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Arterial Wall Hypertrophy and PDGF-A Expression in One-Kidney, One-Clip Hypertension and Angiotensin II Infusion Are Mediated By Elevated Arterial Pressure

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ARTERIAL WALL HYPERTROPHY AND PDGF-A EXPRESSION IN ONE-KIDNEY, ONE-CLIP HYPERTENSION AND ANGIOTENSIN II INFUSION ARE MEDIATED BY ELEVATED ARTERIAL PRESSURE

by

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ABSTRACT

ARTERIAL WALL HYPERTROPHY AND PDGF-A EXPRESSION IN ONE-KIDNEY, ONE-CLIP HYPERTENSION AND ANGIOTENSIN II INFUSION ARE MEDIATED BY ELEVATED ARTERIAL PRESSURE

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These studies were designed to characterize the relative roles of angiotensin II (ANG II) and pressure in the structural alterations that occur in experimental hypertension. Two separate studies were performed in order to differentiate these two mechanisms. First, male Wistar rats were subjected to one-kidney, one-clip (1K1C) hypertension or sham operation. Losartan, an AT1 receptor antagonist, was administered chronically to half of each surgical group beginning one day before the operation and continued until the end of the study. In a second experiment, rats were implanted with osmopumps delivering either ANG II or saline. To maintain pressure in the normotensive range, minoxidil, a vasodilator, was administered to half of each group one day before pump implantation and continued until the animals were sacrificed. In both experiments, systolic blood pressure was monitored and recorded throughout the two week study. Upon completion of both experimental periods, anesthetized rats were perfusion fixed in a vasodilated state and the thoracic aorta, carotid, small mesenteric and external spermatic arteries were extracted, fixed and processed. Morphological analysis revealed that cross-sectional wall area was correlated with elevated arterial pressure, but not with angiotensin II infusion or AT1 receptor activation. Immunohistochemical analysis indicated that

vascular smooth muscle cell hypertrophy, not hyperplasia, contributed to the increase in cross-sectional wall area of the arteries. Also, capillary density was reduced in renal hypertension and this was not affected by AT1 receptor inhibition. In addition, PDGF-A mRNA was elevated in arterial walls of hypertrophied vessels. This effect was associated with elevated arterial pressure, but not with angiotensin II infusion. This PDGF-A expression was partially reduced by AT1 receptor inhibition in the small mesenteric artery.

These studies indicate that arterial hypertrophy in 1K1C hypertension and angiotensin II infusion occurs in response to elevated pressure. This arterial hypertrophy was not associated with hyperplasia or polyploidy of vascular smooth muscle cells. Capillary density is not affected by ANG II infusion, or AT1 receptor inhibition. In addition, PDGF-A expression is correlated with elevated pressure and arterial wall hypertrophy.

I would like to dedicate this work to my family.

To Harry and Betty Moock, my grandparents, for renewing my faith in God, and helping me keep everything in perspective. To Captain Paul Owen, for being the big brother I never had. To my sister, Wendy Taylor Owen, for without her competitive nature and camaraderie, I would not be where I am today.

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LIST OF ABBREVIATIONS

1K1C	One-kidney, One-clip
2K1C	Two-kidney, One-clip
ACE	Angiotensin converting enzyme
ANG I	Angiotensin I
ANG II	Angiotensin II
ANG III	Angiotensin III
AT1	Angiotensin Type-One (receptor)
AT2	Angiotensin Type-Two (receptor)
BRDU	5-Bromo-2′-deoxyuridine
CEI	Converting Enzyme Inhibitor
CTP	Cytosine Triphosphate
DAG	Diacylglycerol
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
DOCA	Deoxycorticosterone Acetate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ETOH	Ethanol
IP3	Inositol (1, 4, 5) tri-phosphate
MAPK	Mitogen-Activated Protein Kinase

MNXS	Minoxidil-O-Sulfate
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate Buffered Saline
PDGF-A	Platelet-derived Growth Factor-A Chain
PDGF-B	Platelet-derived Growth Factor-B Chain
PLC	Phospholipase C
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
SSC	Standard Saline Citrate
SHR	Spontaneously Hypertensive Rat(s)
TGF	Transforming growth factor
tRNA	Transfer Ribonucleic Acid
VSMC	Vascular Smooth Muscle Cell(s)
WKY	Wistar-Kyoto

CHAPTER I

INTRODUCTION

Hypertension, defined as blood pressure greater than 140 mm Hg systolic and/or 90 mm Hg diastolic, is known as the silent killer because physical symptoms typically are not observed until the later stages of end organ disease (1). It is estimated that one quarter of the American adult population has elevated blood pressure (2). Hypertensive individuals have a two to three fold greater risk for cardiovascular diseases compared to their age-matched normotensive counterparts (3). Additionally, the risk of sudden death is increased 3 fold in hypertensive individuals. It is important to understand the underlying pathophysiology occurring in the vasculature in order to develop strategies for treatment of this disease.

In the course of hypertension, the individual arteries adapt to mechanical and hormonal stresses through alterations in medial thickness and/or internal and external diameters, depending upon the size and function of the particular blood vessel. As illustrated in figure 1-1, the large arteries increase wall cross-sectional area with the development of outward hypertrophy (4, 5, 6). The smaller arterioles experience a decrease in lumen size without an increase in wall area, and/or rarefaction, a reduction in the number of functional vessels (5, 7, 8, 9). The lumen reduction in absence of hypertrophy is termed inward eutrophic remodeling and was first found by Short and Thompson (10) on the submucosal arterioles of the intestine in the hypertensive individuals. The smaller arteries are located at the overlap where hypertrophy decreases

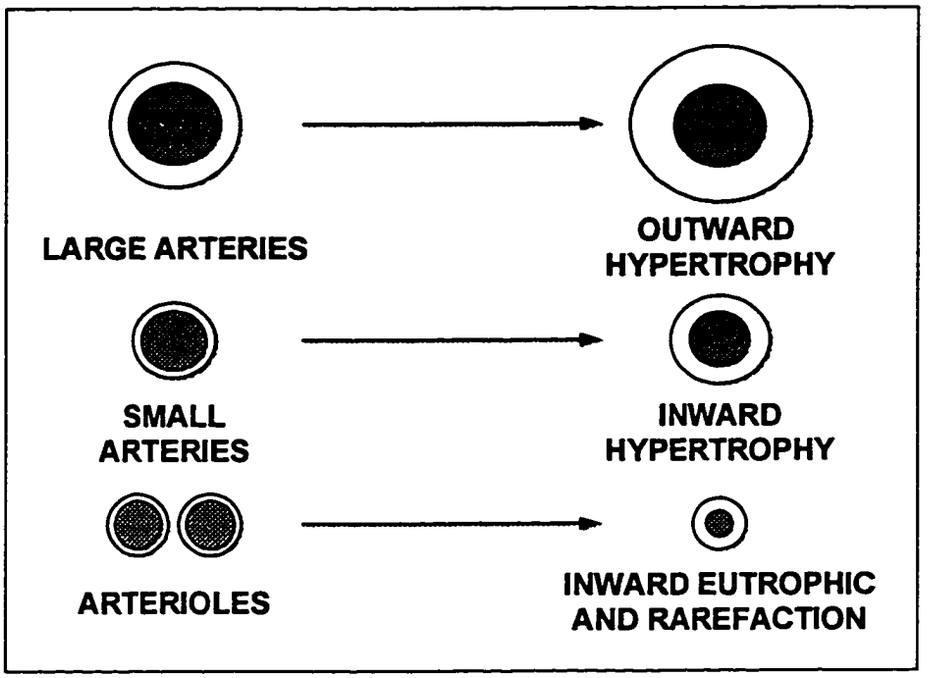


FIG 1-1. Vascular adaptations associated with hypertension.

and eutrophic remodeling begins, wherein they experience either inward eutrophic or hypertrophic remodeling depending on the type of hypertension (11, 12, 13).

Many factors have been proposed to explain the structural alterations, which occur during hypertension. One is the hormone angiotensin II, which is a hypertrophic and hyperplastic stimulus of VSMCs (14, 15), as well as an inducer of PDGF-A chain expression (16). The second is the effects of elevated intravascular pressure. Like ANG II, elevated pressure is also considered a hypertrophic stimulus (4, 18), and is linked to PDGF-A expression (19). The purpose of these studies are to investigate the direct role of ANG II and its indirect role through raised intravascular pressure on the structural changes which occur during hypertension. These changes include VSMC adaptations, alterations in growth factor expression, and rarefaction.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Renin Angiotensin System: An Overview

As a consequence of the effect of ANG II on blood pressure, it is often difficult to distinguish between its direct and indirect effects on vascular hypertrophy associated with hypertension. ANG II is the major active component of the renin-angiotensin system and is principally involved in regulation of blood pressure by promoting sodium retention directly and by stimulating the production of aldosterone. ANG II is also a powerful vasoconstrictor. When the kidneys sense a fall in arterial pressure, prorenin is cleaved to renin which is then released into the blood stream. The adrenals, kidneys, reproductive organs, and the pituitary also produce prorenin, however, the kidneys are the only organs capable of converting it to renin (20). Renin, although not intrinsically vasoactive, will cleave angiotensinogen, a plasma protein, to form Angiotensin I. ANG I, a ten amino acid peptide, possesses only mild vasoconstrictive properties. Two amino acids are then cleaved from ANG I to form ANG II by angiotensin converting enzyme. ACE is most abundant in the lung, but is also present in vascular endothelial cells, the parenchyma of the kidney, brain, adrenal gland and testis (21, 22, 23, 24). ACE is also responsible for the degradation of bradykinin, which is now known to play an important role in the pharmacological effects of converting enzyme inhibitors (25). There are additional enzymes for ANG II conversion, including human chymase. This enzyme is widely distributed throughout human tissue, but is not present in rodents (26). Enzymes from the plasma and local tissue inactivate ANG II. Amino peptidase M serves to hydrolyze ANG

II to Angiotensin III. ANG III sustains some vasoconstrictive properties while playing no role in elevating arterial blood pressure.

Angiotensin II Receptor Subtypes

Like many other hormones, ANG II mediates its effects by binding to and activating a receptor. ANG II receptors are part of the G-protein associated receptors and are divided into two distinct categories, type 1 (AT1) and type 2 (AT2) (27). For the most part, the known functions of ANG II are linked to the AT1 receptor, the predominant receptor in adult vascular smooth muscle and kidney (28). This subtype is defined by pharmacological binding of the AT1 receptor antagonist losartan (formerly termed DUP-753) and may be further divided into 1a and 1b. The AT2 receptor is highly expressed in fetal tissues, and is defined by the binding of the AT2 receptor antagonist PD123319. The AT2 receptor is dominant in the rat aorta during embryonic stages of development, but this is reversed eight weeks after parturition, when the dominant ANG II aortic receptor becomes the AT1 (29). AT1 receptors are thought to mediate the growth-promoting effect of ANG II, while the AT2 receptor may have antiproliferative properties on endothelial cells (30). Therefore studies which evaluate ANG II mediated alterations in VSMC utilize AT1 receptor antagonism.

Angiotensin II Induced Adaptations

Cell culture studies utilizing AT1 receptor antagonism provide evidence that ANG II acts as a growth factor. ANG II [1μM] application in embryonic chick cardiac myocytes increases protein synthesis and total protein content (as measured through ^{3}H-

phenylalanine incorporation). This effect is inhibited by treatment with an AT1 receptor antagonist (31). Mechanical stretch stimulates the release of ANG II from cardiac ventricular myocytes resulting in an increase in c-*fos* expression (32). AT1 receptor antagonism blocks this response. In a related study the immediate-early genes c-*fos*, c-*jun*, *jun* B, *egr*-1 and c-*myc* are induced by the application of ANG II [100 nM] to cell cultures of both myocyte and non-myocytes (33). Again, AT1 receptor antagonism eliminates the early induction of c-*fos* and late gene activation. At the same concentration, ANG II induced c-*fos* mRNA and phosphoinositide turnover in rat VSMCs is blocked by the use of losartan, but not by the AT2 receptor antagonist PD123319 (34). In human VSMCs, ANG II [1μM] induced c-*fos* expression is inhibited by the use of losartan (35). Notably, some of these studies utilize cardiac myocytes in culture. Nevertheless, the results as a whole demonstrate the growth-promoting role of ANG II acting through the AT1 receptor in the cardiovascular system.

In addition to induction of immediate early genes evident through AT1 receptor antagonism studies, ANG II [10^{-6} M] also induces gene expression of PDGF-A, TGFβ1 and basic fibroblast growth factor (*b*FGF) (17, 36). Furthermore, ANG II [10^{-8} M] treatment of endothelial cells results in an increase in preproendothelin mRNA which can also be inhibited by AT1 receptor antagonism (37). These results suggest that ANG II may exert its effects via the increased expression of other growth factors. Nevertheless, the high concentration of ANG II utilized provides a significant liability to these *in vitro* experiments. Plasma concentrations of ANG II measured in rats are 10^{-12} M (38) while these studies use between 10^{-6} to 10^{-9} M. Consequently, utilizing a concentration that exceeds normal endogenous ANG II levels by 1000 fold may result in responses

inconsistent with the *in vivo* environment. Therefore, studies, which utilize concentrations similar to those found in plasma, are important in delineating the effects of ANG II on the vasculature.

In vivo studies to determine the role of ANG II on the vasculature, when compared to cell culture, are much more complex. As a consequence of the effect of ANG II on blood pressure through fluid retention, sodium retention, and increased noradrenaline release, it is difficult to distinguish between its indirect or direct effects on vessel hypertrophy. ANG II infusion at 200 ng/kg/min, although accompanied by a significant increase in pressure, results in a significant increase in wall area of the thoracic aorta and inward eutrophic remodeling of the external spermatic arteriole (39). At a similar dose, ANG II infusion, in the presence of hydralazine to maintain a normal blood pressure, significantly increases media cross-sectional area of small mesenteric arteries (40), suggesting a non-pressor mechanism of ANG II induced structural changes. ANG II infusion, at higher doses, results in an increase in cross-sectional wall area of the mesenteric artery, and fibronectin expression (41, 42). Nevertheless, one concern of these studies listed above is the high level of ANG II infusion. In renal hypertension (43, 44, 45) and salt restriction studies (46) plasma ANG II levels are elevated two to three fold compared to control animals. Infusion of 250 ng/kg/min (41) results in a six-fold increase in plasma ANG II levels, which is substantially higher than endogenous levels during hypertension. Therefore, the previous infusion studies may elicit vascular alterations due to increases in ANG II well above physiological levels.

Other *in vivo* studies, which provide evidence for ANG II acting as a growth factor through a non-pressor mechanism, include those using ACE inhibitors. When SHR

and their respective normotensive controls, WKY, were administered a number of anti-hypertensive agents, converting enzyme inhibitor (CEI) therapy demonstrated a significantly greater decrease in polyploidy of aortic VSMC beyond that expected from the decrease in blood pressure (47). These results suggest that ANG II acts through a non-pressor mechanism to stimulate DNA replication. In another study, a non-depressor dose of captopril during experimental hypertension attenuated the media-intimal area of the abdominal aorta and decreased the wall area of the first order arteriole of the cremaster muscle, further supporting a non-pressor mechanism for ANG II (6). However, the use of CEIs as a tool to determine the role of ANG II in hypertrophy has a complication. It is well established that CEI treatment prevents the degradation of bradykinin, which is known to stimulate the production of nitric oxide and prostaglandins by endothelial cells (figure 2-1). Nitric oxide and prostaglandins have anti-proliferative properties on vascular smooth muscle cells (25). Therefore, experiments using CEIs cannot lead to the conclusion that vascular wall hypertrophy is due solely to the direct effects of ANG II.

The development of a new bradykinin receptor type 2 antagonist, Icatibant (formerly termed HOE-140), allows investigators to examine the role of CEI treatment and regression of hypertrophy. Attenuation of growth in myocardial mass through CEI therapy is blocked by the use of Icatibant, suggesting that the CEI antiproliferative effect is due to bradykinin accumulation (48). Icatibant also decreases the reduction by CEI treatment of neointima formation after balloon injury. Notably, when Icatibant is combined with a CEI there is still some inhibition of neointima formation, which is similar to that observed in losartan treated animals (25). These studies provide evidence that CEI

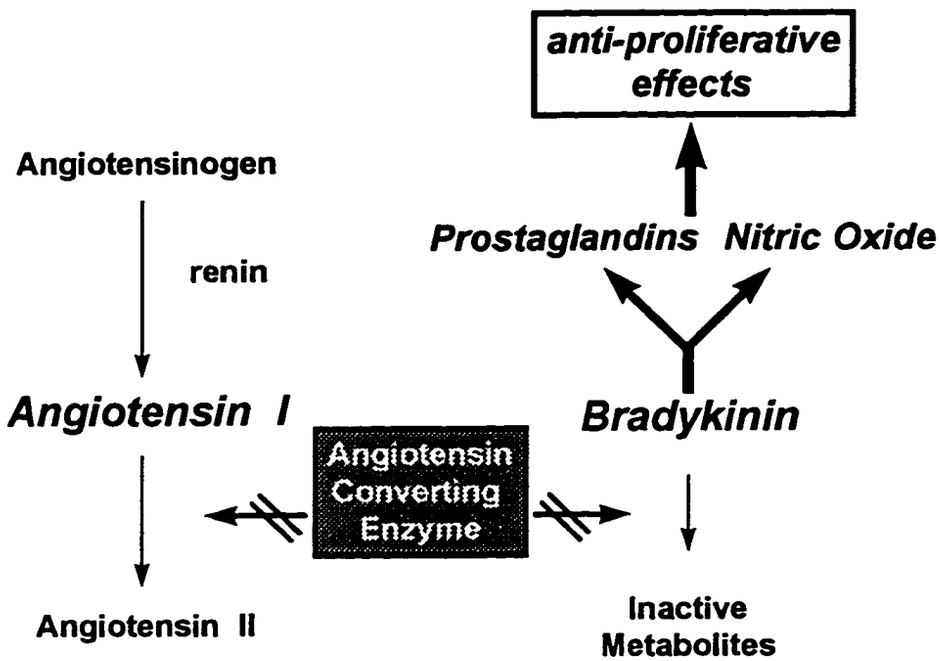


FIG 2-1. Effects of converting enzyme inhibition on bradykinin. Cascade of renin angiotensin system as altered by treatment with CEIs, denoted by hash marks. Converting enzyme inhibition results in significant bradykinin accumulation, as well as prevention of ANG II formation. Bold arrows and italic lettering indicate increased accumulation due to CEI therapy.

therapy alone is not an effective means of evaluating the direct effects of ANG II on vascular growth due to bradykinin accumulation. Additionally, these results support the use of an AT1 receptor antagonist as an alternative method to evaluate the effects of ANG II on vascular changes associated with hypertension.

As discussed previously, through the AT1 receptor, 10^{-6} and 10^{-9} M ANG II elicits growth factor and proto-oncogene expression (34, 35, 36). These changes occur through the induction of many intracellular signaling pathways. As stated earlier, ANG II binds to at least two distinct receptor subtypes, AT1 and AT2. For the most part, the role of the AT2 receptor in VSMC growth is minimal, and the signaling pathway is unknown. However, the steps of intracellular activation through the AT1 receptor are fairly controversial. The classical signal transduction effect includes the activation of PLC-β through the binding of ANG II to the G-protein coupled AT1 receptor (49). This results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP2) to form both inositol (1,4,5) tri-phosphate (IP3) (50) and diacylglycerol (DAG) (51). IP3 then serves to stimulate the release of calcium from intracellular stores (52) and DAG stimulates activation of protein kinase C (53). However, recent evidence suggests that PLC-β isoforms are undetectable in vascular smooth muscle cells (54). Other experiments demonstrate that application of ANG II in cell culture results in the phosphorylation of PLC-γ, through activation of a Src family kinase (pp60c-src), which is regulated by tyrosine phosphorylation (55, 56, 57). In addition, treatment with the tyrosine kinase inhibitor genistein inhibits ANG II induced IP3 production by 75% (54), suggesting PLC-γ mediates activation of IP3. ANG II also induces the MAPK pathway, possibly through the pp60c-src family kinase that promotes association of the linker protein Grb 2 to the

guanine-nucleotide exchange factor son of sevenless (SOS). Sos then successively generates nucleotide exchange on p21ras, which in turn, through activation of Raf, continues the induction of the MAPK pathway (reviewed by 58 and 59).

Mechanically Induced Vascular Adaptations

Like ANG II, there is evidence from the literature that pressure plays a considerable role in the vascular alterations associated with hypertension. There have been numerous attempts to mimic the effects of pressure *in vitro*. Stretch on flexible culture plates results in VSMC synthesis of PDGF (60), and enhances ANG II induced DNA synthesis (61). Mechanical stretch of VSMCs cultured on fibronectin (62) or collagen (63) elicits a significant increase in c-*fos* expression. In addition, uniaxial stretching of cardiac myocytes increases c-*fos* mRNA and inositol phosphate levels (64). In the absence of stretch, VSMCs subjected to static pressure in serum supplemented media results in DNA synthesis (65). These cell studies are important in implicating pressure as a growth stimulus in VSMCs, yet they have limitations. They involve removing the VSMC from its internal environment and disrupting the extracellular milieu. It has been shown that interaction between integrins, matrix proteins and intact endothelium are in part responsible for sensing mechanically simulated changes (66, 67). Therefore studies which evaluate the pressure induced alterations with the vasculature intact would more accurately describe VSMC accommodations associated with hypertension.

In comparison to ANG II, less research is concentrated on *in vivo* models to evaluate the role of pressure on the vasculature. Models of aortic coarctation

hypertension are effective due to the fact that there is a normotensive and hypertensive region in the same animal. Studies utilizing this model demonstrate significant increases in cross-sectional wall area in the arteries subjected to high pressure, while the normotensive regions show no alterations in this parameter (4, 68). Furthermore, the cremaster arterioles found in the normotensive region lack structural changes (68), implying a role for pressure as a stimulus for remodeling. Normalization of hypertrophy in the aorta and remodeling of the arterioles occurs four weeks after clip removal in renal hypertension (5), indicating that pressure may be responsible for the vascular alterations. In addition, sodium loading of SHR to maintain pressure in the presence of CEI therapy, results in wall-to-lumen ratios which correlate with raised blood pressure (12). Correspondingly, interventions that protect the vascular bed from elevated pressure in the SHR, by the use of a ligature, prevent the increase in medial thickness normally associated with the genetically hypertensive rat (69, 70), this implies that structural changes in the vessel may be mediated by elevated pressure. Similarly, reduction of femoral artery pressure, through chronic ligation, in the DOCA-salt hypertensive rat and the SHR results in cross-sectional areas comparable to those in normotensive controls (71). Collectively, these studies provide conclusive evidence for raised intravascular pressure as a factor in the structural alterations associated with hypertension.

The precise mechanism by which pressure elicits alterations in VSMCs is still relatively unknown. In addition to proto-oncogene expression (62), mechanical stretch of VSMCs in culture elicits an increase in phosphoinositol turnover (65). Correspondingly, three and nine days after aortic coarctation, products of inositol lipid hydrolysis and increased levels of c-*fos* and c-*myc* mRNAs are found in the high pressure, but not

normotensive region of the aorta (4, 72). Moreover, elevation of transmural pressure in isolated renal arteries results in a pressure dependent IP3 and DAG production, suggesting that pressure may induce PLC activation (73). In addition to inositol products and their activation, acute restraint induced hypertension results in MAPK activation similar to that of ANG II and phenylephrine generated hypertension (74). This MAPK activation is associated with an upregulation of c-*fos* and c-*jun*. Proto-oncogene expression is also increased when isolated hearts are subjected to acute elevation in wall stress by fluid filled balloons (75). Studies in our laboratory indicate that pressure can induce proto-oncogene expression in the isolated small mesenteric artery (76). This proto-oncogene expression significantly correlates with wall stress (77). In turn, this increased proto-oncogene expression may regulate VSMC growth (78). Thus, in the presence of elevated pressure, the inositol tri-phosphate and MAPK pathways may facilitate an increase in proto-oncogene expression that may lead to pressure induced vascular alterations.

Vascular Smooth Muscle Cell Adaptations

It is now apparent, through the previously evaluated literature, that both ANG II and pressure are capable of eliciting structural changes in vascular smooth muscle during hypertension. These VSMC changes vary depending upon the experimental model. In serum supplemented media, cell culture studies demonstrate that both ANG II and pressure induce hyperplasia (16, 63, 65, 79). However, in serum-free media, ANG II induces an increase in protein content, not DNA synthesis (15). Correspondingly, in intact aortic rings, ANG II induces hypertrophy, not hyperplasia, accompanied with an increase in protein synthesis (14), suggesting VSMCs adaptations *in vitro* are due the factors

present. In addition, SHR, have demonstrated a hyperplastic response to ANG II (80). However, it is suggested that these hyperplastic changes may actually be due to a genetic aberration, since this does not occur in the WKY (81). Hyperplasia was also demonstrated in aortic coarctation, which may be attributed in part to a rapid rise in pressure that was accompanied by endothelial cell synthesis and dysfunction (82). However, it is evident that in the renal hypertensive rat, VSMC adaptation is associated with hypertrophy or polyploidy (11, 18), wherein the smooth muscle cells undergo DNA synthesis without subsequent division. In a concurring study, SHR and WKY rats elevated blood pressure and frequency of polyploidy are highly correlated; indicative of a direct link between pressure and DNA replication without division (47). Altogether, it is evident that the VSMC adaptations which ANG II and pressure generate are dependent upon the experimental model. However, it is well agreed that VSMC changes *in vivo* during hypertension, with the exception of the SHR and rapidly induced hypertension, are not hyperplastic in nature.

Platelet-Derived Growth Factor Expression

In addition to a hypertrophic or hyperplastic response, VSMCs produce a variety of peptides in response to pressure and ANG II including a 28 to 35 KD polypeptide termed platelet-derived growth factor (PDGF) (83). PDGF is composed of two distinct polypeptide chains denoted as A and B, linked together by two sulfide bonds with dimeric combinations AA, AB and BB (reviewed by 84). PDGF mRNA is expressed and secreted by a wide variety of tissues including platelets, VSMCs, endothelial cells, macrophages and fibroblasts (85, 86, 87). Aortic smooth muscle cells of newborn rats express both A

and B chain transcripts, while adult rats express only the PDGF-A chain (88). In the human aortic arch, PDGF-A transcripts are associated with the medial smooth muscle, while B chain mRNA expression is high in the adventitia (89). There are significant differences between the proliferative effects of the dimeric chains on VSMCs; PDGF-BB and PDGF-AB promote DNA synthesis (90), whilst PDGF-AA is a weak mitogen (91). In cell culture, PDGF-BB and -AB chains induce DNA synthesis in a concentration dependent manner in both WKY and SHR VSMCs, while a larger concentration of -AA weakly induced proliferation only in SHR VSMCs (92). Therefore it is suggested that the increased expression of each respective PDGF chain, depending on their mitogenic properties, may be a predictor of whether or not the smooth muscle cell undergoes a proliferative response.

Similar to the dimeric chains of the growth factor, there are three paired receptors, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$. The $\alpha\alpha$ receptor binds all three PDGF dimers, $\alpha\beta$ binds both PDGF-AB and -BB, and $\beta\beta$ binds PDGF-BB (reviewed by 93). PDGF receptor expression is increased by a variety of conditions including application of ANG II (90), hypertension (94) and arterial injury (95). Once bound to the receptor, PDGF causes dimerization of the two subunits and cross phosphorylation on tyrosine residues (96). Prohibiting the dimerization of the receptor pairs inhibits the PDGF induced proliferative response in baboon aortic smooth muscle cells (97), thus providing evidence that PDGF responses require receptor subunit coupling.

Like many growth factors, PDGF induced changes are pleiotrophic. The initial ligand-receptor binding induced tyrosine phosphorylation will activate several enzymes including phosphatidylinositol 3-kinase (98), PLC-γ (99), Phospholipase D (100) as well

as elevation of cyclic adenosine monophosphate (cAMP) levels (101). In cell culture, PDGF-BB rapidly activates the MAPK pathway in VSMCs in a manner similar to ANG II (102). PDGF also induces MAPK activation in rat-1 fibroblasts (100). In addition, PDGF acts through PLC-γ to induce DNA synthesis and c-*fos* expression in fibroblasts (103). Furthermore, PDGF enhances expression of the immediate early gene c-*myc*, suggesting that PDGF induced growth responses are mediated through early gene activation (104, 105). Altogether, these experiments suggest that PDGF stimulates a wide variety of enzymes that can lead to early gene activation, which may conceivably have a role in initiating a growth response.

A number of conditions can elicit PDGF expression including an increase in sheer stress (106, 107, 108), atherosclerosis (89), pulmonary hypertension (109) and arterial injury (95, 110) elicit PDGF expression. In addition, both ANG II and elevated intravascular pressure can increase PDGF expression, implying that PDGF may play a role in the structural alterations associated with hypertension (17, 19, 111). In cell culture, the application of [10^{-6} - 10^{-11} M] ANG II to rat aortic smooth muscle cells in serum free media results in the induction of the proto-oncogene c-*myc* within 30 minutes and PDGF-A expression 6 hours later (17). Moreover, elevated intravascular pressure is also linked to PDGF-A expression, wherein, after pressure normalization of SHR through antihypertensive treatment, PDGF-AA mRNA expression in VSMCs is comparable to those of the normotensive controls. The PDGF-AA mRNA levels correlated with blood pressure, suggesting that blood pressure regulates PDGF gene expression (19). Moreover, cyclical stretch of rat VSMCs results in secretion of both -AA and -BB chains of PDGF, and this effect is reduced by 75% with antibodies specific for the -AA chain (60). These

results demonstrate that pressure alone is a sufficient stimulus for PDGF synthesis, and that pressure may be a plausible mediator of ANG II induced growth factor expression.

Alternatively, there is evidence that α_1 adrenergic stimulation induces PDGF-A expression. At a similar rise in pressure, phenylephrine induced PDGF-A mRNA is greater than ANG II or endothelin induced expression (111). The increased PDGF-A gene expression was accompanied by an increased expression of c-*fos* and c-*myc* but not DNA synthesis. This response correlates with the non-mitogenic properties of PDGF-A. There is now clear evidence that ANG II can increase the release of noradrenaline (41). As a result, α_1-adrenergic stimulation provides yet another means for ANG II to indirectly stimulate an increase in PDGF expression.

Interestingly, in a previous study ANG II infusion significantly increased PDGF-A mRNA levels in the hypertrophied aorta (39). However, in this study, ANG II infusion was accompanied by an increased systolic blood pressure; thus, it did not distinguish between the direct or pressor effect of ANG II. It is evident from the literature that pressure is capable of mediating ANG II induced PDGF synthesis. Collectively, these experiments suggest that PDGF gene expression is affected by both ANG II and pressure, and that this growth factor may play a role in the VSMC changes associated with hypertension. Whether the ANG II regulation of PDGF gene expression is a result of a direct or indirect mechanism, such as elevated pressure or adrenergic stimulation, remains to be clarified.

Microvascular Density

Similar to their effects on PDGF-A expression, both ANG II and raised intravascular pressure have been implicated as mechanisms important in the maintenance of microvascular density. However, unlike the previously discussed literature, pressure is not a proposed propagator of the growth related properties associated with the hormone ANG II on capillary density. In fact, pressure induced alterations on microvascular density are converse to those of ANG II. Pressure is implicated as a potential mediator of vascular rarefaction, the reduction in number of arterioles and capillaries. Rarefaction may be distinguished as two phases, functional and structural or anatomical rarefaction. During functional rarefaction the vessels are present in the microcirculation, but non-perfused. Atrophy and degeneration of the smooth muscle and endothelial cells characterize structural rarefaction (112), which occurs after functional rarefaction. The time course of events is apparent in a study by Prewitt et al., wherein arteriolar functional rarefaction was found in the gracilis muscle of the SHR at 12-14 weeks of age, followed by structural rarefaction at 16-18 weeks (9).

Rarefaction is documented in both human and experimental models of hypertension. In the SHR cremaster muscle, Chen et al. demonstrated a reduction in the number of terminal arterioles and capillaries, with a larger percent of them closed to flow when compared to WKY, demonstrating both structural and functional rarefaction (113). In reduced renal mass, 1K1C and 2K1C hypertension microvessel density in the cremaster decreases after four and eight weeks (7, 8, 114), thus correlating hypertension with rarefaction. Anatomical rarefaction, similar to that seen in experimental hypertension is found in the mesenteric beds of hypertensive patients (10). In addition, a reduction in

capillary density is found in hypertensive patients in the nailfold microcirculation as measured by video-microscopy (115). Capillary rarefaction is also demonstrated in the forearm skin in patients with essential hypertension (116). In the latter study, capillary density was inversely correlated with systolic blood pressure, suggesting again that pressure is inversely related to microvascular density. Altogether, these studies associate alterations in microvascular density with hypertension, and suggest that elevated intravascular pressure is an important factor in both functional and anatomical rarefaction.

Contrary to pressure, previous studies have implicated ANG II as an angiogenic factor in the microcirculation. ANG II is shown to increase the vascular density index in chorio-allantoic membrane of the chick embryo, suggesting a role for ANG II in neovascularization (117). In normotensive rats, treatment with a CEI significantly decreases microvascular density in the cremaster muscle (118), implicating ANG II as a mediator of angiogenesis. Alterations of salt intake are used to evaluate the effect of ANG II on microvascular density. A high salt diet inhibits the renin-angiotensin system through negative feedback, which in turn leads to diminished circulating levels of ANG II. Decreased ANG II levels attenuated arteriolar density in the cremaster muscle of both sham-operated and reduced renal mass hypertensive rats, emphasizing the importance of ANG II effects on vessel density (119). The aforementioned investigators have demonstrated similar results with high salt intake in other experiments (114, 120, 121). The morphological changes associated with high salt diets are structurally different from anatomical rarefaction. These alterations include the loss of vessel integrity by the destruction of the basement membrane and dissociation of smooth muscle and endothelial cells (122). It is evident from previous studies that most of the ANG II induced growth

effects are mediated through the AT1 receptor (33, 34, 35). A recent study demonstrated that losartan treatment significantly lowers the vessel density of ANG II infused rats, but density is still elevated compared to controls, partially implicating the AT1 receptor in angiogenesis (123).

In summary, ANG II, most likely through the AT1 receptor, elicits angiogenesis in the microcirculation. Alternatively, elevated intravascular pressure is correlated with rarefaction. Although ANG II and pressure have opposite effects on capillary density, the ability to detect the effects of ANG II on microvascular density are complicated by corresponding changes in pressure. ANG II infusion results in an increase in blood pressure, and therefore angiogenic properties of the hormone could be antagonized by hypertension induced rarefaction. The proposed experimental models were designed to differentiate between these two aspects and allow collection of information on the effects of ANG II separately from pressure induced changes in microvascular density.

CHAPTER III

SPECIFIC AIMS

It is clearly apparent, in viewing experimental evidence, that there are many structural adaptations in the vasculature associated with human, genetic and experimental hypertension. It is also evident that mechanical factors, such as pressure, as well as chemical factors, including the hormone ANG II, are implicated in these changes. ANG II appears to be a mediating factor in hypertrophy, growth of blood vessels, and growth factor expression. Whether regulation of the vasculature by ANG II is the result of a direct or indirect effect remains to be elucidated. Primarily, our hypothesis is that ANG II is indirectly responsible for the vascular hypertrophy and growth factor expression associated with hypertension through the elevation of intravascular pressure, rather than directly through a non-pressor mechanism. Secondarily, we suggest that elevated arterial pressure may regulate PDGF-A expression, which in turn may contribute to the arterial wall hypertrophy seen in hypertension. The proposed studies allowed for delineation between ANG II and pressure in experimental hypertension.

In order to separate between the effects of ANG II and pressure, two laboratory models of hypertension were utilized. First, animals were subjected to one-kidney, one-clip hypertension, with half the animals receiving losartan, an AT1 receptor antagonist. It is apparent from the literature that AT1 is the predominant receptor on adult vascular smooth muscle cells, and therefore inhibition will allow for removal of the direct effects of angiotensin II on vascular hypertrophy, blood vessel growth and PDGF-A expression. To complement the 1K1C hypertension experiment, a second experiment was utilized to

determine if direct administration of angiotensin II affected arterial hypertrophy, capillary density and growth factor expression. We accomplished this by infusing animals with ANG II through an osmopump and treating them with a vasodilator, minoxidil, to return pressure to a normotensive level.

CHAPTER IV

MATERIAL AND METHODS

One-kidney, one-clip losartan hypertension

Animals and treatment

Six-week-old male Wistar rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Rats were anesthetized with a single intraperitoneal injection of ketamine hydrochloride [80 mg/kg] (Fort Dodge Laboratories; Fort Dodge, IA) and xylazine [12 mg/kg] (Mowbray Corp.; Shawnee, KS). The abdominal area of the rat was shaved and cleaned with antiseptic solution. All surgeries were performed utilizing sterile techniques. Through a midline laparotomy, hypertension was induced by removal of the right kidney along with the placement of a silver clip with a 230 μm gap width on the left renal artery as reviewed by Barger (124). The right renal artery, vein, and the ureter were sutured with 3-0 sterile non-absorbable surgical suture (Ethicon, Inc.; Sommerville, NJ). Control rats were uninephrectomized and the left renal artery was isolated in the same manner as in the hypertensive rats without applying the clip. Abdominal muscle layers were sutured with 4-0 reabsorbable plain gut (Ethicon), and the outer epidermal layer was stapled with wound clips. Animals were then placed on a warm heating pad for recovery. To minimize the risk of infection, 25,000 units of Penicillin G (Marsam Pharmaceuticals; Cherry Hill, NJ) was injected intramuscularly before surgery. Animals were randomly divided into four groups: uninephrectomized control (1KNC, n=10); one-kidney, one-clip hypertension (1K1C, n=10); uninephrectomized control with losartan (1KNC-LOS, n=9); and 1K1C hypertension with losartan (1K1C-LOS, n=8). 1KNC-LOS and 1K1C-

LOS groups were given losartan in their drinking water (120 mg/L), beginning one day before surgery and continuing until the end of the experiment. Based on average daily fluid consumption and preliminary studies, this concentration of losartan results in a dose of approximately 20 mg/kg/day. Losartan was a gift from Ron D. Smith at Dupont Merck Research and Development. The rats were fed Teklad rat chow (Harlan Teklad; Madison, WI) and tap water or losartan water ad libitum, and maintained on a 12 hour light/dark cycle. The Animal Care and Use Committee at Eastern Virginia Medical School approved the animal experiments presented in this study.

To ascertain the proliferative response of smooth muscle cells, 5-Bromo-2'-deoxyuridine (BrdU) (Sigma Chemical Co.; St. Louis, MO), a thymidine analogue, was utilized to label nuclei undergoing DNA replication. BrdU was injected at a dose of 100 mg/kg subcutaneously and 30 mg/kg intraperitoneally 18 hours before killing, and another 30 mg/kg intraperitoneally 6 hours thereafter, as adapted from Yu et al. (125).

Hypertension Assessment

To assess the development of hypertension, indirect systolic tail cuff blood pressures were obtained on rats with a Narco Bio-Systems Electro-Sphygmomanometer (Houston, TX). Tail cuff pressures were recorded from each rat every other day, until the end of the experiment. Rats were acclimatized to this procedure by trial measurements prior to surgery. Blood pressures were taken between 10 a.m. and 1 p.m. in quiet surroundings. Rats were warmed to 35°C for five minutes in a metal box and then placed in plexiglass housing. The mean of three consecutive measurements was recorded from

each rat. 1K1C-LOS (n=3) and 1K1C (n=2) rats whose blood pressure was not elevated over 150 mm Hg by day 8 were eliminated from the study.

Tissue Preparation

After 14 days of treatment, the animals were anesthetized with 60 mg/kg sodium pentobarbital (Abbott Laboratories; North Chicago, IL). The tail artery was cannulated with polyethylene catheter size 10 (Clay Adams; Parsippany, NJ) for measurement of mean arterial pressure. Lidocaine hydrochloride (Astra Pharmaceuticals Products, Inc.; Westborough, MA) was utilized to produce dilation and a small incision was made. The catheter was inserted in the tail artery and heparin (1000units/ml) (Sigma) at the dose of 0.1cc/100grams was slowly injected intra-arterially. Once stabilized, mean arterial pressure was reported through a calibrated pressure transducer on a recorder (model 2200, Gould Brush; Cleveland, OH). To confirm angiotensin II blockade, 25ng/kg ANG II (Sigma) dissolved in 0.9% saline, was injected through a jugular cannula (polyethylene catheter size 10) and the change in pressure was recorded. Prior to sacrifice the right cremaster muscle was dissected and excess fascia removed. Two circular sections, 0.9 mm in diameter, were removed and placed in 0.25% buffered formalin (Fisher Scientific; Pittsburgh, PA) and stored in 4°C for future staining with *Griffonia simplicifolia* I lectin (Sigma). The chest was opened and the inferior vena cava was cut to allow free flow of venous return. The animal was then perfused through a catheter inserted through the root of the aorta with a vasodilator solution of 0.1M sodium nitroprusside, 0.1 M papaverine and 0.1 M verapamil (all from Sigma) in 0.9% saline at 60 mmHg until cleared of blood.

The animal was then perfused at 60 mmHg, measured in the tail artery, with 10% buffered formalin solution for 5-10 minutes.

The left kidney, thoracic aorta, carotid artery, small mesenteric artery and the distal portion of the external spermatic artery (the feeding arteriole to the cremaster) were dissected out from surrounding tissues and placed in 10% buffered formalin. All tissues were fixed by immersion in formalin for four hours. If unable to process immediately following fixation the tissues were placed in PBS. In preparation for processing tissues were labeled and loaded into a processing cassette. The processing unit consisted of graded alcohol solutions including 70%, 95% and 100% ethanol followed by xylene and paraffin (Oxford Labware; St. Louis, MO). After processing, tissues were embedded in paraffin in plastic cassettes and stored at room temperature.

Angiotensin II Infusion

Animals and treatment

Male Wistar rats (200-224 gms body weight) were anesthetized with a single intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (12 mg/kg). Alzet osmotic minipumps (model 2002, Palo Alto, CA) with a fourteen day capacity, an average volume of 230 µl and an infusion rate of 0.5 µl/hr were filled with either 0.9% saline or ANG II in 0.9% saline solution. ANG II was at a concentration adjusted for the rat weight to allow for a delivered dose of 100 ng/kg/min. For example, a rat weighing 200 gms required 2.4 mg/ml concentration of ANG II to deliver the dosage 100ng/kg/min. The back of the neck and scapulae area were shaved and treated with antiseptic solution. Again, sterile surgical techniques were utilized. Subcutaneously, between the scapulae,

the pumps were installed portal ends first and wound clips closed the incision. The animals were then placed on a warm heating pad for recovery. To reduce the risk of infection Penicillin G (25,000 units) was injected intramuscularly before the surgery. Animals were randomly divided into four groups: saline infusion (CON, n=10); saline infusion and minoxidil (CON-MIN, n=10); angiotensin II infusion (ANG II, n=8); and angiotensin II infusion and minoxidil (ANG II-MIN, n=8). CON-MIN and ANG II-MIN animals were given minoxidil fresh in their drinking water at a concentration of 60 mg/L, beginning one day before surgery and continuing until the end of the experiment. Based on average daily fluid consumption and preliminary studies in our laboratory, this dosage was satisfactory to lower blood pressure of ANG II infused animals, without overdosing CON-MIN animals. Minoxidil was a gift from Brenda Ling at Upjohn Laboratories.

To gain insight as to the time and duration of any proliferative response, each group received a 7 day treatment of BrdU to label nuclei undergoing DNA replication during the infusion period. An alzet osmotic minipump (model 1701) with a seven day capacity, an average infusion rate of 1μl/hr and an average volume of 170 μl was utilized for BrdU infusion. Half of the animals in each group received the treatment the first seven days, half received the treatment the second seven days. Therefore, the BrdU pump was removed or implanted one week later, utilizing the surgical techniques described earlier. The concentration of BrdU in the pump (30 mg/ml) was chosen to provide a dosage of 2.5 mg/kg/min (126). The rats were housed three to a cage and fed Teklad rat chow and tap or minoxidil water ad libitum and maintained on a 12 hour light/dark cycle. The Animal Care and Use Committee at Eastern Virginia Medical School approved the animal experiments presented in this study.

Hypertension Assessment

To assess the development of hypertension, indirect systolic tail cuff blood pressures were routinely obtained and recorded as described earlier. ANG II infused rats whose blood pressure was not elevated over 150 mm Hg by day 14 were eliminated from the study (n=1). ANG II-MIN rats whose systolic blood pressure was elevated over 140 mmHg by day 14 were eliminated from the study (n=3).

Tissue Preparation

After 14 days of treatment, the animals were anesthetized with 60 mg/kg sodium pentobarbital, and underwent the same method of perfusion-fixation described earlier, with one exception. The circular sections of the cremaster muscle sections were removed after perfusion-fixation, placed in 0.25% formalin and stored in 4°C for future staining with *Griffonia simplicifolia* I lectin (Sigma). The left kidney, thoracic aorta, carotid artery, small mesenteric artery and the distal portion of the external spermatic artery were dissected out from surrounding tissues, immersion fixed in 10% buffered formalin, processed through graded alcohol solutions and embedded in paraffin, as described earlier.

Morphological Analysis

Four-μm sections of the paraffin embedded tissues were stained with toluidine blue [1 gram of toluidine blue O dye (Eastman Kodak Co.; Rochester, NY), 1 gram of sodium borate in 100 ml dH_2O and filtered], a basophilic dye, to visualize intima-media of the blood vessel. The tissues were deparaffinized (xylene 2 x 3 min), rehydrated (100%

ETOH, 10 x 3 dips; 95% ETOH, 10 dips; dH_2O, 1 x 5 min) at room temperature. The tissues were then stained with toluidine blue at 37°C for one to two minutes. The specimens were then rinsed with distilled water, dehydrated (95% ETOH, 10 dips; 100% ETOH, 3 x 10 dips) and placed in xylene (2 x 3 min) at room temperature. Tissues were then coverslipped using cytoseal (Stephens Scientific; Riverdale, NJ). A video based image system with edge tracking software (JAVA, Jandel Scientific; San Rafael, CA) and a Zeiss 25 standard microscope were used to measure internal and external circumferences from which internal diameter and intimal-medial area were calculated.

Microvascular Density Measurements

The procedures for microvascular density measurements were adapted for our laboratory from Hansen-Smith et al. (127) and Greene et al. (114). Two circular sections, 0.9 mm in diameter were removed from the right cremaster muscle and excess fascia was removed. Sections were rinsed in saline and immersion fixed in 0.25% formalin for 1-7 days in micro centrifuge tubes at 4°C. Tissues were then stained with 10μg/ml rhodamine-labeled *Griffonia simplicifolia* I lectin obtained from Bandeiraea simplicifolia (Sigma), which binds to the basement membrane of capillaries, for three hours and immediately rinsed with saline. This was followed by ten-minute and thirty-minute saline rinses. Tissues were blotted dry and mounted on slides utilizing S\P AccuMount 280 Mounting Medium (VWR Scientific; Bridgeport, NJ). For formalin fixed tissues, prior to mounting, an overnight rinse in physiological saline solution at 4°C was necessary to reduce background fluorescence. Utilizing an Olympus BH-2 series microscope and rhodamine filter, stereological measurements were performed using a 10 x 10 eye-piece

grid to count the capillary intersections at 300X magnification. Each grid square was 0.055 μm wide. Two slides of each muscle were studied, and three fields from each slide were randomly selected and counted. The results of the six fields were averaged to give a single density for each muscle.

Immunohistochemistry for 5-Bromo-2'-deoxyuridine

Tissues were cut into 4μm sections from paraffin embedded tissues, placed on superfrost slides and dried overnight. Tissues were traced on the slide using a diamond point pen and labeled for positive BrdU or negative control. Additionally two kidneys were labeled as positive and negative controls. Slides were heated to 55°C for 1 hour, then deparaffinized in xylene (3 x 4 min) and rehydrated through a series of ethanol concentrations and distilled water (100% ETOH, 3 x 2 min; 95% ETOH, 2 x 2 min; 70% ETOH, 1 x 2 min; dH_2O 1 x 5 min). Endogenous peroxidases were blocked using 0.4% hydrogen peroxide in a methanol solution at 37°C for ten minutes. DNA was denatured after a twenty minute PBS rinse by placing slides in 2N HCL for 30 minutes at 37°C. Afterwards, the sections were rinsed in for twenty minutes in PBS, and then enzymatically pretreated with 0.1% trypsin (GIBCO BRL; Grand Island, NY) for 20 minutes at 37°C. After a twenty minute wash in PBS tissues were incubated with 5% Normal Goat Serum for 15 minutes (Vector Laboratories; Burlingame, CA) in a humidified chamber to reduce background due to hydrophobic interactions. The primary antibody, a mouse monoclonal Anti-BrdU (Sigma), was diluted (1:400 for 1K1C; 1:500 for ANG II infusion) and incubated on the slides for 2 hours at 37°C. The negative controls were incubated with 5% Normal Goat Serum. After washing in PBS, all slides were incubated with the

biotinylated secondary antibody IgG(H+L) (1:500 for 1K1C; 1:600 for ANG II infusion) obtained from Vector Laboratories at room temperature for 30 minutes. Slides were then stained using a Vectastain Elite avidin-biotin complex kit (Vector Laboratories). Slides were then incubated with 0.1% diaminobenzene solution (Sigma) containing 0.1 M imidazole (Sigma), hydrogen peroxide and PBS. Slides were rinsed in running tap water (5 min), and dehydrated (70% ETOH, 2 min; 95% ETOH, 2 x 2 min; 100% ETOH, 2 x 2 min) and placed in xylene (2 x 2 min). Slides were air dried and coverslipped using cytoseal. In order to confirm that all of the animals received BrdU, all kidneys from each animal were tested for BrdU. Rats with negative kidneys were eliminated from analysis (1K1C, n=3; ANG II infusion, n=1).

Subcloning of PDGF-A cDNA

The PDGF-A cDNA was obtained from Tucker Collins (Brigham & Women's Hospital, Harvard) as a 1.8 KB EcoRI insert in PUC19 plasmid. The plasmid was excised from the PUC19 plasmid with Sac II followed by EcoRI. The DNA was then ligated into pSK^+ (bluescript) vector, plated on LB-ampicillin plates, and grown overnight. Clones were selected and were amplified in LB-ampicillin medium. The cDNA was purified using the Qiagen plasmid purification protocol (Qiagen Inc.; Chatsworth, CA). To avoid alkaline hydrolysis the cDNA was further cut with Sal I at the 3' side. Religation resulted in a 368 base pair fragment in pSK^+. Digestion with Sty I along with T_7 polymerase was used to produce an antisense probe, while digestion at the Sal I site along with T_3

polymerase produced a sense probe. After enzymatic digestion, the linearized cDNA was stored at -20°C in TE buffer at pH 8.0 at a concentration of 0.5 μg/μl.

Riboprobe synthesis

^{35}S-CTP labeled riboprobes were synthesized in a method similar to Le Beau et al. (128) and Wilcox (129). The Radiation Safety Committee at Eastern Virginia Medical School approved the use of radioactivity for this purpose. ^{35}S-CTP from Dupont NEN was thawed, and allocated into microcentrifuge tubes centrifuged, dried in the speed-vac and stored at -70°C until further use. All reagents were ordered from Promega Corp. (Madison, WI) unless otherwise specified. The reaction cocktail consisted of 5X transcription buffer (containing 200mM TrisHCl, pH 7.5, 30mM $MgCl_2$, 10 mM spermidine and 50 mM NaCl), 100 mM DTT, RNAsin, nucleotide mix of ATP (1.0 μl), GTP (1.0 μl), UTP (1.0 μl) and CTP (0.2 μl) along with 2 μg of linearized cDNA and DEPC (Sigma) treated H_2O to make a final volume of 18 μl in the microcentrifuge tube containing 90 mCi of ^{35}S-CTP. The reaction cocktail was mixed well and centrifuged briefly. Then T_7, or T_3 Polymerase was added to the reaction cocktail, and then incubated at 37°C for 2 hours. Twenty eight μl DNAse stop buffer and 2 μl DNAse was added and the probe was incubated for 15 minutes at 37°C to halt the transcription reaction and degrade the DNA template.

Incorporation was determined by diluting a small amount of the reaction cocktail and pipeting it on to PEI cellulose F chromatography strip (VWR Scientific). The paper was placed in 1M KH_2PO_4 (Sigma) and the buffer was run to the top of the strip.

Quantitation was determined after exposure to the phosphorimager screen (Molecular Dynamics) for thirty minutes and calculated by volume integration with local background. Incorporation greater than 90% was considered satisfactory.

After DNAse treatment the reaction mix was placed on Sephadex G50 spin columns (5 Prime-3 Prime; Boulder, CO) to remove all unincorporated nucleotides. Ten mg/ml yeast tRNA (GIBCO BRL) 100 mM DTT (Sigma) and DEPC-H_2O was added to the eluted ^{35}S labeled riboprobe to make a final volume of 200 µl. The probe was divided into 5 separate 40 µl aliquots and 4 µl of 3M NaOAc, pH 7.5 (Sigma) and 100 µl of cold ethanol was added and the probe was stored at -70°C.

On the day of hybridization one aliquot of the probe was removed and centrifuged at 4°C, 12,000 rpm, for thirty minutes. The supernatant was discarded into radioactive waste and 200 µl of cold 70% ethanol was added to the probe. After five minutes at bench top, the probe was then spun again at 4°C, 12,000 rpm, for fifteen minutes, and the supernatant was removed and discarded. The pellet was dried in the speed-vac, and resuspended in 10 µl formamide and 10 µl of 20 mM DTT. The activity of the probe was determined by placing 1 µl in 6 ml scintillation fluid and counting on a Beckman LS 8000 scintillation counter. The desired specific activity was 10^6 cpm/µl, which was enough probe to do 20 slides.

In Situ Hybridization

In situ hybridization was primarily based on Wilcox (129) for paraffin embedded formalin fixed tissues.

Reagents and Preparation

Prior to conducting the experiment, all solutions and glassware were made RNAse free by baking at 200°C for 8-10 hours in an oven. The plastic dishware, pH meter and other items that could not be baked were soaked in 0.5M NaOH (RNAse Free) for 45 minutes at room temperature and then rinsed twice with DEPC-H_2O and covered.

DEPC was diluted (1ml/1000ml) in dH_2O and mixed well. The solution sat at room temperature overnight and was autoclaved the next day. Additionally, all chemicals were molecular biology grade without any detectable RNAse activity, and all solutions and/or dilutions were made with DEPC-H_2O.

Tissue sections were cut in 4μm sections from paraffin embedded tissues, floated in 30% ETOH, followed by DEPC-H_2O, mounted on Superfrost Plus slides and dried for 24 hours at room temperature. Tissues were then deparaffinized in 2 changes of xylene for ten minutes each, rinsed twice for one minute in 100% ethanol, and rehydrated through graded ethanol concentrations of 95%, 70%, and 50% at one minute each. The tissues were then washed for ten minutes in 0.5 X SSC [75 mM NaCl (Amresco; Solon, OH) 7.5 mM Sodium Citrate (Sigma), pH = 7.0].

Tissues were permeabilized with 5 μg/ml Proteinase K (Amresco) in buffer [500 mM NaCl, 10 mM Tris (Amresco), pH 8.0] for ten minutes followed by three PBS (Amresco) washes for 5 minutes each. The tissues were then fixed by immersion in 4%

paraformaldehyde for 10 minutes at 4°C, followed by three PBS washes for 5 minutes each.

Hybridization

The prehybridization solution consisted of 792 µl of hybridization stock solution [50% Formamide (Ambion Inc.; Austin, TX), 10% Dextran sulfate (Sigma), 1X Denhardt's solution (Sigma), 1 mM EDTA (Sigma), pH 8.0,10 mM Tris (pH 8.0), and 0.3 M NaCl] to 208 µl DEPC-H_2O. Prehybridization was carried out by the addition of 100-200 µl per slide of the above solution for two to three hours at 42°C. The prehybridization solution was then drained off the edge of the slide.

The hybridization solution consisted of 10 mg/ml yeast tRNA, 1 M DTT, $1x10^7$ cpm/µl probe and DEPC-H_2O to make 208 µl. The probe was then mixed, heated to 95°C for three minutes and 792 µl of ice cold hybridization stock solution was added immediately. 100 µl of the hybridization solution was placed on each slide. The slides were placed in sealed humidified chambers heated at 55.1°C, as determined by the equation below, and saturated with 50% formamide, 20% 20 X SSC, and 30% DEPC-H_2O overnight.

$$T_m = 79.8 + 18.5(\log[Na^+]) + 0.58(\text{fraction G+C}) + 11.8(\text{fraction G+C})^2 - 0.35(\%\text{formamide}) - 820/L$$

For this protocol: $[Na^+] = 0.3M$
% formamide = 50
C+G fraction = 0.61
L = 368 base pairs

Thus: $Tm = 52.63 + 0.58(\text{fraction G+C}) + 11.8(\text{fraction G+C})^2 - 820/L$

The following day the slides were washed twice for 10 minutes with 2 X SSC, 1mM EDTA and 10mM β-Mercaptoethanol (Sigma) at room temperature. The β-Mercaptoethanol/EDTA solution was necessary to keep the ^{35}S-labeled probe in a reduced state throughout the washing steps to reduce background. The slides were then treated with 20-40 μg/ml RNAse A solution (Sigma) in RNAse buffer for thirty minutes to remove single stranded/unhybridized RNA. This was followed by two more washes for ten minutes of 2 X SSC, 1mM EDTA and 10mM β-Mercaptoethanol. A high stringency wash followed containing 0.1 X SSC with 1mM EDTA and 10mM β-Mercaptoethanol four times for 30 minutes each at 55°C. The slides were then washed twice for ten minutes in 0.5 X SSC. Finally the tissues were serially dehydrated in solutions containing 50%, 70% and 90% ethanol with 1 mM DTT and 0.1 X SSC for 2 minutes each. This was followed by 100% ethanol three times, two minutes each. The slides were then air dried in the hood overnight.

Phosphorimager Quantitation

The slides, wrapped in saran wrap, were loaded in a blank phosphorimager screen. The controls, experimentals, as well as sense and antisense were all placed in the same screen. The large vessels were exposed for a minimum of three days, and the small vessels (200 μm) were exposed for at least five days. The screen was scanned on the phosphorimager using 100 μm resolution and 4X magnification. Quantitation of the specimens was performed utilizing volume integration with local background.

Statistics

Results were analyzed using analysis of variance combined with Newman-Keuls multiple-range tests, or the Kruskal-Wallis non-parametric test combined with Dunn's multiple-comparison test for between group comparisons. Probabilities of 0.05 or less were interpreted as being statistically significant. Results are expressed as means ± standard error of the mean. Computer programs utilized for analysis and graphics included: InStat (GraphPad Software; San Diego, CA), Sigma Plot (Jandel Scientific), and Microsoft PowerPoint (Microsoft Corporation).

CHAPTER V

AT1 RECEPTOR INHIBITION DOES NOT REDUCE ARTERIAL WALL HYPERTROPHY NOR PDGF-A EXPRESSION IN RENAL HYPERTENSION

Introduction

As stated in chapter two, both ANG II and pressure are implicated as stimuli for the vascular changes associated with hypertension (4, 6, 40, 69). Previous studies that provide evidence that ANG II acts as a growth factor through a non-pressor mechanism include those using ACE inhibitors, which reduce cross-sectional wall area in the abdominal aorta in the presence of elevated pressure (6). However, as stated earlier, these results are confounded by the fact that ACE is also responsible for the degradation of bradykinin, whose products have anti-proliferative effects in vascular smooth muscle (25). These effects are clarified by studies utilizing bradykinin receptor antagonists in combination with CEI therapy, suggesting that converting enzyme inhibition is not an effective means for evaluating the role of ANG II on vascular growth (48). The development of an antagonist to AT1, the predominant ANG II receptor in adult VSMCs (28, 29) allowed for evaluation of ANG II induced alterations, in the absence of bradykinin accumulation.

The purpose of this study was to investigate the role of ANG II, through the AT1 receptor, on the vascular changes associated with renal hypertension. This was accomplished through the administration of the AT1 receptor antagonist, losartan, to renal

hypertensive rats. We then evaluated alterations including arterial hypertrophy, VSMC adaptations, rarefaction, and modifications in PDGF-A expression, which are frequently encountered during hypertension. A detailed methods section is located in chapter four.

Results

1K1C-LOS animals ingested 20.8 ± 0.8 mg/kg/day of losartan, while 1KNC-LOS animals ingested 17.8 ± 0.7 mg/kg/day. Tail systolic blood pressures were significantly elevated in both 1K1C and 1K1C-LOS animals (figure 5-1) above those of the 1KNC and 1KNC-LOS groups beginning four days after surgery (day zero) and continuing for the duration of the study. There were significant differences between the 1K1C and 1K1C-LOS groups on Day 6 and Day 12 of the study, but as these differences reversed direction, there is no physiological significance to them. Losartan lowered the systolic blood pressure in 1KNC-LOS from day 8 through day 12 when compared to the 1KNC group.

Mean arterial blood pressures measured through an indwelling catheter in the tail artery were significantly elevated in clipped animals (151.3 ± 9.1 mmHg, 1K1C; 149.7 ± 12.9 mmHg, 1K1C-LOS) when compared to the uninephrectomized control groups (112.3 ± 6.3 mmHg, 1KNC; 102.8 ± 6.9 mmHg, 1KNC-LOS). Infusion of 25 ng/kg ANG II in the losartan treated animals demonstrated a blunted pressor response (-0.5 ± 0.8 mmHg, 1K1C-LOS; -3.67 ± 1.6 mmHg, 1KNC-LOS) when compared to tap water groups (28.5 ± 6.7 mmHg, 1K1C; 36.5 ± 9.3 mmHg, 1KNC). There were no significant differences in the body weights of all four groups ($p>0.05$).

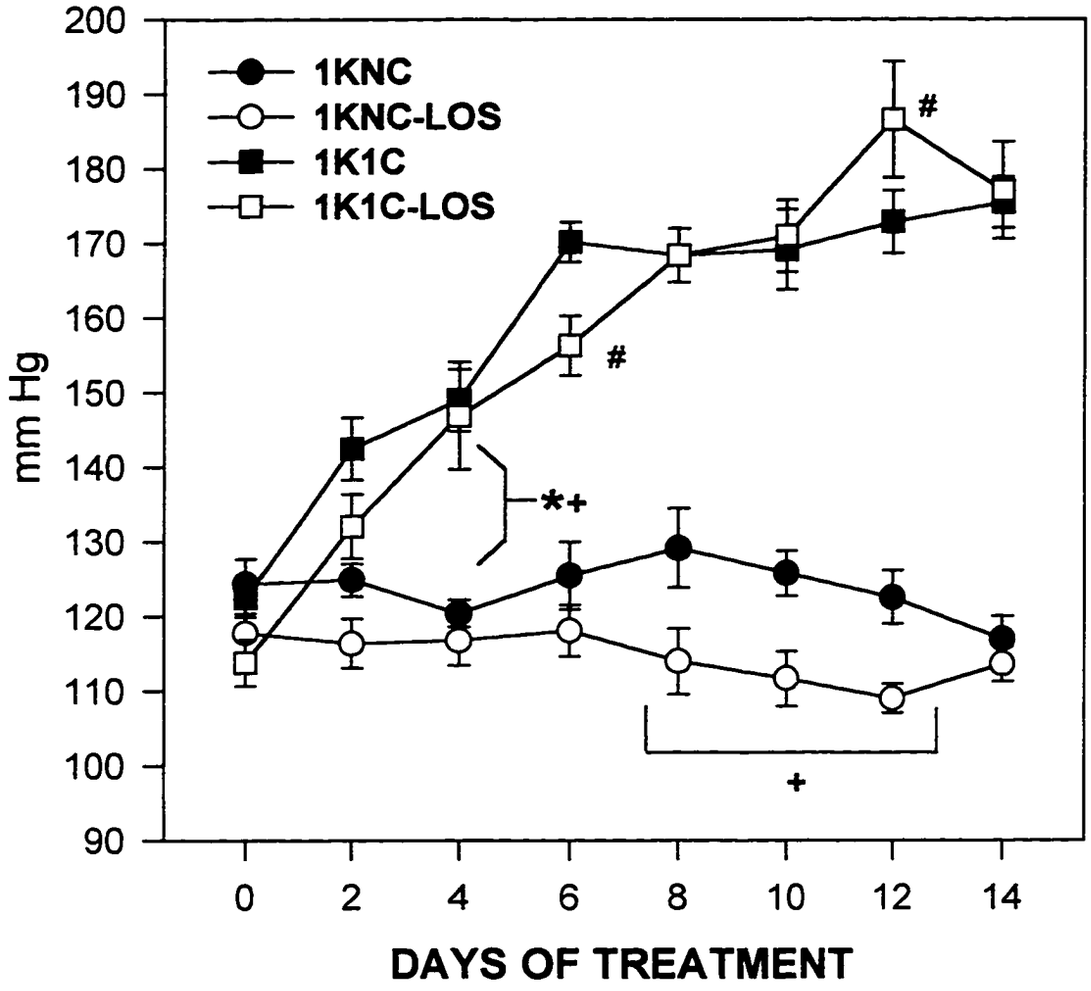


FIG 5-1. Tail cuff systolic blood pressures. Line graph showing systolic blood pressure for control groups (1KNC and 1KNC-LOS, circles) or renal hypertensive groups (1K1C and 1K1C-LOS, squares). Filled figures depict tap water consumption, while open figures depict losartan consumption. Values are expressed as mean ± SEM. Surgery was performed on day zero. Symbols indicate statistical significance at $p<0.05$ versus 1KNC (*), 1KNC-LOS (+), and 1K1C-LOS (#).

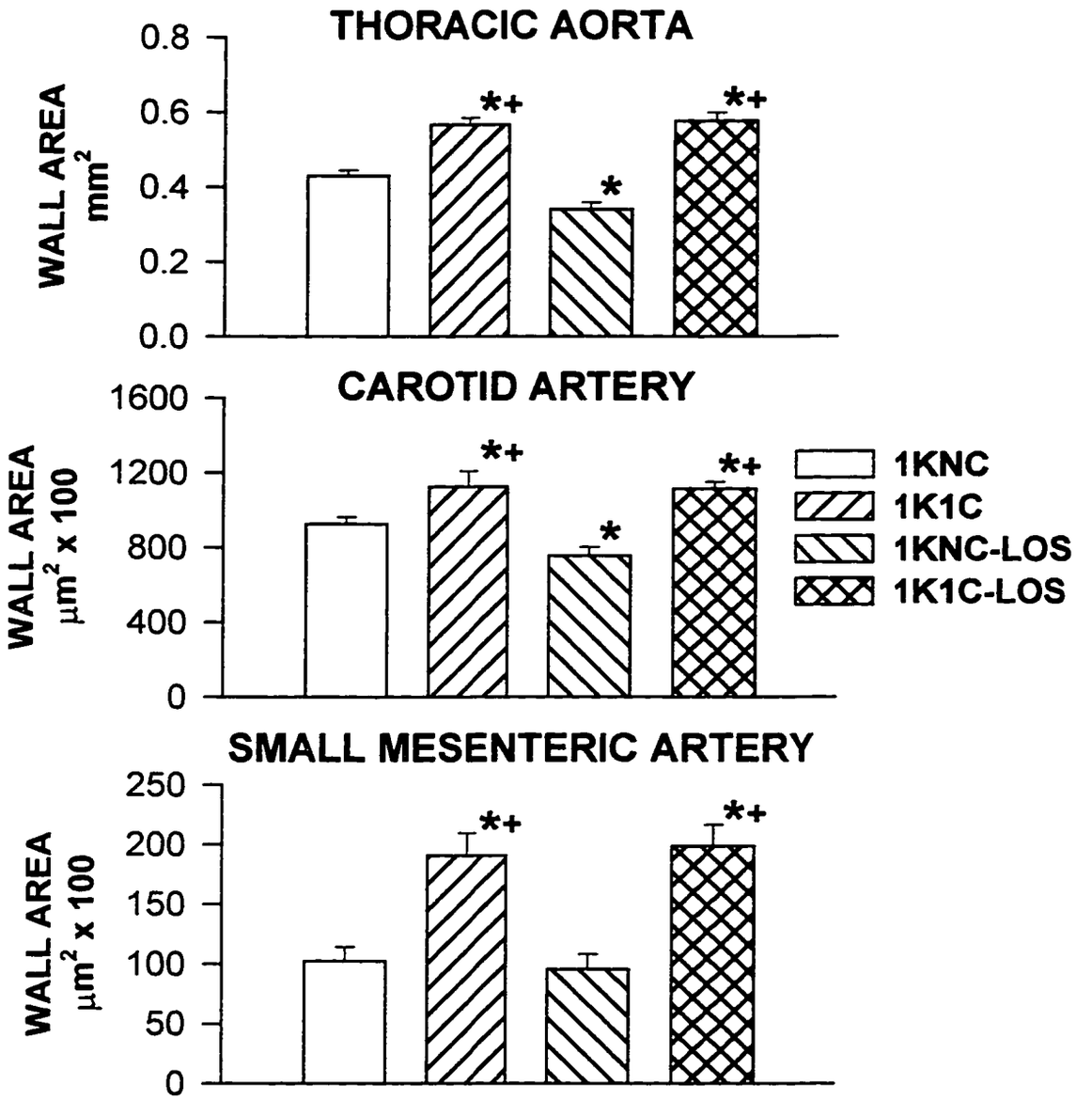


FIG 5-2. Arterial cross-sectional wall area. Bar graph showing cross-sectional wall area of the thoracic aorta, carotid and small mesenteric arteries for 1KNC, 1K1C, 1KNC-LOS, and 1K1C-LOS treated groups. Wall area was determined by the use of a video based image system with edge tracking software on toluidine blue stained tissues. Values are expressed as mean ± SEM. Symbols indicate significance levels at $p<0.05$ versus 1KNC (*), and 1KNC-LOS (+).

Cross-sectional wall area was significantly elevated, in the thoracic aorta, carotid artery and small mesenteric artery of the 1K1C animals, and these results were not affected by treatment with losartan (figure 5-2). The 1KNC-LOS animals demonstrated a significant decrease in cross-sectional wall area in the thoracic aorta and carotid artery when compared to the 1KNC. There was no significant difference among clipped animals in cross-sectional wall area of the external spermatic artery (1773.0 ± 153.6 μm, 1K1C; 1859.3 ± 246.1 μm, 1K1C-LOS) when compared to the uninephrectomized controls (1819.0 ± 118.7 μm, 1KNC; 1538.5 ± 158.98 μm, 1K1C-LOS) . There were no significant differences among the clipped animals in the internal diameter of the aorta, carotid and small mesenteric arteries (table 5-1). The internal diameter of the external spermatic artery was significantly reduced in the 1K1C animal when compared to the 1KNC and 1KNC-LOS.

TABLE 5-1. Arterial Lumen Diameters

Rat Groups	Thoracic Aorta mm	Carotid Artery μm	Small Mesenteric Artery μm	External Spermatic Artery μm
1KNC	1.67 ± 0.03	774.4 ± 16.5	201.3 ± 10.1	112.5 ± 2.7
1K1C	1.65 ± 0.03	743.7 ± 24.7	238.1 ± 14.8	97.8 ± 4.1*+
1KNC-LOS	1.61 ± 0.03	759.5 ± 33.7	217.4 ± 20.1	116.5 ± 4.3
1K1C-LOS	1.75 ± 0.03*	758.2 ± 16.6	236.7 ± 20.3	105.4 ± 5.6

Values are mean ± SE.
* $P<0.05$ vs. 1KNC, +$P<0.05$ vs. 1KNC-LOS.

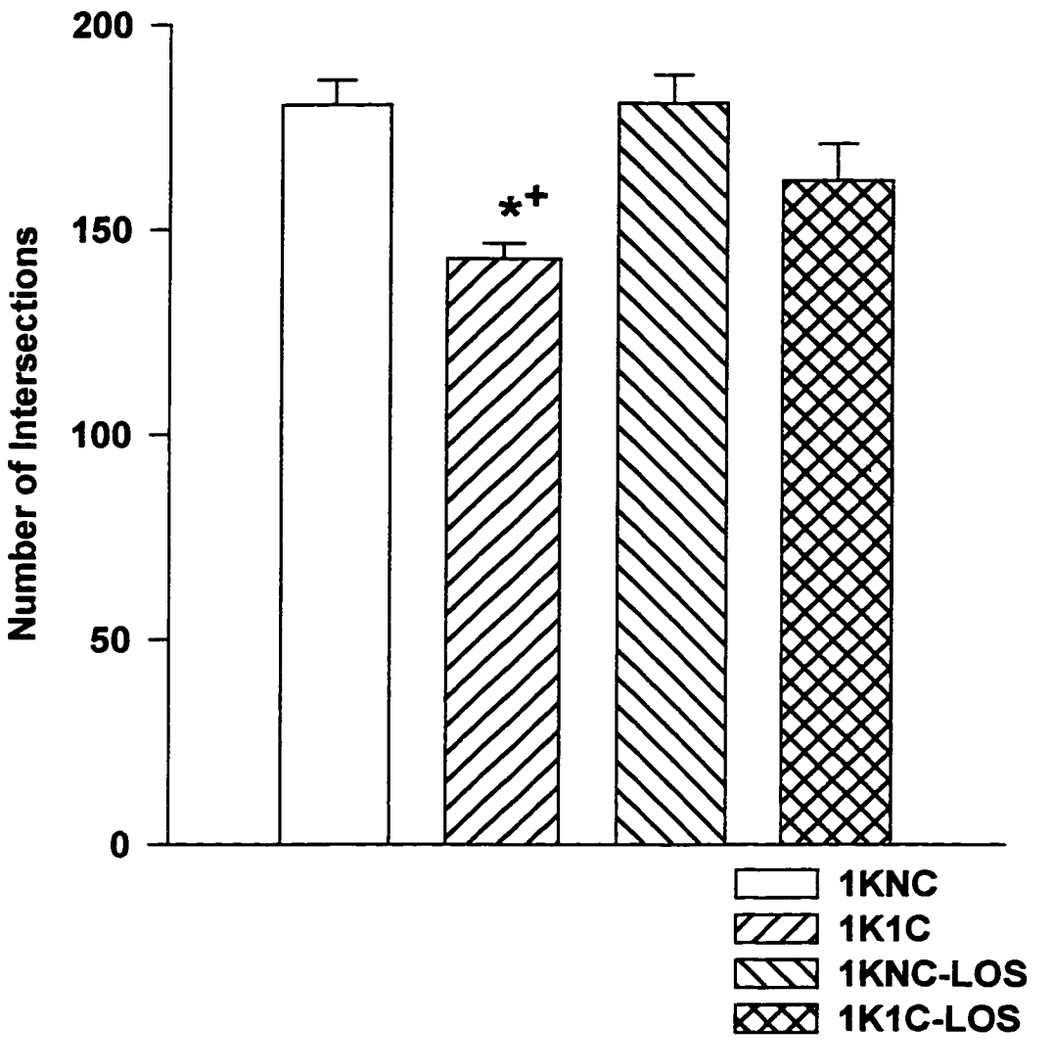


FIG 5-3. Microvascular density. Bar graph showing the microvascular density for 1KNC, 1K1C, 1KNC-LOS, and 1K1C-LOS treated groups. Microvascular density was determined by staining with *Griffonia simplicifolia* I lectin along with fluorescence microscopy. Values are expressed as mean ± SEM. Symbols indicate significance levels at $p<0.05$ versus 1KNC (*), and 1KNC-LOS (+).

Staining for BrdU was low in the carotid and small mesenteric arteries of all the groups ($p>0.05$). The only significant difference was between the 1K1C-LOS (1.6 ± 0.4 nuclei) and 1KNC-LOS (0.2 ± 0.1 nuclei) positively stained nuclei in the thoracic aorta, which were not different from the 1K1C (0.5 ± 0.2 nuclei) and 1KNC (1 ± 0.3 nuclei) groups. There was no positively staining tissue in the external spermatic arteries in any of the four groups. All kidneys with the exception of three animals had positive staining nuclei confirming BrdU delivery through injection.

Staining with *Griffonia simplicifolia* I lectin, which binds to the basal membrane of the capillaries, revealed a significant decrease in microvascular density of the 1K1C animals when compared to both uninephrectomized controls (figure 5-3). 1K1C hypertension reduced capillary density by approximately 22%. Density in 1K1C-LOS animals was not significantly different from either 1K1C or both uninephrectomized control values.

Expression of PDGF-A mRNA, quantitated by phosphorimager, was significantly elevated in both the 1K1C and 1K1C-LOS thoracic aorta when compared to 1KNC (figure 5-4 and figure 5-5). In the carotid artery, PDGF-A mRNA expression was significantly elevated in 1K1C and 1K1C-LOS when compared to both uninephrectomized control groups. Expression of PDGF-A mRNA was significantly elevated in the small mesenteric arteries of the 1K1C animals when compared to both uninephrectomized controls, and treatment with losartan blunted the response.

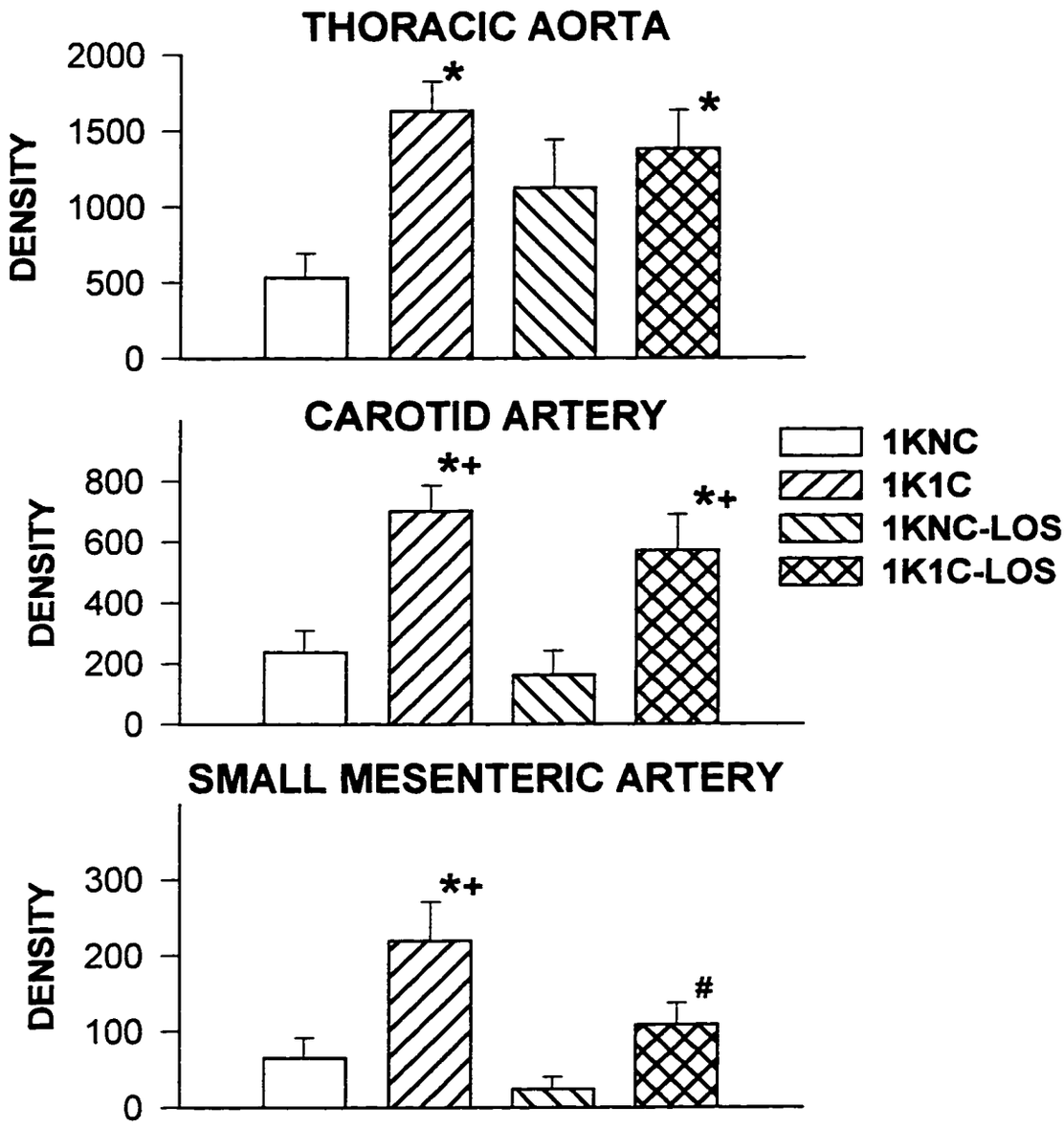


FIG 5-4. PDGF-A expression. Bar graph showing results of *in situ* hybridization for PDGF-A mRNA on thoracic aorta, carotid and small mesenteric arteries for 1KNC, 1K1C, 1KNC-LOS, and 1K1C-LOS treated groups. mRNA content was quantitated on the phosphorimager. Values are expressed as mean ± SEM. Symbols indicate significance levels at $p<0.05$ versus 1KNC (*), 1KNC-LOS (+), and 1K1C-LOS (#).

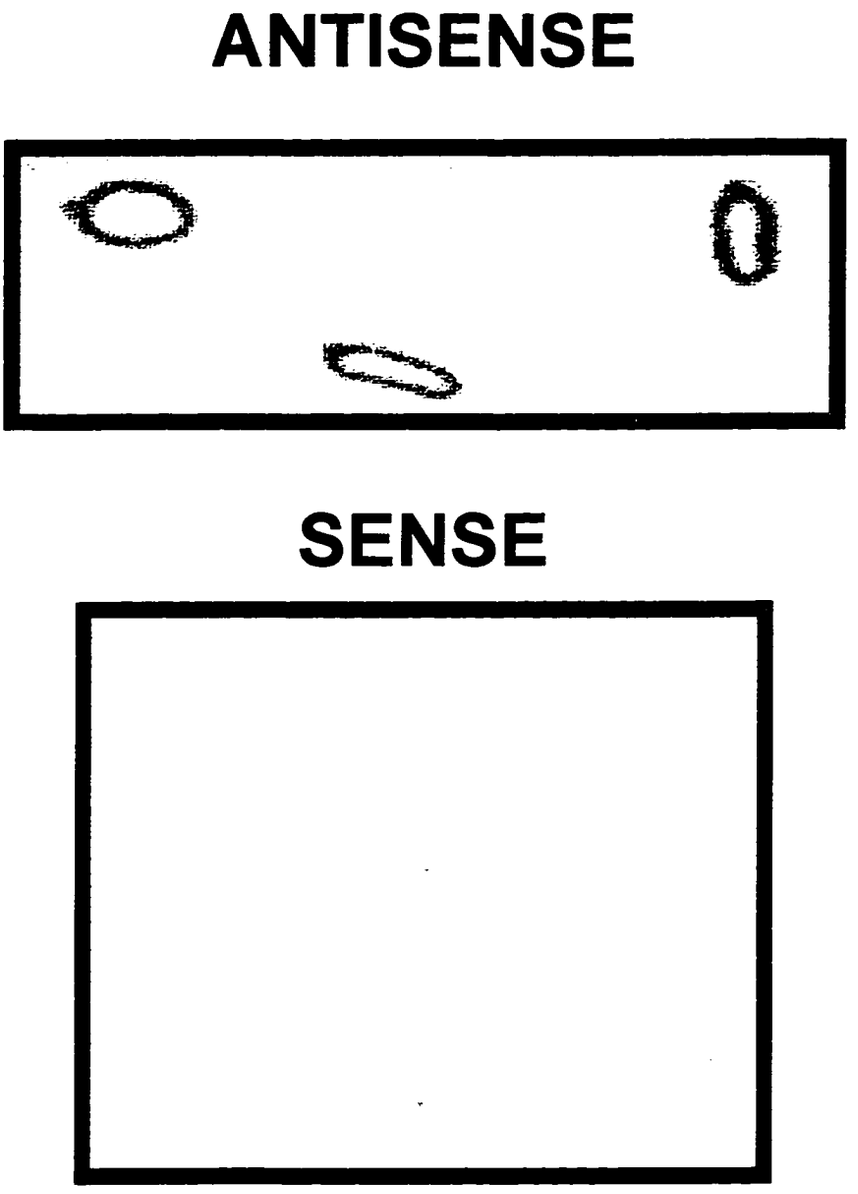


FIG 5-5. Representative photographs of PDGF-A expression in 1K1C thoracic aorta.

Discussion

This present study was an attempt to evaluate the role of ANG II through the AT1 receptor, independent of arterial pressure on the vascular changes associated with 1K1C hypertension. Renal hypertension induced a significant rise in blood pressure four days after surgery and continued for the duration of the study (figure 5-1). Treatment with losartan, an AT1 receptor antagonist, did not prevent the rise in blood pressure confirming that 1K1C hypertension is a renin-independent experimental model (130). AT1 receptor blockade was confirmed in the losartan treated groups by the lack of pressor response to a bolus infusion of ANG II. Therefore this experimental model allowed for the separation of the effects of ANG II, through the AT1 receptor, and elevated arterial pressure.

1K1C hypertension elicited a significant increase in cross-sectional wall area in the thoracic aorta, carotid and small mesenteric arteries (figure 5-2). This increase was not effected by treatment with losartan, implying that pressure mediates arterial hypertrophy in this particular model of hypertension. As stated earlier in chapter two, there is considerable evidence that pressure is capable of eliciting the structural changes associated with hypertension. Experiments on aortic coarctation demonstrated an increase in cross-sectional wall area in only the hypertensive region of the animal (4, 72). In addition, elevated blood pressure is correlated with increased wall-to-lumen ratio in mesenteric arteries of sodium loaded SHR during CEI therapy, implying pressure as a stimulus for vascular alterations (12). Interestingly, the 1KNC-LOS groups demonstrated a significant decrease in cross-sectional wall area in the thoracic aorta and carotid artery when compared to 1KNC. This, however, may be explained by the decrease in systolic blood pressure between the two groups for almost half the duration of the study. These results

suggest that if pressure is a stimulus for arterial hypertrophy, then a decrease in pressure, may result in attenuation of hypertrophy.

Corroborating results are seen in chronic ligation experiments; wherein, protecting the vascular bed from elevated pressure in DOCA-salt hypertensive rat and SHR resulted in a medial thickness similar to their normotensive controls (71), implying that attenuation of structural changes is mediated by lowering pressure. Similar results are seen in ligation experiments by Bund et al. (69) and Folkow et al. (70). There are, however, experiments that implicate ANG II through a non-pressor mechanism as a stimulus for arterial hypertrophy. CEI treatment in 1K1C hypertension resulted in a significant decrease in aortic wall area in the presence of elevated arterial pressure (6). However, as stated earlier, these results are confounded by CEI potentiation of bradykinin. Another study, utilizing ANG II receptor antagonism in SHR, demonstrated a significantly decreased media-to-lumen ratio in the small mesenteric artery (131). This decreased ratio, however, was accompanied by a significant decrease in systolic blood pressure, which correlated with the artery morphological changes, implying pressure as a mediator of this response. Altogether, this study provides convincing evidence that regulation of arterial hypertrophy in renal hypertensive animals during AT1 receptor blockade is mediated by elevated blood pressure.

Arterial hypertrophy did not occur in the external spermatic artery in the hypertensive animals regardless of drug treatment. Yet there was a significant decrease in the internal diameter of the 1K1C group when compared to both uninephrectomized controls, demonstrating inward eutrophic remodeling (table 5-1). In hypertension, these characteristic structural changes occur in vessels smaller then 150 μm in diameter, in

which the lumen decreases in diameter in the absence of arterial hypertrophy (7, 8). Pressure has been implicated as a stimulus for this remodeling by the lack of structural alterations in cremaster arterioles in rats subjected to aortic coarctation (68). The lack of hypertrophy in the smaller arteries may be explained by the law of LaPlace; in which, wall stress (σ) is directly proportional to pressure (P) and radius (R) and inversely proportional to wall thickness (ω). The equation is as follows: $\sigma = PR/\omega$. This law implies that as the thickness of the vessel wall increases, there will be a corresponding decrease in wall stress. This in fact does occur in the larger arteries; wherein, conditions that elevate pressure, such as hypertension, the wall stress can be normalized by increasing the wall thickness. However, the smaller arteries (<150 μm in diameter) will decrease in radius as a result of enhanced vasoconstriction to maintain wall stress. This decrease in radius of the smaller arteries was seen in this experiment, as well as other experimental hypertensive models (7, 8); in which, the external spermatic artery, when subjected to renal hypertension remodels inwardly, without an increase in arterial hypertrophy.

Typically, the mechanism of increased cross-sectional wall area in the larger arteries is through VSMC hypertrophy with or without polyploidy (11, 18, 47, 132). The results of this study demonstrate little or no staining for BrdU in the carotid and small mesenteric arteries for all of the groups, implying that the increased cross-sectional wall area in clipped animals was mediated by VSMC hypertrophy. In addition, in the thoracic aorta, no significant positive staining was found between the two hypertensive groups, and the 1KNC group. There was a small but physiologically insignificant, increase in 1KNC-LOS staining versus the 1K1C-LOS groups in the thoracic aorta. Comprehensively, these results suggest that VSMC hypertrophy occurred during renal hypertension. While

pressure is thought to mediate this response, ANG II is also a plausible factor in the hypertrophy of VSMC *in vitro*. Application of the hormone to VSMCs and intact aortic rings induces an increase in protein content, but not DNA synthesis (14, 15). In addition, CEI treatment of SHR and WKY rats reduces polyploidy of aortic VSMCs beyond the expected decrease in pressure (47). However, in the present study, in the presence of AT1 blockade, pressure elicited the increase in cross-sectional wall area, suggesting that pressure mediated VSMC the hypertrophic response.

This study also demonstrates a significant decrease in microvascular density of hypertensive rats utilizing the staining technique of *Griffonia simplicifolia* I lectin (figure 5-3). Similar results are seen in SHR, 2K1C and 1K1C models of hypertension (7, 8, 9, 113). In addition, rarefaction is well documented in patients with essential hypertension (10, 115, 116). It has been shown that arterioles in the 1K1C hypertensive rat demonstrate an increase in basal tone when compared to controls (133). In addition, studies demonstrate that arteriolar vasoconstriction may lead to structural rarefaction in both the SHR, 2K1C and 1K1C hypertensive models (7, 8, 9). The increase in tone may be mediated through an autoregulatory mechanism to prevent over-perfusion of the tissue, in order to resist increasing flow. Chronic ANG II application does increase tone in renal arteries (134). Therefore, it is possible that AT1 blockade in the 1K1C-LOS group may have attenuated vascular tone, and therefore prevented to some extent rarefaction seen in the 1K1C group.

Another finding in this study was the significant increase in PDGF-A expression in the wall of the hypertrophied aorta, carotid and small mesenteric arteries (figure 5-4). Photographs of *in situ* hybridization for PDGF-A mRNA on a 1K1C thoracic aorta is

located in figure 5-5. There is evidence in the literature that pressure mediates PDGF-A expression. Cyclic stretch of VSMCs grown on fibronectin elicits PDGF-A synthesis (60). In addition, attenuation of PDGF-A expression is correlated with decreased blood pressure in SHR (19), suggesting that pressure mediates PDGF-A expression. ANG II, nonetheless, is also implicated as a stimulus for PDGF-A expression in cell culture (17) as well as ANG II infusion (39). In the latter study, however, ANG II infusion was also accompanied by a significant increase in blood pressure, therefore not distinguishing between the two as a stimulus for PDGF-A expression. Adrenergic stimulation is shown to increase PDGF-A expression from VSMCs (111). ANG II also increases sympathetic outflow, which may be another indirect means of inducing PDGF-A expression. Experiments have demonstrated that ANG II induced epinephrine secretion from the adrenal medulla is partially reduced by losartan (135). In addition, losartan attenuated WKY noradrenergic neurotransmission in the mesenteric vascular bed (136). These results suggest that in part, ANG II mediated adrenergic stimulation may be mediated through the AT1 receptor. This provides a possible explanation for the losartan mediated decrease in PDGF-A expression in the small mesenteric arteries. It should be noted, however, that no significant differences in PDGF-A expression were found in the larger arteries between the two hypertensive groups, regardless of drug treatment. Collectively, these results suggest that the increase in PDGF-A expression is mediated by pressure, and to a smaller magnitude AT1 receptor activation.

In summary, this experiment has three important findings. First, the results of these studies indicate that ANG II, acting through the AT1 receptor, is not necessary for vascular hypertrophy in 1K1C hypertension, implying that pressure is the mediator of this

response. Second, AT1 receptor inhibition does not effect capillary density. Third, these results suggest that PDGF-A expression, which may contribute to this hypertrophic response, appears to be related primarily to elevated pressure and to a lesser extent to AT1 receptor antagonism.

CHAPTER VI

ANGIOTENSIN II INDUCED ARTERIAL WALL HYPERTROPHY AND PDGF-A EXPRESSION IS MEDIATED BY ELEVATED PRESSURE

Introduction

The previous study described in chapter five, demonstrated indirectly, through AT1 receptor inhibition, that pressure mediates vascular changes such as arterial hypertrophy and PDGF-A expression associated with renal hypertension. Yet, there is evidence that ANG II acts directly as growth factor on smooth muscle cells, wherein ANG II infusion at 200 ng/kg/min, in the presence of hydralazine to maintain a normal blood pressure, significantly increases medial cross-sectional area of the small mesenteric artery (40). Nonetheless, a major concern of this study is the high level of ANG II infusion. In other experimental models, such as salt restriction, 1K1C, and 2K1C hypertension (43, 44, 45, 46) plasma ANG II levels are elevated two to three fold compared to controls. However, infusion of 250 ng/kg/min ANG II (41), results in a six fold increase in plasma levels. Thus a 200 ng/kg/min infusion rate may give rise to higher plasma levels than those ever reached by endogenous concentrations of ANG II. A reduction in infusion dose, creating increases in plasma ANG II levels similar to those seen in renal hypertension, may more accurately describe the direct effects of ANG II on smooth muscle cell adaptations.

The purpose of this study was to investigate the direct role, if any, of ANG II on the vascular changes associated with hypertension. In order to distinguish between direct

and indirect roles of ANG II during hypertension, animals were infused with ANG II at 100 ng/kg/min and treated with minoxidil to prevent a rise in blood pressure. We then evaluated modifications that often accompany hypertension. These changes included hypertrophy or hyperplastic responses of VSMC as well as alterations in PDGF-A expression. A detailed methods section is located in chapter four.

Results

Tail systolic blood pressures, were significantly elevated in ANG II animals (figure 6-1) compared to the other three groups beginning four days after implantation and continuing for the duration of the study. Consumption of minoxidil prior to surgery initially lowered the blood pressure of treated animals. ANG II-MIN animals ingested 9.4 $\pm$ 0.2 mg/kg/day of minoxidil; while the CON-MIN animals ingested 8.7 $\pm$ 0.3 mg/kg/day. Mean arterial blood pressures measured through an indwelling catheter in the tail artery were significantly elevated in the ANG II animals (117.4 $\pm$ 11.2 mm Hg) when compared to the control groups (88.7 $\pm$ 6.7 mm Hg, CON; 73.9 $\pm$ 4.2 mm Hg, CON-MIN) and ANG II-MIN animals (90.3 $\pm$ 9.3 mm Hg). The body weight of CON animals (329.5 $\pm$ 8.3 gms) was significantly lower then CON-MIN animals (366.5 $\pm$ 8.6 gms), but not different from ANG II (352.1 $\pm$ 4.9 gms) or ANG II-MIN (343.4 $\pm$ 6.8 gms) groups.

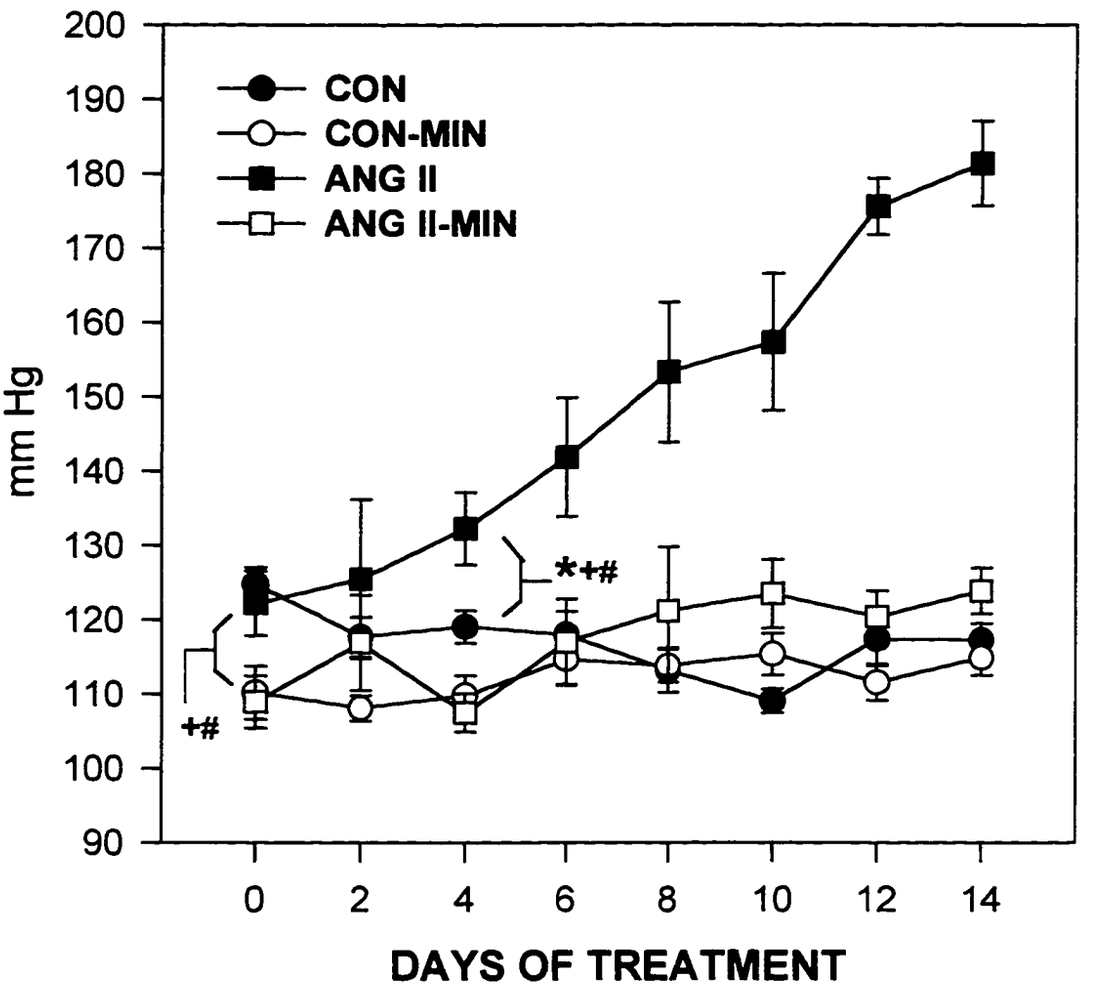


FIG 6-1. Tail cuff systolic blood pressures. Line graph showing systolic blood pressures for physiological saline groups (CON and CON-MIN, circles) or angiotensin II groups (ANG II and ANG II-MIN, squares). Filled figures depict tap water consumption, while open figures depict minoxidil consumption. Values are mean ± SEM. Pump implantation was performed on day zero. Symbols indicate significance levels at $p<0.05$ versus CON (*), CON-MIN (+), and ANG II-MIN (#).

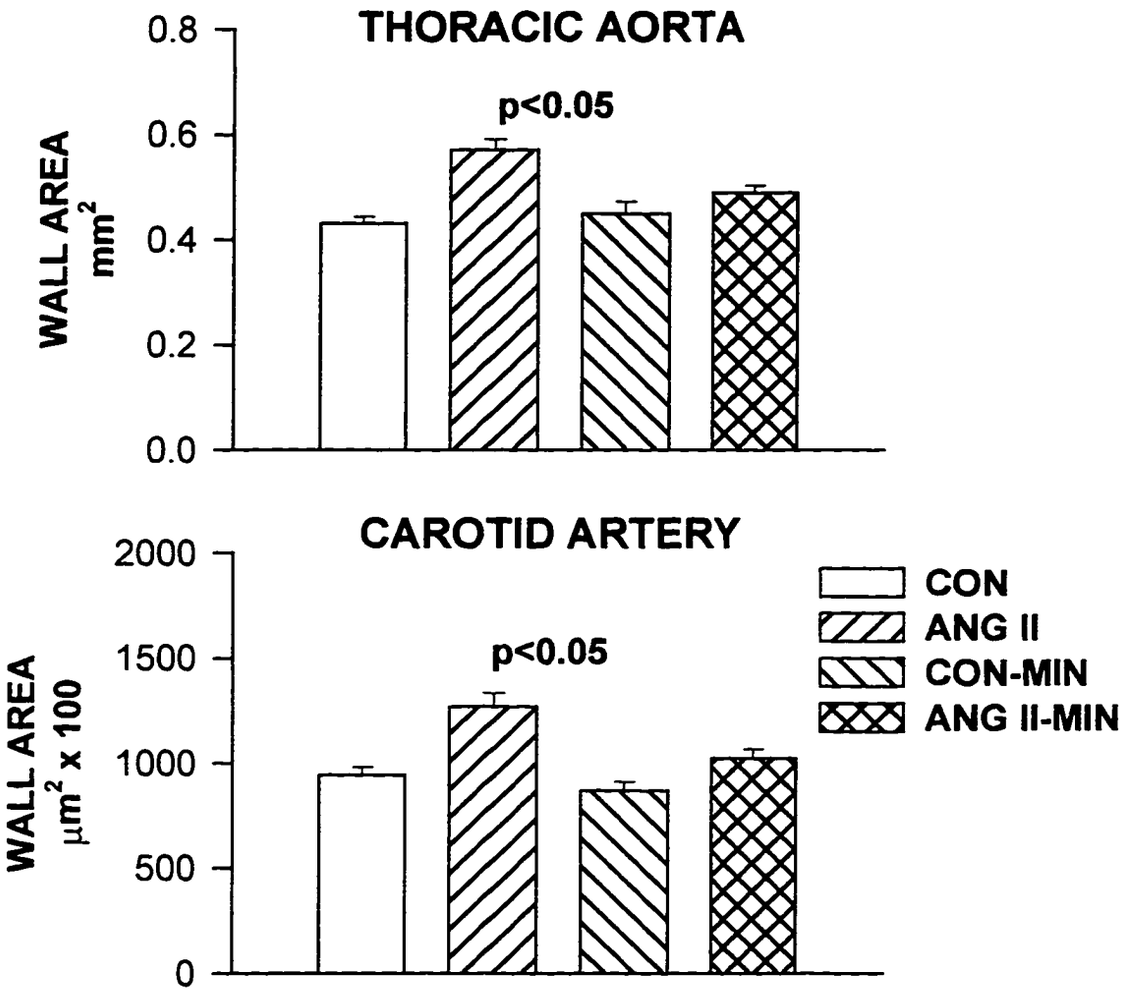


FIG 6-2. Cross-sectional wall area in thoracic aorta and carotid artery. Bar graph showing cross-sectional wall area of the thoracic aorta and carotid artery for CON, ANG II, CON-MIN and ANG II- MIN treated groups. Wall area was determined by the use video based image system with edge tracking software on toluidine blue stained tissues. Values are mean ± SEM. Statistical significance levels were set at $p<0.05$.

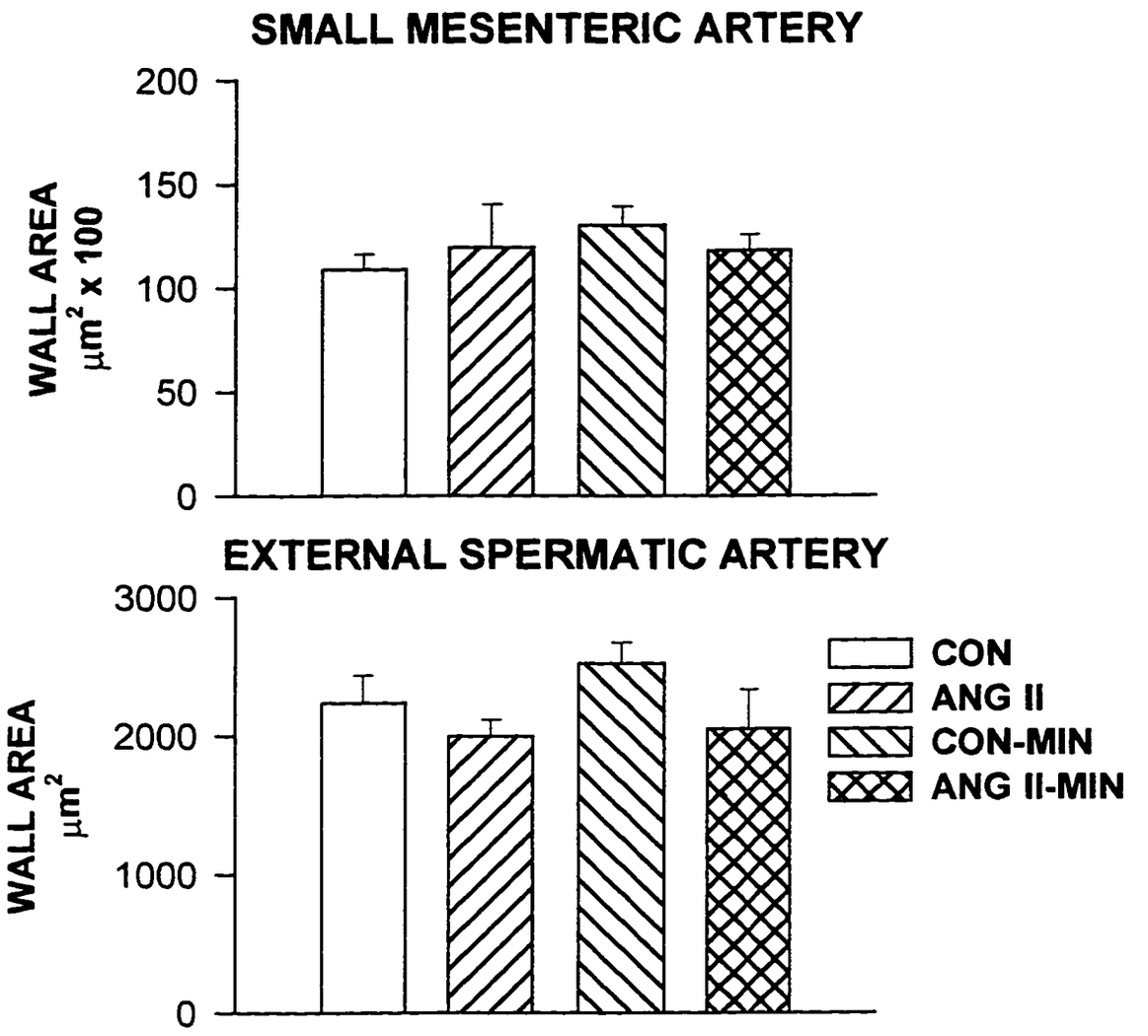


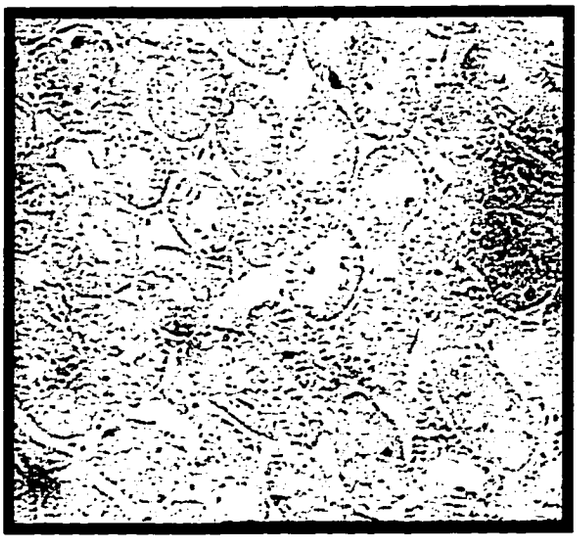
FIG 6-3. Cross-sectional wall area in small mesenteric and external spermatic arteries. Bar graph showing cross-sectional wall area of the small mesenteric and external spermatic arteries for CON, ANG II, CON-MIN and ANG II-MIN treated groups. Wall area was determined by the use of a video based image system with edge tracking software on toluidine blue stained tissues. Values are mean ± SEM. Significance levels were set at $p<0.05$.

Cross-sectional wall area in the thoracic aorta and carotid artery was significantly elevated in the ANG II group when compared to both control groups and ANG II-MIN animals (figure 6-2). There were no significant differences among the groups in the cross-sectional wall area of the small mesenteric artery and external spermatic artery (figure 6-3). The internal diameter of the thoracic aorta was significantly increased in the ANG II, ANG II-MIN and CON-MIN animals when compared to CON (table 6-1). The internal diameter of the carotid artery in the ANG II-MIN group was significantly elevated above the CON group. The small mesenteric artery internal diameter was significantly reduced in the ANG II animals compared to CON-MIN and ANG II-MIN arteries. The internal diameter of the external spermatic artery was significantly reduced in the ANG II group when compared to the CON-MIN arteries.

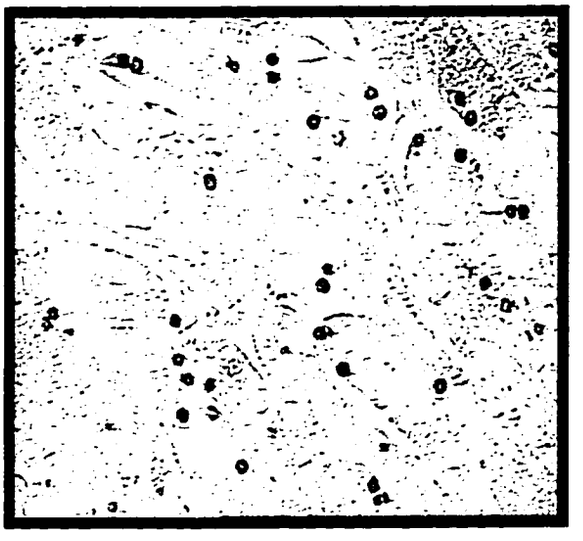
TABLE 6-1. Arterial Lumen Diameters

Rat Groups	Thoracic Aorta mm	Carotid Artery μm	Small Mesenteric Artery μm	External Spermatic Artery μm
CON	1.70 ± 0.02	779.3 ± 10.4	228.5 ± 6.8	112.8 ± 5.0
ANG II	1.82 ± 0.05*	819.3 ± 25.8	210.6 ± 11.0+#	99.5 ± 3.1+
CON-MIN	1.87 ± 0.04*	820.7 ± 15.7	253.8 ± 9.0	121.0 ± 4.8
ANG II -MIN	1.90 ± 0.04*	863.6 ± 23.3*	245.6 ± 6.8	104.8 ± 5.0

Values are mean ± SE.
* $P<0.05$ vs. CON, +$P<0.05$ vs. CON-MIN, #$P<0.05$ vs. ANG II-MIN.



NEGATIVE CONTROL



POSITIVE CONTROL

FIG 6-4. Representative photographs of control and positive renal tubules in immunohistochemistry for BrdU.

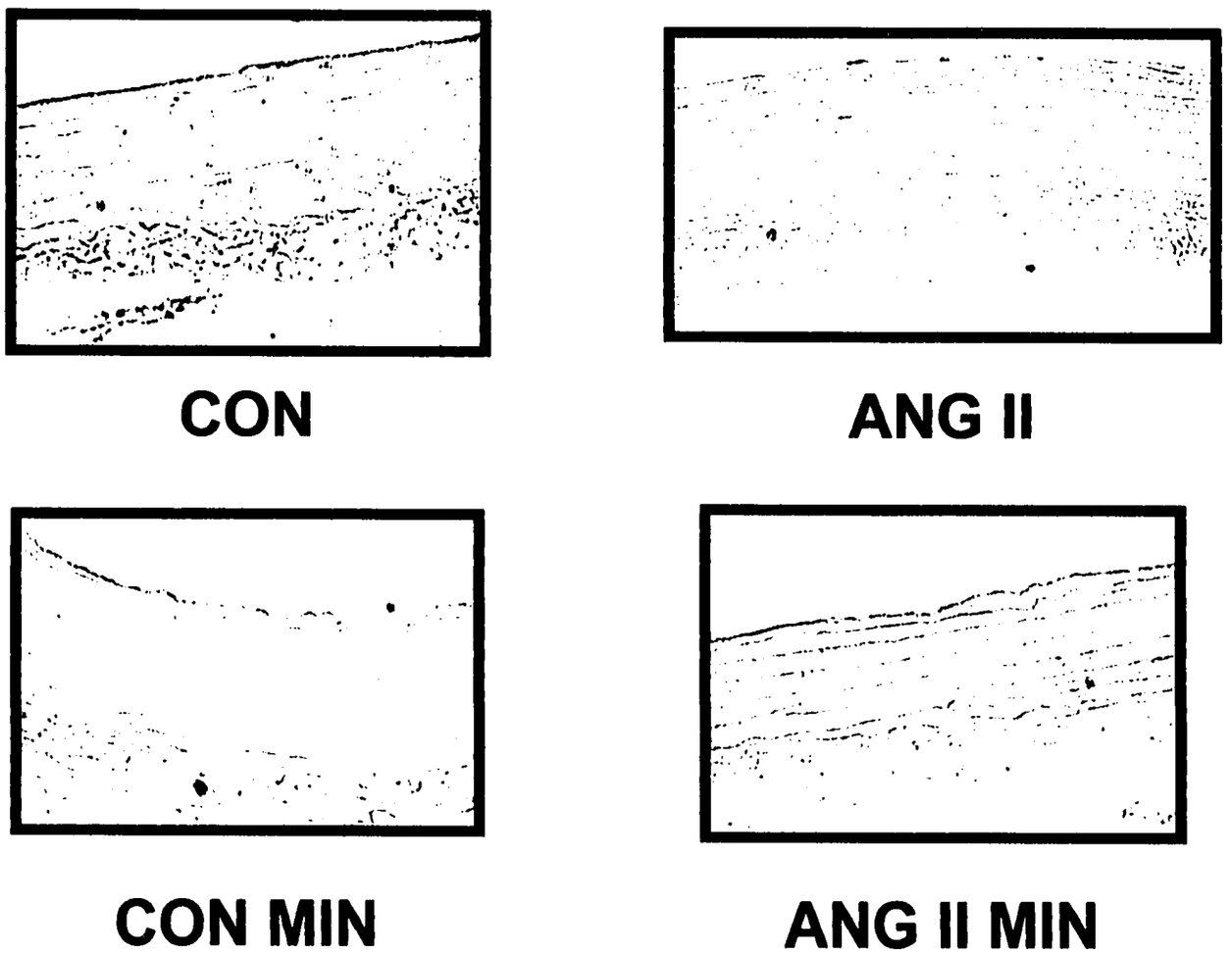


FIG 6-5. Representative photographs of thoracic aortas treated with primary antibody for BrdU.

Immunohistochemistry for BrdU, revealed no positively staining nuclei in the thoracic aortas, carotid or small mesenteric arteries among all four animal groups, regardless of whether BrdU was infused the first or second week. All kidneys with the exception of one animal had positive staining nuclei, confirming BrdU delivery through the osmotic minipump. Representative photographs of positively stained kidneys and thoracic aorta, are shown in figures 6-4 and 6-5.

Microvascular density was measured by staining with *Griffonia simplicifolia* I lectin and fluorescence microscopy. Statistical analysis revealed no significant difference between all four groups (table 6-2).

TABLE 6-2. Microvascular Density

	CON	ANG II	CON-MIN	ANG II-MIN
Number of Intersections	166.7 ± 4.0	158.2 ± 9.4	167.5 ± 10.5	162.0 ± 10.5

Values are mean ± SE.
Significance levels were set at $P<0.05$.

In situ hybridization with the ^{35}S labeled riboprobe for PDGF-A chain was quantitated through the use of a phosphorimager. Expression of PDGF-A mRNA was significantly elevated in the thoracic aorta of the ANG II group compared to both control groups and the ANG II-MIN group (figure 6-6 and figure 6-7).

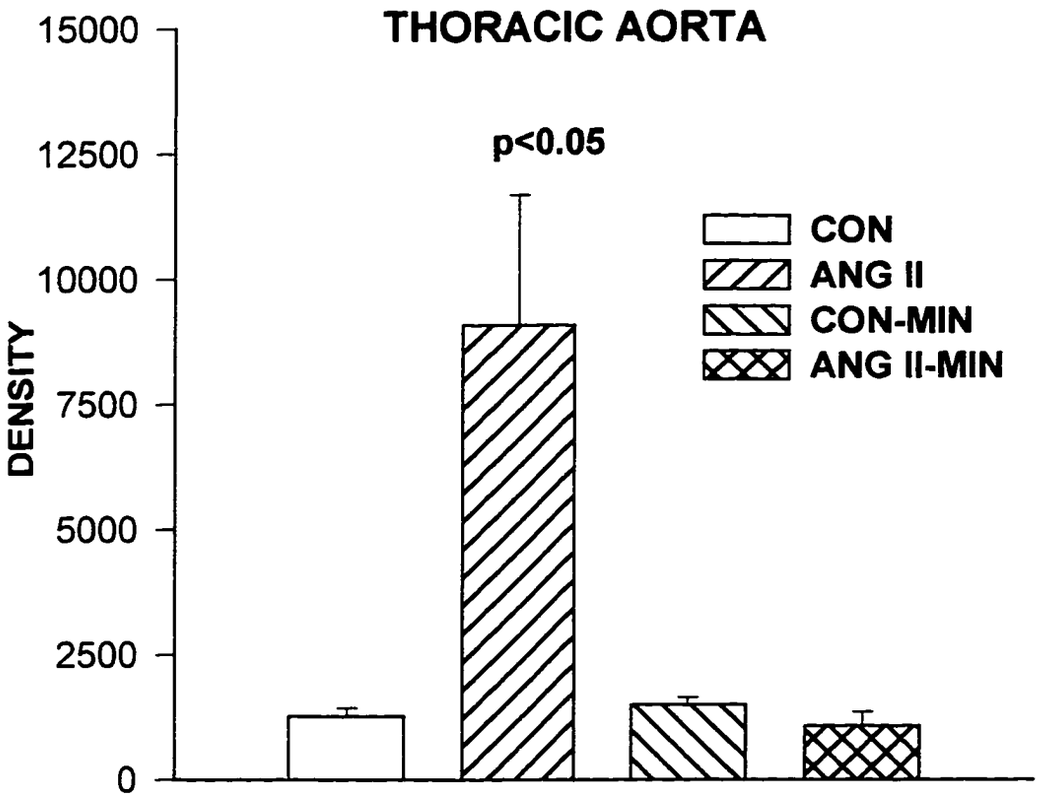


FIG 6-6. PDGF-A expression. Bar graph showing results of in situ hybridization for PDGF-A mRNA on thoracic aorta for CON, ANG II, CON-MIN and ANG II-MIN groups. mRNA content was determined by in situ hybridization with ^{35}S-labeled riboprobe and quantitated on the phosphorimager. Values are mean $\pm$ SEM. The significance levels were set at $p<0.05$.

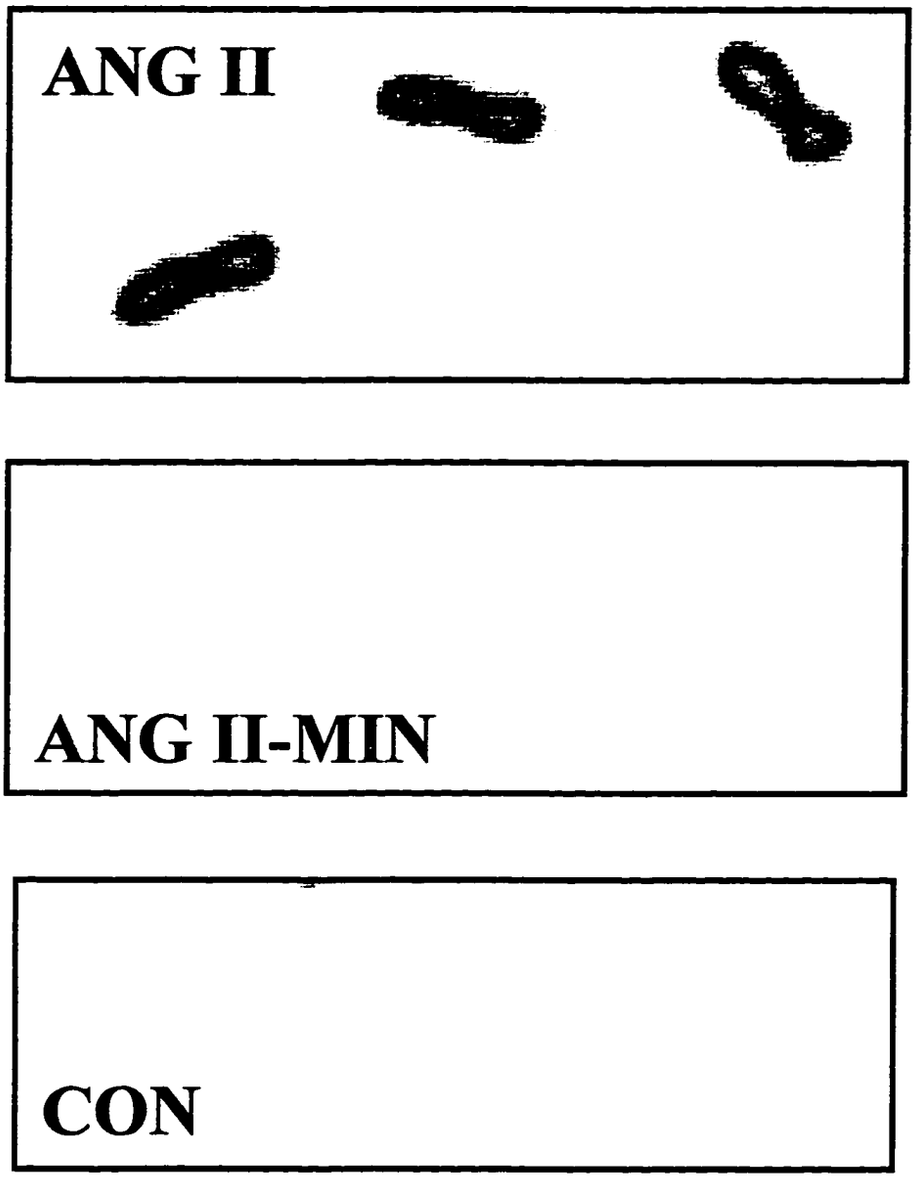


FIG 6-7. Representative photographs of PDGF-A mRNA expression in thoracic aortas.

Discussion

The present study was an attempt to differentiate between the direct and indirect effects of ANG II on structural changes in the vasculature. ANG II infusion at 100 ng/kg/min was sufficient to elicit a significant rise in systolic blood pressure four days after pump implantation that continued for the duration of the study (figure 6-1). Treatment with minoxidil, a K-ATP channel activator, maintained blood pressure in a normal range in ANG II infused animals. This allowed us to evaluate the direct effects of ANG II in the absence of a confounding increase in arterial pressure.

Infusion of ANG II alone caused a significant increase in cross-sectional wall area in the thoracic aorta and carotid arteries (figure 6-2). This effect was prevented when blood pressure was maintained in the normal range by minoxidil, suggesting that pressure was the mediator of the arterial changes at this dose of ANG II infusion. As stated earlier, evidence from the literature suggests that pressure plays a considerable role in vascular alterations associated with hypertension. An increase in medial thickness of the vasculature in the SHR is attenuated by the placement of a ligature that prevents a rise in pressure (69, 70). Similar results are seen in DOCA-salt hypertension (71). In aortic coarctation hypertension, there were significant increases in cross-sectional wall area in the arteries subjected to high pressure, while the normotensive regions showed no significant alterations (4, 68), implying pressure as a stimulus for arterial hypertrophy. Other studies, nonetheless, have suggested a non-pressor role for ANG II in these hypertrophic changes. CEI therapy, albeit confounded by bradykinin potentiation, significantly attenuated the cross-sectional wall area of the aorta in the presence of elevated pressure (6). In addition, ANG II infusion caused a significant increase in wall

area of the thoracic aorta (39). However, these changes were also associated with an increase in systolic blood pressure, leaving elevated pressure and ANG II indistinguishable from one another. Our data provides strong evidence that regulation of wall structure in hypertensive animals during ANG II infusion is mediated by elevated pressure.

The internal diameter of the larger arteries in both minoxidil treated groups significantly increased (table 6-1). Typically, in many models of hypertension, the lumen of the larger arteries does not change in spite of an increase in cross-sectional wall area (5, 6). Minoxidil treatment induces a significant increase in cardiac index as well as skeletal muscle blood flow (137). Changes in blood flow can regulate arterial lumen size through changes in shear stress (138, 139). Consequently, it is quite possible that the diameter changes seen in the minoxidil treated animals are due to the increase in flow initiated by the vasodilator treatment.

Minoxidil elicits its hypotensive effects through the activation of the ATP sensitive potassium channel (140). The antihypertensive effects of minoxidil are thought to occur by the sulfated form Minoxidil-O-Sulfate (MNXS) which was shown to relax isolated arterial rings from rabbit mesentery (141). MNXS also inhibits norepinephrine induced contractions of VSMCs, and tetraethylammonium, a potassium channel antagonist, prevents the MNXS hyperpolarization (142). In addition, the prevention of VSMC contraction may be due to the ability of MNXS to inhibit norepinephrine induced calcium influx (143). Minoxidil treatment, at twice the dosage utilized in this study, significantly prevented the progression of medial hypertrophy of mesenteric arteries in the SHR, when compared to their untreated counterparts. This attenuation of arterial hypertrophy was accompanied by a significant decrease in mean arterial pressure and noradrenaline

turnover, correlating the vasodilator induced changes with decreased pressure (144). At the same dosage in normotensive rats, minoxidil treatment resulted in no significant changes in the medial area of the carotid, basilar, large mesenteric and small mesenteric arteries, along with no significant changes in noradrenaline levels or mean arterial pressure (145). These results suggest that minoxidil may elicit arterial changes in wall area, but these alterations appear to be related to reductions in mean arterial blood pressure. Thus the ability of minoxidil to attenuate the increase in cross-sectional wall area in the ANG II infused animal is probably associated with a lowered mean arterial pressure.

In the present study, there were no significant changes in the cross-sectional wall area of neither the small mesenteric artery nor the external spermatic artery. However, in the ANG II group these vessels exhibited a trend, although not statistically significant, towards inward eutrophic remodeling (table 6-1). Characteristic structural alterations during renal hypertension include hypertrophy of smaller mesenteric arteries (11), and lumen reductions in the first and second order arterioles (7, 8). The lack of structural changes in cremaster arterioles in rats subjected to aortic coarctation hypertension (68) indicates a role for pressure as a stimulus for remodeling. ANG II, nevertheless, is also implicated as a direct hypertrophic factor in the small mesenteric resistance vessels. In a study by Griffin et al. (40) ANG II infusion at 200 ng/kg/min induced a pressure independent, hypertrophy of the small mesenteric artery. Our infusion dose was half of that used by Griffin et al. resulting in a dose the concentration of ANG II that may have been too low to have a direct effect on vascular structure and pressure was not high enough to induce remodeling.

The lack of hypertrophy in the large arteries with combined with ANG II and minoxidil treatment, is best explained by a decrease in mechanical stress caused by lowering blood pressure. As stated previously in chapter two, aortic coarctation studies demonstrated a significant increase in products of inositol lipid hydrolysis and proto-oncogene expression in the hypertensive region of the aorta (4, 72). In addition, acute hypertension induces MAPK activation and proto-oncogene expression (74). Pressure can induce proto-oncogene expression (76), which is shown to correlate with wall stress (77). This increase in proto-oncogene expression may regulate smooth muscle cell growth (78). Thus, inositol phospholipids and the MAPK pathway as well as proto-oncogene expression are viable signaling pathways for pressure induced changes.

Hypertension is associated with hypertrophy of VSMC and alterations of the extracellular matrix (18, 47, 146, 147). This hypertrophic, not hyperplastic, smooth muscle cell adaptation is found in many models of hypertension (11, 47, 72). Arterial hypertrophy may also be due to VSMC polyploidy (132). The lack of any BrdU staining in our vessels suggests that polyploidy did not occur during hypertrophy. Photographs of positively stained kidneys and thoracic aortas are seen in figures 6-4 and 6-5. Although pressure appears to be the cause of this response in the present study, ANG II also is implicated as a hypertrophic stimulus. ANG II induced an increase in protein synthesis, not DNA synthesis in intact rat aortic segments (14). Outside of cellular adaptations, ANG II also induces an increase in fibronectin expression, independent of pressure (148). In the present study, however, ANG II infusion did not elicit hypertrophy of the arterial wall unless pressure was also elevated.

An additional finding of this study is that there was no significant decrease in microvascular density in the ANG II infused animal (table 6-2). As stated earlier, both experimental models and patients with essential hypertension demonstrate a significant decrease in microvascular density (7, 8, 9, 10, 115, 116). The rise in pressure, at this dose of ANG II, was probably not high enough to induce rarefaction. It should be noted that ANG II did not influence microvascular density in the normotensive animal. Previous studies implicate ANG II as factor that mediates an increase in capillary density (114, 119). However, ANG II infusion results in downregulation of ANG II receptors (149). This reduction in the availability of binding sites may be a plausible explanation for the lack of ANG II-induced angiogenesis, in the normotensive animal. In addition, it is quite possible that only a certain number of capillaries are necessary to maintain normal function in the cremaster muscle, and as a result, no significant increase in capillary density was found at this infusion dose of ANG II.

Another outcome of this study is that the hypertrophic response of the aortic wall, mediated by elevated pressure, was accompanied by an increase in PDGF-A expression (figure 6-6). Photographs of thoracic aortas are found in figure 6-7. Pressure has been linked to PDGF-A expression, in a previous study the reduction of blood pressure of in the SHR significantly decreased PDGF-A expression (19). Furthermore, cyclical stretch of VSMCs cultured on fibronectin, has been shown to increase PDGF-A expression (60). Alternatively, application of ANG II in cell culture also induced PDGF-A expression (17). ANG II infusion, accompanied with an elevated systolic blood pressure, caused a significant increase in PDGF-A expression in the thoracic aorta (39). In addition to pressure and ANG II, adrenergic stimulation is implicated in PDGF-A expression. A

study by Majesky et al. demonstrated that phenylephrine induced PDGF-A mRNA was significantly greater than ANG II induced expression at a similar pressure rise (111). It has been shown that ANG II infusion increased plasma noradrenaline levels (41). This suggests that ANG II induced PDGF-A expression may be due to an increase in circulating noradrenaline levels. However, our study demonstrated no increase in PDGF-A expression when the pressure was returned to normal values in ANG II infused animals. These results suggest that at this dosage of ANG II, the increase in PDGF-A expression is mediated through elevated pressure, and PDGF-A may be part of the signaling pathway to transduce an elevation of pressure into a hypertrophic response.

In summary the results of these studies indicate that the hypertrophic response of arterial VSMCs to ANG II infusion is mediated through an increase in arterial blood pressure. Secondly, ANG II infusion does not elicit changes in microvascular density. Thirdly, PDGF-A expression, which may be a mediator of the hypertrophic response, is also associated with the elevation in pressure rather than a direct effect of ANG II.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

The objectives of this study were to delineate the relative roles of angiotensin II and pressure on arterial hypertrophy, VSMC adaptations, capillary density and growth factor expression in experimental hypertension. Our hypothesis was that angiotensin II is indirectly responsible for the arterial wall hypertrophy and PDGF-A expression through the elevation of intravascular pressure, rather than through a direct non-pressor mechanism. These studies provide convincing evidence that pressure is the primary mediator of arterial wall hypertrophy and PDGF-A expression in experimental hypertension. The main conclusions of these studies are as follows:

1. The hypertrophic response of arterial VSMCs in both 1K1C hypertension and angiotensin II infusion is mediated through arterial blood pressure.

2. PDGF-A expression, which may contribute to the hypertrophic response, appears to be related primarily to elevated arterial pressure.

3. Capillary density in the cremaster muscle is reduced in renal hypertension, but is unaffected by AT1 blockade, or ANG II infusion.

In viewing the summary graph (figure 7-1), we propose that the increased cross-sectional wall area seen in these hypertension models may be mediated, in part, by PDGF-A. The weak mitogenic properties of PDGF-A may be correlated with VSMC hypertrophy that was seen in the large arteries of these hypertensive models. The exact mechanism in which pressure elicits growth factor expression is still relatively unknown. Pressure, either through *in vivo* or *in vitro* experiments, has been shown to increase the production of immediate early genes in both VSMCs and cardiac myocytes. In aortic coarctation, MacIver et al. (72) demonstrated a significant increase in proto-oncogene expression in the hypertensive, but not the normotensive region of the aorta. Acute, restraint induced hypertension resulted in an upregulation of c-*fos* and c-*jun* expression (74). *In vitro*, mechanical stretch on collagen and fibronectin coated flexible culture plates induced c-*fos* expression (62, 63). Increased expression of immediate early genes including c-*fos*, c-*myc*, c-*jun* and *egr*-1 occurred in isolated hearts subjected to acute elevation in wall stress (75). Our laboratory has shown that pressure can increase expression of c-*fos*, c-*myc* and ribosomal RNA in isolated mesenteric arteries (76). In addition, this c-*fos* expression is correlated with wall stress (77). These immediate early genes encode for transcription factors, which may ultimately control the growth of VSMCs (78). In vascular endothelial cells increased *egr*-1 expression is followed by an increase in PDGF-A expression (150). The promoter region for PDGF-A contains recognition elements for the immediate early gene *egr*-1 (151). Therefore, it is quite possible to propose that *egr*-1, as well as other immediately early genes, may be responsible for the

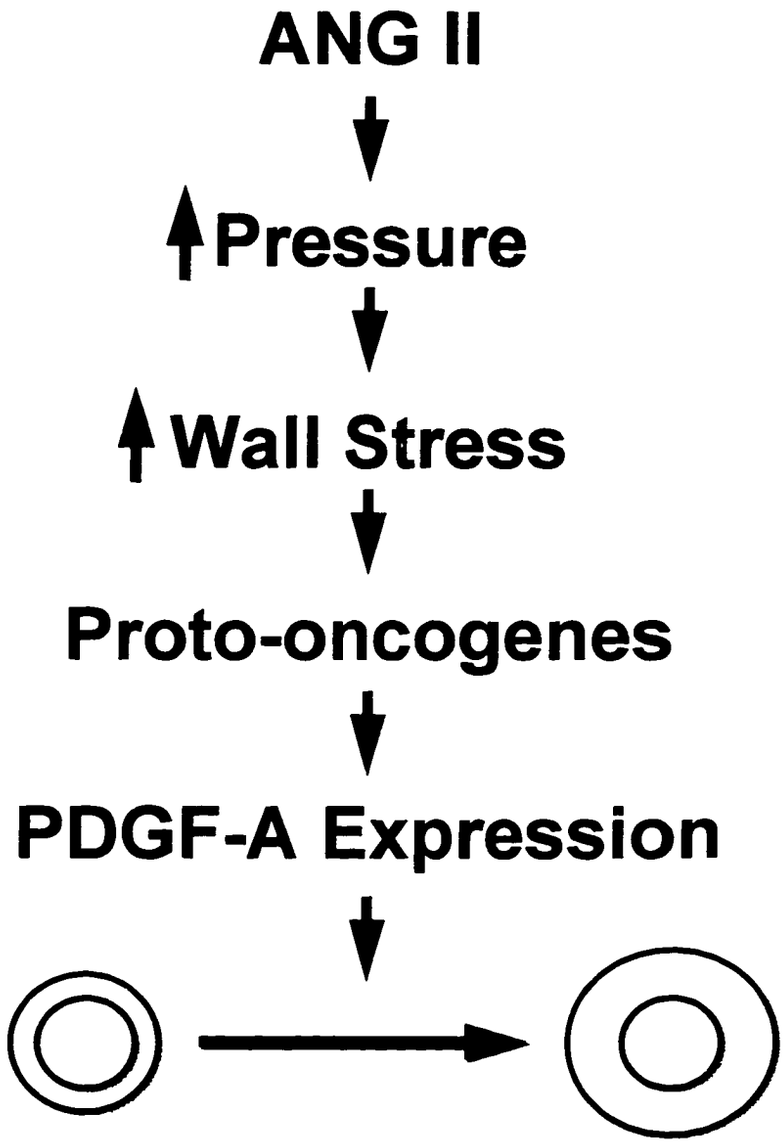


FIG 7-1. Proposed mechanism for pressure induced hypertrophy.

pressure induced increase in PDGF-A expression. Further studies are needed to determine the mechanism of pressure induced PDGF-A mRNA expression and protein translation.

Finally, it should be noted that this study does not account for alterations in the extracellular matrix proteins. Experimental models of hypertension are shown to increase production of fibronectin, collagen and elastin in both VSMCs and cardiac myocytes (152, 153, 154). Mechanical tension on intact pulmonary arteries elicits synthesis of collagen, elastin as well as increased procollagen mRNA (67). In Dahl-salt sensitive rats, increased elastin production in the aorta is correlated with increased blood pressure (152). Furthermore, in aortic coarctation, alterations in collagen and elastin in the splenic and ileal arteries were associated with elevated blood pressure (146). In addition, TGF-β1 is shown to increase fibronectin and collagen types I, IV, and V in VSMCs (reviewed by 155). Moreover, it has been shown that interactions between the extracellular matrix and their integrin receptors are partially responsible for sensing mechanically stimulated changes (66). As a result, it is conceivable that some of the increase in cross-sectional wall area may be attributed, in part, to an increase in matrix deposition or a lack of matrix degradation. In addition, inward eutrophic remodeling may be due to alterations in smooth muscle cell and extracellular matrix alignment. An interesting follow up to this study would be to evaluate some of the alterations in matrix components as well as their potential mediators including TGF-β1, in this as well as other hypertensive models. *In situ* hybridization and immunohistochemistry techniques would be useful tools in evaluating extracellular matrix changes in hypertension.

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