was designed to eliminate the possibility of deformation during investigator-handling, dislodgement after implantation, and allow for repeated use with a material that can be autoclaved. In this initial study, we determined whether the application of these new clips improved the success rate of 2K1C hypertension in rats nine days following clip placement.

MATERIALS AND METHODS

This investigation conforms to The Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all protocols were approved by the University of Georgia Animal Care and Use Committee.

Experimental Groups

Thirty two male Sprague Dawley rats (380-400 g) were assigned to four study groups (n = 8 per group): sham-operated or one of three 2K1C groups (0.27, 0.25, or 0.23 mm gap widths).
Vascular Clip Design and Manufacture

The vascular clip design is shown in Figure 1. Clips were manufactured by first cutting gap widths of 0.27, 0.25 or 0.23 mm, at a depth of 2.0 mm, into a medical grade titanium rod (3 mm diameter; M. Vincent & Associates, LTD.) using carbide saw blades of varying thickness (RobbJack Corp, Lincoln, CA). Once the internal cuts were made, a 0.254mm hole was drilled using a twist drill (Greenfield Industries, Seneca, SC) through the outer edge of the titanium rod. The edges of all cuts were then deburred using a 90° double angle milling cutter (Niagara Cutter, LLC., Amherst, NY) and individual clips cut from the rod using a 0.79mm slitting saw (RobbJack Corp, Lincoln, CA). The outer edges of the resultant clips were then chamfered using a lathe (HLV-H Hardinge, Elmira, NY). All clips were manufactured with the use of computer-aided design and manufacturing (CAD/CAM) systems, thus eliminating any clip-to-clip gap width variability.(19)

Surgical Procedures

All rats were weighed and anesthesia induced and maintained with 2% isoflurane delivered in 95% O₂ and 5% CO₂. The left abdominal wall was shaved and coated with 70% alcohol and povidone-iodine. Body temperature was monitored and maintained at 37ºC with a digital anal thermometer and heating pad, respectively. A left paracostal celiotomy was performed, the left kidney exteriorized, and the renal artery and vein carefully isolated by blunt dissection. In each of the rats in the 2K1C groups, a clip was placed on the left renal artery with the aid of an Olympus SZ40 dissecting microscope. A nylon suture (5-0 Ethilon, Ethicon, Inc.) was then passed through the pre-drilled hole in the clip and tied to prevent clip dislodgement.
The kidney was carefully placed back in its original location and the abdominal wall and skin closed using interrupted sutures (5-0 Ethilon, Ethicon, Inc.) and 9mm staples (Reflex9, Cellpoint Scientific Inc.). A few drops of local anesthetic (0.1 mL of 2% Lidocaine HCl) and bacitracin, polymixin and neomycin ointment were applied to the incision site. For rats in the sham group, surgeries were performed as described above, except renal clips were applied to the renal artery then removed prior to the kidney being placed back in its original position. All rats were observed for 4h following surgery, then individually housed for 24h and allowed access to standard rat chow and water ad libitum.

**Blood Pressure Measurements**

Nine days following clip application, the rats were weighed, anesthetized as above and the left medial thigh and inguinal area shaved and swabbed prior to surgery. The left femoral artery was exposed and isolated by blunt dissection. A polyethylene catheter (PE/3, Scientific Commodities Inc.) was advanced inside the femoral artery, such that the tip of the catheter was close to the aortic bifurcation. Mean arterial pressure (MAP) and peak systolic blood pressure (PSBP) were measured via a pre-calibrated pressure transducer (Radnoti LLC, Monrovia, CA) and recorded using LabChart software (version 7.0, AD Instruments, Colorado Springs, CO). MAP and PSBP were measured for \( \geq 15 \) min.

**Plasma Renin Concentration**

Immediately following blood pressure measurement, 3 mL of blood was collected in EDTA (7.2 mg, Vacutainer, BD, Franklin Lakes, NJ) via the arterial catheter. Within 30 min of collection, EDTA-plasma samples were prepared by centrifugation, frozen and stored at -20°C.
until analysis. Plasma renin concentrations were determined using a modification of a commercial fluorometric kit (SensoLyte® 520 Rat Renin Assay Kit, AnaSpec, Inc., Fremont, CA). (21) Briefly, stock rat renin substrate and recombinant rat renin were diluted at 1:50 and 1:500 with assay buffer, respectively. Recombinant rat renin was then diluted in series to obtain a 9-point standard curve. All samples, including standards, were analyzed in triplicate. Each well contained 90 µL of serum (or 90 µL of standard) and 10 µL of diluted substrate. The 96-well, flat-bottom, plate was centrifuged at 3000 rpm for 3 min and incubated at 37°C for 4h. Fluorescence was measured with a Fluoroskan Ascent 96-well plate reader (Thermo Fisher Scientific Inc.) at excitation/emission of 490/520 nm and recorded using Ascent software (version 2.6, Thermo Fisher Scientific Inc.). Plasma renin concentration was determined by comparing the plasma samples to the standard curve. There was an excellent linear relationship ($R^2 = 0.975$) between relative fluorescence units and renin concentration in the standard curve.

Organ Weight Measurements

Following blood collection, rats were killed by decapitation while still under general anesthesia. Heart weight, and clipped and non-clipped kidney weights were recorded and clip location relative to the renal artery was determined for all rats in the 2K1C groups.

Statistical Analysis

Results are expressed as mean ± SEM. All data were analyzed using repeated measures ANOVA with post-hoc analysis using the Tukey-Kramer multiple comparison procedure.
Comparison of means between sham-operated and 2K1C rat groups were determined by use of Student’s paired t-test. A value of $P < 0.05$ was deemed to be significant. (26)

RESULTS

Peak systolic blood pressure (PSBP) in all 2K1C groups were significantly higher than in the sham-operated group as determined 9 days after placement of the clips. PSBP in rats with clips of gap widths of 0.27, 0.25 and 0.23 mm were 121 ± 5, 136 ± 6 and 150 ± 8 mmHg respectively whereas the PSBP in the sham-operated group was 102 ± 3 mmHg ($P < 0.05$, for all comparisons between the 2K1C groups and the control group, Figure 2). Rats with renal clips of widths of 0.27, 0.25 or 0.23 mm had significantly higher MAP values (112 ± 4, 121 ± 6 and 135 ± 7 mmHg, respectively, Figure 2) than those of the sham-operated rats (95 ± 2 mmHg). Hypertension was observed in 8 of 8 rats in the 0.23 mm and 0.25 mm clip groups, whereas 7 of 8 rats became hypertensive in the group with the 0.27 mm clips (the MAP of normotensive rat in the 0.27 mm group was 95 mmHg whereas the other rats had MAP values between 104 and 125 mmHg). Rats with renal clips of widths 0.27, 0.25 or 0.23 mm had significantly higher heart rates (392.5 ± 7.1, 373.0 ± 12.7, and 395.3 ± 6.1 beats per minute, respectively) compared to sham-operated rats (343.3 ± 14.3 beats per minute). Upon post-mortem examination, all clips were found to be firmly in place in all 2K1C rats at the end of the 9 day experimental period.

As summarized in Table 1, nine days after clip placement, body weight changes in all 2K1C rats were significantly lower when compared to sham-operated rats (there was no significant difference in initial body weight, Day 0, between sham and 2K1C rats). There was no
significant difference in non-clipped and clipped kidney weights in the sham-operated group. In contrast, the clipped kidney weights were significantly lower than those of the non-clipped kidney in all 2K1C groups, and non-clipped kidney weights from all 2K1C rats were significantly higher than non-clipped kidney weights from sham rats. Rats in the 0.23mm 2K1C group had significantly higher heart weights than sham-operated rats (Table 1).

As summarized in Figure 3, plasma renin concentrations were higher in all 2K1C groups when compared to sham-operated rats. Plasma renin concentrations for 0.27, 0.25 and 0.23 mm-clip 2K1C rats were 114 ± 4, 126 ± 11 and 108 ± 8 µg/mL, respectively compared to 86 ± 4 µg/mL for sham rats (P < 0.05, for 0.27 and 0.25 mm-clip 2K1C rats compared to sham values; PRC was not significantly increased in the 0.23mm group, P = 0.057).

DISCUSSION

Although the 2K1C model has provided valuable insights into the pathogenesis of renovascular hypertension, its continued use and adoption by cardiovascular researchers is hindered by the relatively low success rates of development of hypertension in rats (6, 11, 23, 27) and mice. The most likely reason for the unreliability of the 2K1C model in eliciting hypertension is that the clip design and materials used in this model have several inherent flaws. Specifically, the traditional use of an open U-shaped clip made from silver leads to inaccurate and inconsistent reduction of renal artery diameter and frequent clip displacement from the renal artery.
The aim of this study was to determine whether consistent levels of hypertension could be produced in 2K1C rats while addressing the flaws in traditionally-used clips. We chose an experimental period of nine days as this is sufficient to detect significant changes in blood pressure (14) and is similar to other studies where novel clip or cuff designs have been evaluated. Initially, our first approach was to use a similar design to traditional clips, but using titanium as the material to eliminate problems with the malleability and the inflammatory effects of silver or stainless-steel. However, when we used open U-shaped titanium clips with gap widths of 0.25mm, only 2 of 7 rats (29%) became hypertensive one week after clip implantation (data not shown). We determined that the lack of reproducible hypertension produced by our first generation of clips was due to the clips becoming dislodged from the renal artery as clips were no longer in place in 5 of 7 rats (71%) when post-mortems were performed. Although these clips were much more robust than similar clips made from silver in terms of maintaining the gap widths during investigator handling, the fine deburring of the titanium appeared to render the surfaces extremely smooth and the clips, therefore, slipped off the artery in the days following placement.

Our next iteration of the design included a 0.254 mm diameter hole at the outer edge of the clip. This allowed the investigator to pass a nonabsorbable suture through the hole and then tie the suture in a knot to prevent the clip from becoming dislodged from the artery. This design element also prevented investigator-induced changes in internal gap width as the application of the suture did not deform the titanium clip. This is in contrast to the methods of Lorenz et al., where a ligature is tied around a ductile piece of polyurethane tubing, which was reported to result in variable cuff widths by these investigators.(17) The novel clips produced hypertension
in 100% of rats 9 days following placement of clips with gap widths of either 0.25 or 0.23 mm. In the 0.27 mm 2K1C group, 7 of 8 rats developed hypertension.

Since RAAS activation is fundamental to renovascular hypertension, we assayed plasma renin concentration and determined that 0.27 and 0.25mm 2K1C groups had significantly higher plasma renin concentrations than those in the sham group. However, whereas the 0.23 mm 2K1C group had the highest MAP and PSBP values, rats in this group had lower plasma renin concentrations than either of the other 2K1C groups. The lack of a further increase in plasma renin concentration in the 0.23 mm 2K1C group may be because this level of reduction in renal artery diameter may be too severe and thus damage the kidney, as was demonstrated in one clipped kidney in this group which appeared to develop acute ischemia. This is supported by the literature where gap widths of 0.25 mm are most commonly adopted for rodent 2K1C studies and are associated with consistent increases in RAAS.(2, 3, 11) The increases in non-clipped kidney weight over time (i.e. 9 days) and lack of increase in clipped kidney weight over time found in the present study were consistent with previous findings in 2K1C rats.(11, 20) While 0.23mm 2K1C rats were the only 2K1C group which had a significant increase in heart weight compared with sham-operated rats, all 2K1C rats had an increase in heart-to-body weight ratios consistent with the onset of cardiac hypertrophy.(5) The difference between body weights of 2K1C rats compared to sham rats on day 9 in this study was similar to that reported by Muller et al.(18)

The results of the current study using the new clip design are consistent with those found in 2K1C models in the rat, namely (i) hypertension, (ii) RAAS activation, (iii) lack of weight gain in the kidney ipsilateral to the clipped renal artery, and (iv) reduced gain in body weight. Cardiac hypertrophy, another common finding in 2K1C hypertension, was only significant in rats from the 0.23mm gap width group in this initial study. The major difference between this and
previous studies utilizing silver and stainless-steel U-shaped clips is the improvement in the reliability of hypertension. Although Lorenz et al. has demonstrated improved success with a modified polyurethane cuff in mice, these investigators reported marked variability in cuff diameter (i.d. 0.25 to 0.29mm) upon post-mortem examination. Our experiments directly addressed two main factors that plague studies using traditional silver clips, namely (i) malleability, and (ii) maintenance of clip placement, which were overcome through the use of titanium and the addition of a tie-in-place hole that resulted in 100% hypertension when clips of gap widths 0.25 or 0.23 mm were used.
REFERENCES


Table 1. Body and tissue weight parameters in sham and 2K1C groups at 9 days.

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<th>Group</th>
<th>∆BW (g)</th>
<th>NCKW (g)</th>
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<th>CKW (g)</th>
<th>CKW (mg/g BW)</th>
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The data are presented as Mean ± SEM. ∆BW, change in body weight from Day 0; NCKW, non-clipped kidney weight; CKW, clipped kidney weight; HW, heart weight. There were 8 rats in each group. *P < 0.05, 2K1C groups versus sham. †P < 0.05, CKW versus NCKW.
**Figure 1.** Design of the new clip. Left Panel: Dimensions are shown in millimeters. Right Panel: Illustrations of the new clip design (Patent(s) Pending).
Figure 2. Mean arterial pressure (MAP) and peak systolic blood pressure (PSBP) in sham and 2K1C groups (n = 8) at 9 days. *denotes significant difference from sham group.
Figure 3. Plasma renin concentrations (PRC) on day 9 from each group (n = 8); data are presented as mean ± SEM. *denotes significant difference from sham group.
CHAPTER 5

OCCIPITAL ARTERY FUNCTION DURING THE DEVELOPMENT OF 2-KIDNEY, 1-CLIP HYPERTENSION IN THE RAT

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ABSTRACT

Renovascular hypertension (RVH) is the most common cause of secondary hypertension. Recent reports have provided evidence that circulating factors can gain access to the cell bodies of the nodose ganglion via the occipital arteries (OA), thereby influencing blood pressure regulation. The aim of this study was to characterize the contractile responses of isolated OA to angiotensin II (AII), arginine vasopressin (AVP), and 5-hydroxytryptamine (5-HT) from 2-kidney, 1-clip (2K1C) and sham-operated rats 9 days after clip implantation. Rat OA (250-400µm diameter) were isolated and bisected such that segments proximal to the external carotid artery (proximal segment) or nodose ganglion (distal segment) could be mounted separately on myographs. 2K1C rats had significantly higher mean arterial blood pressure (119.8 ± 3.8 mmHg, n = 8) compared with sham-operated controls (96.4 ± 1.0 mmHg, n = 8). Additionally, 2K1C rats had significantly higher heart rates and plasma renin concentrations than sham-operated rats. Maximum responses of OA to AII (100pM-100nM), from sham-operated rats, were significantly higher in proximal (E\text{max}: 66.8 ± 10.1, n = 8) than distal segments (E\text{max}: 44.9 ± 7.8, n = 8). The responses of both segment types were significantly higher compared to maximal responses from 2K1C rats (proximal segment E\text{max}: 18.5 ± 3.8, distal segment E\text{max}: 14.0 ± 2.9, n = 8 for both). Distal segments were significantly more sensitive to 5-HT than proximal segments in both 2K1C and sham-operated rats, though sham-operated rats elicited significantly higher maximal responses to 5-HT (1nM – 10µM) at proximal segments compared to 2K1C rats. Distal segments elicited significantly higher maximal responses to AVP than proximal segments from both 2K1C and sham-operated rats, however there was no significant difference between experimental
groups. The results of this study are consistent with the concept that OA function may be altered in 2K1C rats in an agonist and anatomical specific manner.

INTRODUCTION

Renovascular hypertension (RVH) is the most common cause of secondary hypertension,(1, 28) resulting from a decrease in renal blood flow and activation of the renin-angiotensin-aldosterone system (RAAS).(11) RVH is associated with increases in circulating vasoconstrictors, such as angiotensin II (AII),(25) and arginine vasopressin (AVP),(6, 7) which act to decrease artery diameter, thereby raising vascular resistance and blood pressure.(14, 19)

Given the prevalence of this disease, it is vital that experimental models accurately and reliably reflect the pathogenesis of RVH. One of the most commonly-used models to study RVH is the two-kidney, one-clip (2K1C) technique. First described by Goldblatt et al.,(9, 10) this technique decreases kidney perfusion, thereby activating granular cells to release renin.(11) Furthermore, AII influences renal absorption of sodium and water via AVP and aldosterone release, thereby increasing blood volume and blood pressure.(2)

In addition, renal hypoperfusion decreases renal-afferent nerve activity, which in turn, increases renal-efferent sympathetic nerve activity, thereby increasing renin secretion via β1-adrenergic receptor activation.(15-17) In 2K1C hypertension, renal baroreceptors (BR) are stimulated and relay the change in blood pressure to nucleus tractus solitarii in order to regulate blood pressure.(15) Furthermore, the cell bodies of vagal- and glossopharyngeal-afferent nerves, such as the nodose (NG) and petrosal ganglion (PG), respectively, (4) play a vital role in blood
pressure regulation via the NTS(5, 27), and thus the vasculature that supplies them is also important. The occipital (OA) and internal carotid artery (ICA) supply blood to the NG and PG, however Lacolley et al. demonstrated that the OA-NG complex lacks a tight blood ganglion barrier, compared with the ICA-NG complex.(18) Specifically, Lacolley et al. demonstrated that microinjections of 5-HT in the OA elicited immediate reductions in blood pressure and heart rate in the rat, however these findings were absent when OA were ligated.(18) In addition, Jacobs et al. demonstrated that hypotension and bradycardia ensues after 5-HT injections in the CCA of cats, whereas these findings were absent in cats with prior OA ligations.(13) Therefore, this is consistent with the concept that the OA may play an important role in the regulation of NG and PG function and, in turn, blood pressure homeostasis.

Vascular reactivity plays a central role in total peripheral resistance and thus blood pressure,(19) and several studies have detailed vascular changes in vessels isolated from 2K1C rats. However, to date no studies exist examining the possibility that the contractile properties of OA may be altered during the development of 2K1C-assocaited RVH. The aim of this study is to provide initial insights into the contractile properties of the OA isolated from sham and 2K1C rats, and provide the basis from which to elucidate the role of this potentially important blood vessel in the development of hypertension.

MATERIALS AND METHODS

This investigation conforms to The Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all protocols were approved by the University of Georgia Animal Care and Use Committee.
**Experimental Groups**

Sixteen male Sprague Dawley rats (380-400 g) were assigned to two experimental study groups (n = 8/group): sham-operated or 2K1C. Renal clips were manufactured from a medical grade titanium (0.25 mm internal gap width) as described previously.(3)

**Surgical Procedure**

All rats were weighed and anesthesia induced and maintained with 2% isoflurane delivered in 95% O₂ and 5% CO₂. A left paracostal celiotomy was performed, the left kidney exteriorized, and the renal artery and vein carefully isolated by blunt dissection. In each of the rats in the 2K1C groups, a clip (0.25mm, internal diameter) was placed on the left renal artery and a nylon suture (5-0 Ethilon, Ethicon, Inc.) was then passed through the pre-drilled hole in the clip and tied to prevent clip dislodgement, as described previously.(3) For rats in the sham group, surgeries were performed as described above, except renal clips were applied to the renal artery then removed prior to the kidney being placed back in its original position.

**Blood Pressure Measurements**

Nine days following clip application, rats were weighed, anesthetized as above, and a polyethylene catheter (PE/3, Scientific Commodities Inc.) was advanced inside the left femoral artery such that the tip of the catheter was close to the aortic bifurcation. Mean arterial pressure (MAP) and peak systolic blood pressure (PSBP) were measured via a pre-calibrated pressure
transducer (Radnoti LLC, Monrovia, CA) and recorded using LabChart software (version 7.0, AD Instruments, Colorado Springs, CO). MAP and PSBP were measured for ≥ 15 min.

**Plasma Renin Concentration**

Immediately following blood pressure measurement, 3 mL of blood was collected in EDTA (7.2 mg, Vacutainer, BD, Franklin Lakes, NJ) via the arterial catheter. Within 30 min of collection, EDTA-plasma samples were prepared by centrifugation, frozen and stored at -20°C until analysis. Plasma renin concentrations were determined using a modification of a commercial fluorometric kit (SensoLyte® 520 Rat Renin Assay Kit, AnaSpec, Inc., Fremont, CA), as described previously. (3, 26) Plasma renin concentration was determined by comparing the plasma samples to the standard curve. There was an excellent linear relationship (R² = 0.9979) between relative fluorescence units and renin concentration in the standard curve.

**Organ Weight Measurements**

Following blood collection, rats were killed by decapitation while still under general anesthesia. Heart weight, and clipped and non-clipped kidney weights were recorded and clip location relative to the renal artery was determined for all rats in the 2K1C group.
Occipital Artery Isolation and Small Vessel Myography

Following euthanasia, heads were immediately placed in cold physiological saline solution (PSS) containing (in mM): NaCl 118, NaHCO₃ 24, KCl 4, glucose 5.6, MgSO₄ 1, NaH₂PO₄ 0.435, CaCl₂ 1.8. Care was taken to isolate the OA (300 – 400 µm internal diameter) from behind the digastricus muscles of the neck and bisected into proximal (proximal segment) and distal (distal segment) segments and mounted separately on small vessel myographs (Model 500A, Danish Myo Technology, Denmark). After equilibrating for 30 mins in PSS gassed with 12% O₂, 5% CO₂, and 83% N₂ (pH 7.4, 37°C), OA were stretched using the methods described by Mulvany et al. for systemic arteries.(22) The maximum responses of OA segments to a depolarizing stimulus were then established by exposing them to 80 mM K⁺ (KPSS; isotonic replacement of Na⁺ by K⁺; 3 x 2 min exposures, 15-20 mins apart), as described previously.(24)

Experimental Protocols

Concentration response curves for AII, AVP and 5-HT were performed on OA. Contractile responses are expressed as a percent of that observed to the final KPSS exposure (%Tₖ).

Agonists and Antagonists

[AVP⁸]-Vasopressin acetate salt (AVPR agonist) and 5-hydroxytryptamine hydrochloride (5-HTR agonist) were purchased from Sigma Chemical Co. (St. Louis, MO); and Angiotensin II (AIIR agonist) was purchased from the American Peptide Co. (Sunnyvale, CA).
Statistical Analysis

Heart and kidney weight data were expressed as milligrams of organ weight divided by grams of body weight. Data are presented as mean ± S.E.M. Data were analyzed by 2-way analysis of variance (ANOVA) with post hoc analysis using the Bonferroni correction for multiple comparisons procedure. A value of $P < 0.05$ was deemed to be significant.(32) Contractile responses were expressed as a percentage of the maximal contractile response to KPSS (% $T_K$) for each vessel. Differences between maximal contractile response ($E_{\text{max}}$) and vasoconstrictor potency ($EC_{50}$) for small vessel myography data were determined by Student's modified $t$-test.

RESULTS

Nine days after clip implantation, 2K1C rats had significantly higher MAP (119.8 ± 3.8 mmHg) and PSBP (133.5 ± 4.9 mmHg) compared with sham-operated controls (MAP: 96.4 ± 1.0 mmHg; PSBP 104.8 ± 1.8 mmHg, Figure 1). Hypertension was observed in 8 out of 8 2K1C rats (MAP range: 109 – 141 mmHg), while sham-operated rats remained normotensive (MAP range: 93 – 101 mmHg). Furthermore, 2K1C rats had significantly higher heart rates (396.4 ± 8.7 beats per minute) compared to sham-operated controls (340.2 ± 11.7 beats per minute). As summarized in Figure 2, plasma renin concentrations were significantly higher in 2K1C rats (105.6 ± 12.8 µg/mL) compared to sham-operated rats (67.6 ± 2.0 µg/mL).

As summarized in Table 1, 2K1C rats demonstrated a significant loss in weight gain when compared to sham-operated rats, after nine days of clip implantation. There was no
significant difference in initial body weight, Day 0, between sham and 2K1C rats. Clipped kidney weights (2.4 ± 0.09 mg/g BW) were significantly lower than those of the non-clipped kidney weights (3.3 ± 0.09 mg/g BW) in 2K1C rats and clipped kidney weights from sham rats (2.8 ± 0.08 mg/g BW). In contrast, there was no significant difference in non-clipped and clipped kidney weights in the sham-operated group, though non-clipped kidney weights from 2k1C rats were significantly higher than sham-operated rats. In addition, 2K1C rats had significantly higher heart weights than sham-operated rats (Table 1).

Maximum contractile responses of the OA to AII, AVP, and 5-HT are shown in Figures 3, 4 and 5, respectively. The proximal and distal segments of the OA contracted robustly to AII, AVP, and 5-HT in a concentration-dependent manner. However, in sham-operated controls, the distal and proximal segments of the OA exhibited significantly higher maximal constrictions to AII ($E_{\text{max}}$: 44.9 ± 7.8% T_K; and 66.8 ± 10.1% T_K, n = 8 for both, respectively, Table 2) compared to the distal and proximal segments of the OA from 2K1C rats ($E_{\text{max}}$: 14.0 ± 2.9% T_K; and 18.5 ± 3.8% T_K, n = 8 for both, respectively, Figure 3). Distal segments exhibited significantly stronger maximal constrictions to AVP ($E_{\text{max}}$: 72.3 ± 8.6% T_K, n = 8) compared with proximal segments ($E_{\text{max}}$: 25.7 ± 5.0% T_K, n = 8) from 2K1C rats. This was also the case for AVP-induced contractions in sham-operated rats (distal segment $E_{\text{max}}$: 79.2 ± 7.3% T_K; proximal segment $E_{\text{max}}$: 28.8 ± 4.0% T_K n = 8 for both, Table 3). Proximal segments, from sham-operated rats, elicited significantly higher maximal responses to 5-HT and were significantly more sensitive to 5-HT than proximal segments from 2K1C rats. There was no significant difference in maximal response of distal segments compared with the proximal segments from 2K1C rats, as was the case in sham-operated rats. However, distal segments were significantly more sensitive to 5-HT than proximal segments for both 2K1C and sham-operated rats.
DISCUSSION

Reliably inducing hypertension is vital in the study of RVH-induced changes in OA function. In this study, the use of our novel clips produced hypertension in 100% of 2K1C rats 9 days following clip placement around the left renal artery and was associated with significantly higher MAP, PSBP, and heart rates values compared with sham-operated controls. This is in contrast to many previous studies that reported much lower success rates.(8, 12, 29, 31, 33)

RAAS activation, due to renal hypoperfusion, is a core component of RVH and in the current study, 2K1C rats had significantly higher PRC values than sham-operated controls. Furthermore, increases in non-clipped kidney weight, lack of increase in clipped kidney weight, and onset of cardiac hypertrophy at the end of this study are consistent with previous findings from 2K1C rats.(7, 12, 23) Upon gross examination, kidneys from either experimental group showed no signs of scarring, damage or acute ischemia. The finding that 2K1C rats demonstrated impaired weight gain compared to sham rats in this study is similar to that reported in rats by Muller et al.(21) In addition, poor weight gain has also been demonstrated in adolescents with unilateral renal arterial stenosis, high renin and aldosterone levels, and high blood pressure.(30)

Lacolley et al. (18) and Jacobs et al. (13) have recently demonstrated the unique permeability of the OA-NG complex. Specifically, microinjections of 5-HT (molecular weight, MW = 212) into the OA, elicited a pronounced drop in heart rate and MAP. Lacolley et al. demonstrated that the small molecular weight tracer Basic Blue 9 (BB9, MW = 374) was taken up by NG cell bodies, 30 mins after injection into the jugular vein of rats with open OA, however in rats with OA ligations NG cell bodies lacked BB9 staining.(18) Therefore, the lack of a tight BGB at the OA-NG complex may allow for circulating factors, such as AII, AVP and 5-HT, to
gain access to, and affect the function of, NG cell bodies. However, to our knowledge, there are no current reports concerning the contractile properties of the OA during RVH.

Proximal and distal segment responses to 5-HT were similar within each experimental group and between distal segments of OA isolated from sham and 2K1C rats. However, proximal segments from 2K1C had significantly lower maximal responses to 5-HT when compared to proximal segments isolated from sham-operated rats, in contrast to the increase in responsiveness to 5-HT of tail arteries isolated from 2K1C rats.(20) AVP elicited significantly higher maximal contractions in distal segments of the OA compared to the proximal segments in both 2K1C and sham rats. However, there were no differences in contractile responses of OA segments to AVP between experimental groups.

In this initial study, we found marked differences in contractile responses of OA segments to three agonists relevant to blood pressure regulation. Moreover, RVH was associated with both segment and agonist-specific alterations in OA contractile function. Although proximal segments exhibited significantly higher maximal responses to AII compared to distal segments of the OA in sham rats, there was no significant difference between segment types isolated from 2K1C rats. Interestingly, OA segments isolated from 2K1C rats had significantly lower AII-induced maximal contractile responses compared to OA isolated from sham rats. At first glance, this finding would appear to be consistent with the concept that increased circulating AII, as a result of 2K1C, down-regulates AII receptors on OA; thereby reducing the contractile effect of AII in vitro. However, on closer examination, it is apparent that the responses of OA from sham-operated rats was much higher than that observed for OA isolated from control rats (i.e., rats that have not undergone any surgical procedure - see Chapter 4 for details). Hence, it would appear that the "sham" procedure may in itself increase OA reactivity, which is then reduced during
RVH. The precise reasons for the alteration in OA function associated with the sham procedure remain obscure, but are likely related to either the anesthetic used, the physical disruption of the adventitia surrounding the renal artery, or any inflammatory response to the latter. Further studies will be required to define the roles of these potential variables and also to determine whether the reduction of OA responsiveness to AII during 2K1C is real.
REFERENCES


Table 1. Body and tissue weight parameters in sham and 2K1C rats (n = 8/group) at 9 days.

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<tr>
<th>Group</th>
<th>(\Delta BW) (g)</th>
<th>NCKW (g)</th>
<th>NCKW (mg/g BW)</th>
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<td>Sham</td>
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The data are presented as Mean ± SEM. \(\Delta BW\), change in body weight from Day 0; NCKW, non-clipped kidney weight; CKW, clipped kidney weight; HW, heart weight. There were 8 rats in each group. *\(P < 0.05\), 2K1C groups versus sham. †\(P < 0.05\), CKW versus NCKW.
Table 2. Contractile effects of AII on the proximal and distal segments of the occipital artery.

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<td>$EC_{50}$ (nM)</td>
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Data are presented as mean ± S.E.M, n = 8/group/segment. Significance set at $P < 0.05$; *2K1C versus sham; †significant difference between segment type within group.
Table 3. Contractile effects of AVP on the proximal and distal segments of the occipital artery.

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</tbody>
</table>

Data are presented as mean ± S.E.M, n = 8/group/segment. Significance set at $P < 0.05$; *2K1C versus sham; †significant difference between segment type within group.
Table 4. Contractile effects of 5-HT on the proximal and distal segments of the occipital artery.

<table>
<thead>
<tr>
<th>Segment</th>
<th>2K1C</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E\textsubscript{max} (%T\textsubscript{K})</td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>100.3 ± 6.2</td>
<td>113.3 ± 6.5*</td>
</tr>
<tr>
<td></td>
<td>EC\textsubscript{50} (nM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,852 ± 120</td>
<td>1,941 ± 119*</td>
</tr>
<tr>
<td>Distal</td>
<td>E\textsubscript{max} (%T\textsubscript{K})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>112.2 ± 6.2</td>
<td>121.3 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>EC\textsubscript{50} (nM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>941 ± 113†</td>
<td>844 ± 110†</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M, n = 8/group/segment. Significance set at $P < 0.05$; *2K1C versus sham; †significant difference between segment type within group.
Figure 1. Mean arterial pressure (MAP) and peak systolic blood pressure (PSBP) from sham and 2K1C rats (n = 8/group) at 9 days; data are presented as mean ± SEM. *denotes significant difference from sham group.
Figure 2. Plasma renin concentrations (PRC) on day 9 from sham (n = 7) and 2K1C (n = 8) rats; data are presented as mean ± SEM. *denotes significant difference from sham group.
Figure 3. Mean ± SEM responses of proximal (□ sham; △ 2K1C) and distal (■ sham; ▲ 2K1C) segments of the OA to AII, n = 8/group.

segments of the OA to AII, n = 8/group.
Figure 4. Mean ± SEM responses of proximal (□ sham; △ 2K1C) and distal (■ sham; ▲ 2K1C) segments of the OA to AVP, n = 8/group/segment.
Figure 5. Mean ± SEM responses of proximal (□ sham; Δ 2K1C) and distal (■ sham; ▲ 2K1C) segments of the OA to 5-HT, n = 8/group/segment.
CHAPTER 6

CONCLUSION

High blood pressure is a major cause of morbidity and mortality and increases a patient’s risk of developing heart disease and stroke, the first and third leading cause of death in the United States. (6, 19) Hypertension affects more than one-third of adults in the United States and The World Health Organization estimates that over 17 million people in the world die from cardiovascular diseases every year. (9) Renovascular hypertension (RVH) is the most common cause of secondary hypertension, resulting from renal hypoperfusion and activation of the renin-angiotensin-aldosterone system (RAAS). RVH is associated with increases in blood volume, via sodium and water retention, and circulating vasoconstrictors which decrease resistance artery diameter, thereby raising vascular resistance and arterial blood pressure. Given the prevalence of this disease, it is vital that experimental models accurately and reliably reflect the pathogenesis of RVH.

One of the most commonly-used models to study RVH is the 2K1C technique. First described by Goldblatt et al. in 1934, application of silver clips to partially reduce the diameter of renal arteries (3, 4) results in decreased kidney perfusion and renin release. Renin cleaves angiotensinogen into AI, which is subsequently converted into the potent vasoconstrictor, AII, by ACE. Circulating AII increases blood pressure by eliciting smooth muscle constriction in resistance arterioles and raising blood volume through renal absorption of sodium and water via stimulating AVP and aldosterone release from the pituitary and adrenal glands, respectively.
Additionally, decreased renal perfusion activates renal-afferent baroreceptors (BR) innervating renal afferent arteries. Specifically, in 2K1C hypertension, decreased renal perfusion causes a reduction in action potential frequencies sent to the NTS via renal-afferent sensory neurons.(7) Furthermore, BR in cell bodies of the vagus and glossopharyngeal nerves, such as the nodose (NG) and petrosal ganglion (PG), respectively, monitor changes in blood pressure.(1, 18, 22)

The function of the vagal and glossopharyngeal cell bodies is vital for the maintenance and regulation of blood pressure and blood flow and thus the vasculature that supplies them is equally important. Occipital arteries (OA) arise from the external carotid arteries (ECA), proximal to the bifurcation of the common carotid arteries (CCA) into the internal carotid arteries (ICA) and ECA. Blood supply to the NG and PG is provided by the ICA and OA, however, recent research has demonstrated that low molecular weight markers can gain direct access to the cells bodies of the NG via the OA, but not via the ICA.(8) This is consistent with the concept that the OA may play an important role in the regulation of NG function and, in turn, blood pressure homeostasis. Neuromodulators synthesized by, or transported to, NG cell bodies affect NG membrane potential and thus regulate action potential firing to the NTS. Therefore, if circulating factors gain access to the cell bodies of the NG, via the OA, they could potentially contribute to the regulation of baroreflex activity by influencing the resting potential of NG axon hillocks. Additionally, cell bodies of the NG have a wide variety of receptors, such as glutamate, AII, AVP and 5-HT.(22) Of these, it is known that AII, AVP and 5-HT can be up- or down-regulated by organ culture, reactive oxygen species, cardiovascular diseases, neuronal damage, and/or hypertension.(10, 16, 17, 20)

Vascular reactivity plays a central role in the control of blood pressure, and several studies have detailed vascular changes in blood vessels from 2K1C rats, including aorta,(11, 14,
common carotid,(15, 21) and mesenteric(10, 12, 16, 17) arteries. However, there have been no in vitro studies regarding the contractile properties of OA isolated from rats. Therefore, the overall aim of this project was to determine the contractile properties of OA isolated from control, sham and 2K1C hypertensive rats. It is hoped that these data will help elucidate the role of this potentially important blood vessel in the development of hypertension.

Initially, rat OA were isolated and bisected in order to maximize the number of vessels under study. However, this led to the serendipitous discovery of differences in OA sensitivity and maximal contractile responses between proximal and distal segments. Specifically, maximum responses to AVP and the AVP₁ receptor agonist, oxytocin, were significantly higher in distal than proximal segments, and were abolished by the AVP₁ receptor antagonist, V2255. Furthermore, distal segments were significantly more sensitive to either 5-HT or the 5-HT₂ receptor agonist, α-CH₃-5-HT, than proximal segments. These results are consistent with the concept that the contractile properties of rat OA may vary depending on the agonist studied and the specific segment of the OA under investigation. In summary, this study is the first to characterize the contractile responses of OA isolated from the rat to AII, AVP and 5-HT, which appear to be mediated by AII₁, AVP₁ and 5-HT₂ receptors, respectively.

In order to determine whether OA function changes in disease states, such as hypertension, successful induction of RVH via renal artery clipping was vital. As previously mentioned, the 2K1C technique has provided many insights into the pathogenesis of RVH. However, 2K1C studies often report low success rates of hypertension (~40-60%) and/or only include data from animals in which successful 2K1C-induction occurs. Therefore, we hypothesized that these low success rates were due to flaws in the clip’s design. Specifically, gap widths from traditional silver clips may either undergo investigator-induced alterations during
handling or be easily dislodged from the renal artery during the experimental period. As such, we designed and tested a novel vascular clip possessing design elements to maintain both gap width and position around the renal artery. Indeed, the results of the second study confirm that application of these novel clips produced reliable and consistent levels of hypertension in rats. Clips with gap widths of 0.27, 0.25, and 0.23 mm elicited higher mean arterial blood pressures of 112 ± 4, 121 ± 6, and 135 ± 7 mmHg, respectively (n = 8/group) than those of sham-operated controls (95 ± 2 mmHg, n = 8). Moreover, plasma renin concentrations were also increased in all 2K1C groups as compared to sham-operated controls.

The third, and overall, aim of this study was to determine RVH-induced changes in OA function, and thus reliable induction of 2K1C was vital for this project. As such, the above data confirms that novel clips with gap widths of 0.25mm produced reliable hypertension in 100% of 2K1C rats 9 days following clip placement, while clips with gap widths of 0.27 or 0.23mm produced either moderate levels of hypertension or resulted in renal ischemia of the clipped kidney, respectively. Therefore, the final aim of this project utilized novel renal clips with gap widths of 0.25mm which was associated with significantly higher MAP, PSBP, and heart rates values compared with sham-operated controls. Additionally, given that RAAS activation via renal hypoperfusion is essential in the study of RVH, we assayed PRC and demonstrated that 2K1C rats had significantly higher PRC values than sham-operated controls. Furthermore, increases in non-clipped kidney weight, lack of increase in clipped kidney weight, and onset of cardiac hypertrophy at the end of this study are consistent with previous findings from 2K1C rats.(2, 5, 13)

The results obtained during this study provide evidence that RVH is associated with alterations in the contractile properties of OA. Specifically, maximal contractile responses of OA
to 5-HT were significantly greater in proximal segments from sham-operated rats compared to proximal segments from 2K1C rats. Maximal responses to AII in proximal and distal segments of OA from 2K1C rats were significantly lower than responses from sham-operated rats. However, when comparing the data obtained from the studies using OA from control rats that had not undergone surgeries with those obtained in the RVH study, it is possible that the reduction in AII-induced responses in OA isolated from 2K1C rats may be artefactual. Specifically, maximal contractile responses of OA from sham-operated rats were almost three fold higher than those observed in OA from control rats. Hence, the procedures associated with the "sham" operation may in themselves induce changes in OA function. These findings highlight the importance of sham-operated controls in studies involving surgical procedures when interpreting the resulting data. Identifying the precise causes for the increases in OA reactivity in sham-operated rats will require further study, but are likely related to either the prolonged period of anesthesia or the physical manipulation or disruption of the tissue surrounding the renal vasculature.

Collectively, these studies represent the first steps in understanding the role of the occipital artery in the regulation of blood pressure in health and disease. Central to the successful completion of these studies was the refinement of the 2K1C model, coupled with the use of the small vessel myograph to provide the first characterization of the contractile properties of the OA in normotensive and hypertensive rats. It is hoped that these studies will be valuable additions to the field of hypertension research, and that they will provide the foundation for future studies concerning this potentially crucial blood vessel.
REFERENCES


