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A STRUCTURAL BASIS FOR THE GENESIS OF HYPERTENSION IN THE SPONTANEOUSLY HYPERTENSIVE RAT

By

JEFFREY G. DICKHOUT, B.SC. (HONS)

A Thesis

Submitted to the School of Graduate Studies

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for the Degree

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HYPERTENSION DEVELOPMENT IN THE SHR

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ABSTRACT

The spontaneously hypertensive rat (SHR) was used as a model of human essential hypertension. The overall hypothesis was that hypertrophy of the smooth muscle layer of small muscular arteries in essential hypertension results in greater contractility of these vessels that then results in elevated total peripheral resistance and higher blood pressures. Elevated total peripheral resistance and small artery hypertrophy are well documented in the SHR, however, it remains unknown if these changes are the cause or result of elevated blood pressure. For this reason, we have focused our studies on young SHR during the initiation of hypertension to attempt to separate cause from effect.

Studies were done to determine when SHR's blood pressure begins to differ from its normotensive control the Wistar-Kyoto rat (WKY). We found that blood pressure began to diverge between SHR and WKY at four weeks of age. Structural and functional differences between small muscular arteries from the mesenteric vascular bed of 4-week old SHR and age matched WKY controls were studied using a new morphometric protocol involving confocal microscopy and a pressurized artery myograph. Arteries from SHR had a larger medial volume, increased number of smooth muscle cell layers, but similar lumen size when compared with WKY in the maximally relaxed condition. Functional studies showed that SHR arteries contracted more in response to stimulation by KC1 and norepinephrine, resulting in significantly smaller lumen size in these vessels

as compared to WKY. We concluded that structural and functional differences in SHR arteries were primary changes which may contribute to the development of hypertension.

Further studies were conducted to determine if a differential incidence of apoptosis during the development of SHR and WKY arteries contributes to the structural differences. One to two week old animals were used for these studies since at this time the structure was similar between the strains . To measure the incidence of apoptosis, we used both DNA laddering and end labeling. It was found that SHR had a significantly decreased incidence of apoptosis over WKY. The cellular nature of the medial layer hypertrophy in SHR at 4-weeks was also assessed. Numerical density of smooth muscle cell nuclei in the medial layer was measured with a three dimensional disector method under confocal microscopy. We found that the numerical density of medial smooth muscle cells was significantly less in SHR than WKY, and the number of smooth muscle cells was similar between the strains. The smooth muscle cell length from SHR was significantly longer than WKY. We concluded that increased smooth muscle cell length in prehypertensive SHR is responsible for their increased medial volume.

These studies have shown that medial layer hypertrophy due to smooth muscle cell lengthening in the small muscular arteries of SHR which increases their contractile ability, occurs at the initiation of hypertension. This evidence demonstrates that structural and functional changes in these SHR arteries can not be the result of increased blood pressure but may be a factor causing hypertension by increasing the total peripheral resistance in these animals.

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This thesis begins with a general literature review discussing human essential hypertension as a disease process and the nature and relevance of the spontaneously hypertensive rat as a model of this disease. Subsequent chapters will be comprised of manuscripts submitted for publication or published papers. The final chapter will summerize the conclusion from the earlier studies and make recommendations for future research. The submitted and published papers are coauthored with my supervisor Dr. R.M.K.W. Lee who provided me with general direction for the studies from which I designed the experiments, preformed all the experimental work in each of the studies, and produced drafts of the manuscripts. Dr. Lee then aided me in preparation of the finished manuscripts for publication. The only other contributors to this work were Dr. S.L. Kyone who assisted me with physiological studies in Chapter 2; Dr. Geoff R. Norman who aided me in statistical analysis for Chapter 3; and Dr. J. Smeda who created Figure 6 of Chapter 5.

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LIST OF ABBREVIATIONS

1K1C	one-kidney, one-clip
2K1C	two-kidney, one-clip
3`-OH	three prime hydroxyol
ACE	angiotensin converting enzyme
ANOVA	analysis of variance
AUC	area under the curve
BP	blood pressure
BSS	basic salt solution
CO	cardiac output
Dahl S	Dahl salt-sensitive rats
DOCA	deoxycorticosterone acetate
DPI	dots per inch
DNaseI	deoxyribonuclease I
ED_{50}	effective dose 50
HPLC	high performance liquid chromatography
IP	intra-peritoneal
KCl	potassium chloride

LSD	least sum difference
LSM	laser scanning microscope
mmHg	millimeters of mercury
NE	norepinephrine
NIH	National Institute of Health (U.S.A.)
PRA	plasma renin activity
SAS	Statistical Analysis Society
SC	subcutaneous
SEM	scanning electron microscopy
SHR	spontaneously hypertensive rats
SMC	smooth muscle cell
SV	stroke volume
TdT	terminal deoxytransferase
TPR	total peripheral resistance
TTX	tetrodotoxin
V_g	total genetic variance
WKY	Wistar-Kyoto rats

Chapter 1. Introduction

1.1 Background

Human Essential Hypertension

Essential hypertension is a sustained elevation of blood pressure (BP) of unknown etiology. It results from the culmination of a series of pathological changes in the body that lead to this sustained elevation of BP. The current physiological definition of hypertension used in many large epidemiological studies of the human population is a systolic BP between 140 and 159 mmHg and/or a diastolic BP between 90 and 94 mmHg for border line hypertensives, and a systolic BP over 160 mmHg and/or a diastolic BP over 95 mmHg for hypertensives (Dannenberg et al.,1988; Garrison et al.,1987). The incidence of hypertension as reported in the Framingham heart study cohort based on 30 years follow-up of 5,209 subjects reveals a rate increasing with age in men from 3.3% at ages from 30 to 39 to 6.2% at ages from 70 to 79 and in women from 1.5% at ages from 30 to 39 to 8.6% at ages from 70 to 79 (Dannenberg et al.,1988).

BP is a quantitative, not a qualitative trait, and is under the influence of both genetic and environmental factors. The genetic nature of human essential hypertension is one of a complex inheritance involving many genetic loci (Kurtz et al.,1993). However, not all essential hypertensives are the same. Essential hypertension represents a cluster of syndromes of varying genetic type (Sever, 1995).

Amongst humans with essential hypertension some different categories of disease can be defined and these different categories of disease are grouped in populations with different ethnic or racial origin. Native American populations face higher rates of hypertension as shown in a study of 780 adults on the Navajo Reservation in the United States. This study revealed a rate of 24% among men and 15% among women for systolic BP above 140 or diastolic BP above 90 mmHg (Percy et al.,1997). In these populations the higher prevalence of hypertension is strongly associated with non-insulin dependent diabetes mellitus, and a higher body mass index (Percy et al.,1997; Haffner et al.,1994). African Americans are another group that have been well studied and reveals a prevalence and characteristics of hypertension that are markedly different from white populations. In comparison to Caucasians, African Americans showed lower plasma renin activity (PRA), more sodium retention, and poor response of PRA to sodium depletion and loading (Somova et al.,1996). In fact, salt sensitive hypertension is much more common in blacks than in whites (Folkow, 1992; Anderson et al.,1989; Wright, 1988). Higher body mass, however, is a less important factor for the development of hypertension in blacks and whites than in Native Americans (Berenson et al.,1989).

The Need for an Animal Model

The long course of hypertension development in humans and its genetic nature makes an animal model of this disease indispensable. In an animal model such as the rat, inter-generational breeding studies to map the genetic nature of the disease and detailed studies of disease development are possible (Rubattu et al.,1995). Moreover, invasive procedures which are of too great a risk or unethical in human subjects are possible and may lead to a

greater understanding of the mechanisms behind the disease.

Various animal models of the disease exist and differ in their characteristics. Secondary forms of hypertension are usually modelled by manipulation of the kidney. In the kidney mass reduction model, 2/3 to 7/8 of the kidney mass is surgically removed and hypertension results (Cowley, 1997a). This manoeuver also increases salt sensitivity of the animals (Cowley, 1997a). In the Goldblatt model, renal perfusion is reduced by the application of a clip to a renal artery, with (one-kidney, one-clip, 1K1C) or without (two-kidney, one clip, 2K1C) the removal of the contra-lateral kidney. This clip decreases kidney perfusion and results in the release of renin, thus increasing plasma levels of angiotensin II and thereby increasing systemic BP (Orlowski, 1993).

The 1K1C model is characterised by an early elevation of renin in the acute phase and normal levels of plasma renin in the chronic phase (Hoobler et al.,1973). Anti-renin antibodies were shown to lower BP in 1K1C rabbits during the acute phase, 7 days after nephrectomy (Nx), but not in the chronic phase, 30 days after Nx (Hoobler et al.,1973). In the 2K1C model, while plasma renin activity was not elevated even in the acute phase, anti-renin antibodies were shown to lower BP in both the acute phase and to a lesser degree in the chronic phase (Hoobler et al.,1973).

Other secondary models include the DOCA/salt hypertensive model where the mineral cortical steroid, deoxycorticosterone acetate (DOCA), is given to normotensive animals and results in the development of hypertension through kidney damage (Brownie, 1990). The level of hypertension is augmented by salt loading and the renin-angiotensin system is suppressed (Brownie, 1990).

Primary models of hypertension, models where hypertension is of genetic origin, include the spontaneously hypertensive rat (SHR) and the Dahl salt sensitive rat (Dahl S). The Dahl S shares many similarities with hypertension in African American populations (Campese, 1994; Grim et al.,1990). This includes salt sensitivity (Rapp, 1982), insulin-resistance (Buchanan et al.,1991), and hyperlipidemia (Reaven et al.,1991). They are low renin hypertensives (Rapp, 1982), and angiotensin converting enzyme (ACE) inhibitor treatment is less effective in lowering BP in these animals (von Lutterotti et al.,1991). SHR have low cholesterol (Iritani et al.,1977), and show good BP response to ACE inhibition (Lee et al.,1991; Lee et al.,1997). ACE inhibitor treatment of SHR at an early age results in a sustained lowering of BP even after withdrawal of treatment (Harrap et al.,1990). Adult SHR show normal to depressed PRA (Nakamura et al.,1995). However, 10-week old SHR showed an elevated response in kidney renin mRNA after exposure to stimulators such as salt depletion when compared to normotensive Wistar Kyoto rats (WKY) (Kitami et al., 1989). Young SHR also have a lower body weight than age-matched WKY controls (Dickhout et al.,1998b).

The Origin of the SHR Model

In these studies we have chosen to use the SHR as a model of human essential hypertension. SHR had its origin from Wistar rat stock at the animal centre in Kyoto, Japan (Okamoto et al.,1963). As such the WKY rat stock from whence it was bred represents a normotensive control with the most closely matching genetic heritage. SHR was developed in the early 1960s by full sibling inbreeding of brother/sister pairs with higher than average BP until 100% of the progeny had naturally occurring hypertension (Trippodo et al.,1981).

Introduced by Okamoto and Aoki (Okamoto et al.,1963), it represented a genetic model of hypertension requiring no physiological or pharmacological intervention to produce the disease (Trippodo et al.,1981).

Physiology of Hypertension

BP is generated by the heart on the arterial side. This pressure allows perfusion of the tissues. As such, the heart is the mechanism doing the physical work which maintains a pressure gradient from larger arteries to capillaries by ejecting a certain volume of blood to perfuse the tissues. Cardiac output (CO) is the total volume of this blood ejected over some length of time (Volume/Time) (Lund-Johansen et al.,1990). The arteries and arterioles which the blood flows through act as a resistance in the circuit and absorb the work done by the heart. Total peripheral resistance (TPR) can be defined as the resistance to flow generated by all the peripheral vessels and is expressed as the pressure drop per flow rate (mmHg x Time/Volume) (Lund-Johansen et al.,1990). BP develops in the vessel under the force exerted on the blood by the heart in opposition to the resistance of the circuit. This relationship is summarized in Equation 1.1.

$$BP = CO \times TPR \quad \text{....................(1.1)}$$

From this simple relationship, it is clear that an increase in either CO or TPR will increase BP. In the short term, an increase in sympathetic nervous activity will increase BP by increasing TPR due to the constriction of sympathetically innervated small muscular arteries. Further, since CO is determined by the force of contraction of the heart and its

degree of filling producing a certain stroke volume (SV), and the heart rate (HR), any factor which increases these parameters will also increase BP. For example, an increase in blood volume, which would increase the degree of filling of the heart, would increase CO and increase BP.

The central role the kidneys play in the development and maintenance of hypertension has been suggested by many researchers, due to their influence on blood volume. Coleman and Guyton had put forth in the 1960s the concept that the kidneys could provide the site for long-term BP regulation through a mechanism of pressure-natriuresis-diuresis to adjust extracellular fluid and blood volume. This concept is well described in the recent review by Cowley (Cowley, 1997b). The pressure-natriuresis-diuresis relationship predicts that any increase in BP would result in a compensatory decrease in blood volume due to increases in renal perfusion pressure and glomerular filtration rate. However, since maintenance of fluid and electrolyte balance are critical factors to homeostasis, shifts in the pressure-natriuresis-diuresis relationship are necessary to maintain homeostasis at higher pressures. The renin/angiotensin/aldosterone system has evolved to prevent electrolyte and fluid loss beyond certain limits. Also, changes in the pressure-natriuresis-diuresis relationship may initiate higher BP as an adaptive response to maintain renal perfusion and fluid and electrolyte balance. In either case, changes in the pressure-natriuresis-diuresis relationship are found in all forms of experimentally induced secondary forms of hypertension, and in primary genetic hypertension in animals and in humans (Cowley et al.,1996).

The hypothesis that an increase in TPR brings about hypertension has been long

advocated by Folkow and his colleagues (Folkow, 1990). Decreased vascular lumen diameters in the pre-capillary blood vessels may be the mechanism behind this increase in TPR. The reason behind this hypothesis is clear from an examination of the biophysics of the system. Fluid flow (Q), as described by Poiseuille's Law (Equation 1.2)(Berne et al.,1988a) is proportional to the product of the pressure difference (P_i - P_0) and the fourth power of the vessel radius, r, and inversely proportional to the viscosity of the fluid, η, and the length of the vessel, l.

$$Q = \frac{\pi (P_i - P_0) r^4}{8 \eta l} \qquad \text{(1.2)}$$

Therefore, a decrease in the internal diameter of the blood vessel would result in a exponential decrease in flow. In order to maintain the necessary flow for organ perfusion, a much larger pressure differential must be generated to overcome the decrease in blood vessel diameter.

Since blood vessel diameter on the arterial side has such a large influence on TPR and thus BP, it is important to look in greater detail at the structure of arteries. A generalized diagram of the artery is illustrated in Figure 1.1. This diagram shows all the tissue layers in a large artery: intima, internal elastic lamina, media, external elastic lamina, and adventitia. However, not all these tissue layers exist in all arterial vessels. As the lumen diameter of the arteries decrease, so do the vessel wall components. Large caliper arteries are known as

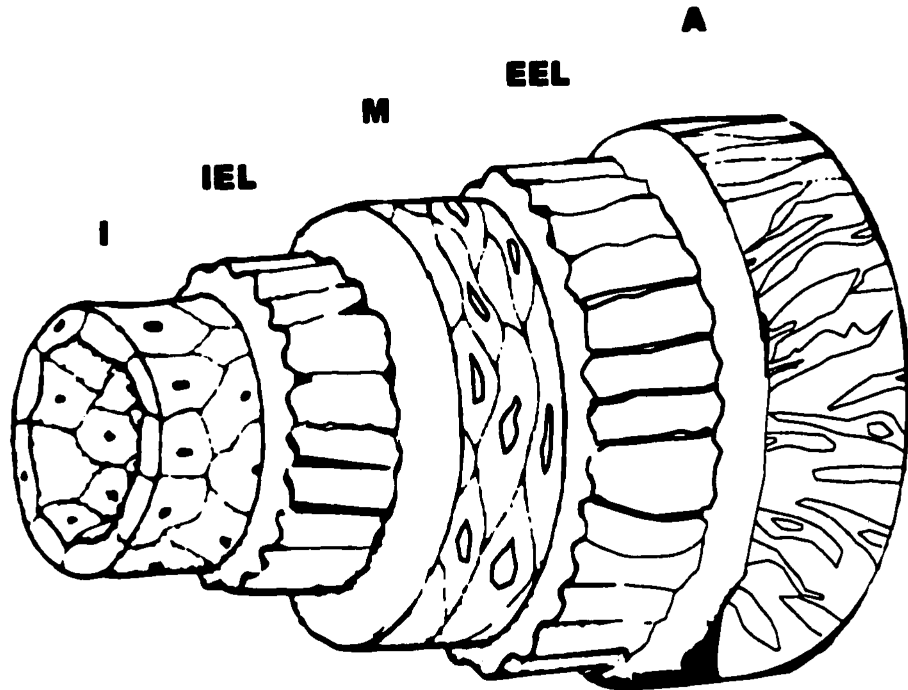

Figure 1.1. Illustration of idealized artery with all potential tissue layers shown being labeled as; I : Intima, IEL : Internal Elastic Lamina, M : Media, EEL : External Elastic Lamina, A : Adventitia.

elastic arteries and serve mainly as passive conduits of the blood absorbing the energy of the pulse during systole and releasing it again at diastole. They are composed of all tissue layers illustrated in Figure 1.1 and have media and adventitial layers consisting of many elastin as well as collagen fibers. As the caliper decreases, elastic arteries change into muscular arteries. The elastin content is greatly reduced and the smooth muscle cells of the media play a much larger role in control of the blood vessels diameter to regulate blood flow (Wheater et al.,1987). Smaller muscular arteries lack an external elastic lamina. As diameters still decrease arterioles are found which contain a media of only a single smooth muscle cell layer and an adventitia layer which merges with the surrounding connective tissue (Wheater et al.,1987).

Evidence first began to accumulate concerning the "resetting of peripheral resistance" in essential hypertension from animal models (Folkow et al.,1970a; Folkow et al.,1970b; Folkow et al.,1970c). Studies of the SHR found increased resistance to perfusion of the hindquarter in adult hypertensive animals (Folkow et al.,1970c). Further, the finding of increased vascular resistance in human essential hypertensive (Mulvany, 1996) with narrowing of the internal diameter of subcutaneous arteries (Aalkjaer et al.,1995) suggests that resetting of the peripheral resistance is operating in human essential hypertensives as well. This type of structural change in the resistance blood vessels may be an adaptive response to pressure increase and so form a positive feedback loop where pressure increase causes reduction in lumen radius through vessel wall hypertrophy, thereby causing further pressure increase (Folkow, 1990).

However, vessel wall hypertrophy may not be necessary for internal radius reduction.

The term vascular remodeling was first introduced by Baumbach and Heistad (Baumbach et al.,1989) to describe a reduction in the external and internal diameter of the pial arterioles of the stroke-prone SHR. Here, reduction in lumen radius occurs without the hypertrophy of the blood vessel wall but instead a rearrangement of vessel wall components. This appears to be a process that is also operating in the female SHR where total vessel wall area was similar between SHR and WKY for the large mesenteric arteries, however, lumen area was significantly less in SHR (Lee et al.,1991). Similarly, the small mesenteric arteries of 4-week old (Lee, 1985) and 5-month old (Warshaw et al.,1979) SHR had a smaller lumen diameter than age-matched WKY.

Alternatively, in the absence of encroachment of the vessel wall on the lumen under passive conditions, an increase in the ability of the vascular smooth muscle to contract against pressure would result in a reduction of lumen diameter under active tension. Support for this idea comes from the findings in the large mesenteric arteries of male SHR of wall thickening with similar lumen areas in the maximally relaxed state (Lee et al.,1983; Lee, 1985), combined with smaller lumen diameters in the SHR arteries under agonist stimulation (Dickhout et al.,1997).

Similarities of the SHR Model to the Human Disease

The SHR is the most widely used model of human essential hypertension (Pravenec et al.,1989). The SHR is a suitable model to study hypertension development because it is similar to humans with essential hypertension. It is a model of the disease with a multi-factor genetic origin (Trippodo et al.,1981). This has been demonstrated through backcrossing with progenitor strains of the SHR and examination of the frequency distribution of BP in the

recombinant strain (Pravenec et al.,1989). Here BP was continuously distributed between values for the normotensive and SHR parents of the cross indicating the multi-allelic origin of the high BP trait (Pravenec et al.,1989). Among the specific factors of the SHR that bear similarity to human essential hypertension are: a genetic predisposition to high BP without specific etiology, salt sensitivity, increased total peripheral resistance without volume expansion, cardiovascular adaptation in response to disease, and similar responses to drug treatment (Frohlich, 1986).

High BP in adult SHR is associated with increased total peripheral resistance. In humans with essential hypertension this is also true. The reason for this increased peripheral resistance in the SHR can be traced to the small muscular arteries and arterioles (Trippodo et al.,1981). These vessels have been shown to have an increased reactivity to a variety of contractile agonists (Trippodo et al.,1981; Dickhout et al.,1997). This has also been shown to be true in humans with essential hypertension (Folkow et al.,1973). In both humans and the SHR there is evidence that the increased reactivity is related to structural change found in small muscular arteries (Folkow et al.,1973; Trippodo et al.,1981; Dickhout et al.,1997). Since our overall hypothesis is that structural change in the small muscular arteries of SHR is responsible for increasing total peripheral resistance and thus causing elevated BP, these similarities of the animal model to the human disease are of particular importance.

In regards to SHR's response to drug treatment, ACE inhibitors are particularly effective in the reduction of BP in this animal model. Moreover, ACE inhibitor treatment is accompanied by a significant reduction in vessel wall hypertrophy in the SHR (Morishita et al.,1992; Lee et al.,1991). ACE inhibitors also show a large anti-hypertensive effect in a

significant percentage of human essential hypertensives and this has been shown to be accompanied by reduction of small muscular artery wall hypertrophy as in SHR (Schiffrin, 1996).

1.2 Objectives

The overall objective of this work is to define more closely the process of pathological change that leads to the development of hypertension in the SHR as a model of human essential hypertension. To this end, contrasting the development of the SHR with it's normotensive control, WKY, will allow us to determine some of these developmental differences. At the outset of our work we pose the following hypothesis about the development of hypertension in the SHR:

Hypotheses

1) Structural change in the small muscular arteries of the spontaneously hypertensive rat is a primary process in the development of hypertension in this animal model leading to an increase in TPR and high BP.

2) This structural change involves an increase in the smooth muscle of the medial layer in small muscular arteries and may be the result of hypertrophy or hyperplasia of the smooth muscle cells.

3) An increase in the medial smooth muscle volume causes an increased contractile response of these blood vessels to contractile agonists thereby occluding the lumen to a greater degree thus increasing TPR.

4) Apoptosis is a process that occurs in these blood vessels during their development. A

difference in the incidence of apoptosis leads to structural differences between hypertensive and normotensive vessels.

1.3 Methodology

Structural Studies of Blood Vessels

The structural study of blood vessels is complicated by the fact that they are not rigid but elastic tissues and exist in the body under both longitudinal and radial stress. Stress is a force applied to a given area of the blood vessel's wall and the application of this force results in deformation of the tissue. The ratio of the change in length of an elastic object to its original size is defined as strain. Once the stress acting on a blood vessel is removed, by removal of intra-lumen pressure or excision from the body, the elastic vessel recoils and losses both length and radius. The elastic modulus of a blood vessel is defined as the ratio of stress over strain. The arterial wall's elastic modulus is considerably less in the longitudinal direction. In canine femoral artery it was found to be five times lower in the longitudinal direction (McDonald, 1974). This means that at a given stress the blood vessel deforms to a much greater degree in the longitudinal direction. Thus removal of the *in vivo* stress on a blood vessels results in mainly shortening of the vessel. Therefore, techniques must be developed to preserve the *in vivo* structure of the blood vessels for study outside the body.

Fixation of blood vessels is one way to preserve their *in vivo* structure by increasing their elastic modulus and so reducing their deformation when the *in vivo* stress is removed. Therefore, we have developed fixation techniques using a combination of the aldehyde

fixatives formaldehyde and glutaraldehyde. These fixatives function by cross linking protein molecules and increasing the structural rigidity of the blood vessels (Dawes, 1988). This was either applied to vessels pressurized on micro-pipettes after excision (Dickhout et al.,1997) or through perfusion (Dickhout et al.,1998a) to stabilize vessel structure before excision. These procedures allowed us to make microscopic observations of the blood vessels without distorting their structure.

In conventional microscopy the depth of field is very small. Thin or ultra-thin sections must be cut due to the optical properties of light or electron microscopes. This allows only a two dimensional representation of the tissue under study. For this reason, we have chosen to employ laser scanning confocal microscopy which allows serial optical sectioning without the distortion that may be introduced by microtomy. Computer aided image reconstruction of these optical sections permitted assessment of the three dimensional structure of the blood vessels.

Functional Studies of Blood Vessels

Once we have established a structural difference between hypertensive and normotensive blood vessels, it is necessary to demonstrate that this structural difference has some repercussions in the physiological function of these arteries. Since it is difficult to study the function of arteries *in vivo* while manipulating pressure and agonist concentration, methods have been developed to study arteries *in vitro* where pressure and agonist concentrations can be reliably controlled. Three main methods have been employed. They are the wire myograph, the McGregor prefusion preparation, and the blind sac pressurized myograph.

In the wire myograph method, a blood vessel is stretched between two fine wires. One of these wires is attached to a force transducer to measure the radial tension applied to the blood vessel wall. This tension (T) is then converted to effective pressures (P) by the Laplace relationship, Equation 1.3 (Berne et al.,1988b).

$$T = P \times \text{Radius} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1.3)$$

Thus, the response of the vessel to agonist stimulation can be measured at given effective pressures. This method has been extensively applied by Mulvany's group to measure contractility of small muscular arteries from young and old SHR versus normotensive WKY with the finding of greater contractility in the SHR vessels (Warshaw et al.,1979). This technique only applies force to the vessels radially from the stretching induced by the two wires. This radial force is not uniformly distributed throughout the vessel wall since it originates from two point sources: the wires. Pressure distributes force uniformly over the surface area of the vessel wall and in the *in vivo* condition along the length of the vessel, as the vessel offers some resistance to blood flow.

The blind sac myograph preparation which uses the pressure of a physiological saline solution within the vessel lumen most accurately mimics the *in vivo* condition and significantly differs from the wire myograph technique. The two techniques applied to SHR and WKY vessels have revealed that the functional response from the same artery differs in either apparatus at the same applied pressures, actual pressures for the blind sac and calculated effective pressures for the wire myograph (Lew et al.,1992). This difference in

response between the two preparation methods demonstrates the need to apply actual pressure within the vessels lumen to obtain results that easily translate into the *in vivo* physiological function of the blood vessels.

The McGregor preparation differs from both the above technique in that it sets a flow rate for a given vascular bed in the *in vitro* preparation and measures response in perfusion pressure while manipulating the resistance to flow through stimulation by various agonists (McGregor, 1965). This technique has been successfully applied to the study of young SHR vessels (Tsuji et al.,1989). Unfortunately this technique suffers from the lower than normal perfusion pressures in relaxed arteries due to the low viscosity of the physiological saline perfusate. Furthermore, results are not localized to a given caliper of vessel but rather are aggregate measurements for the entire bed. This fact makes it difficult to compare the structural change we may find in a given vessel to the functional response of the same vessel. Given the above facts, we have chosen the blind sac myograph technique as the method to assess the physiological function of structurally modified vessels.

We have used three different contractile agonists which act through different mechanisms to assess the functional response of the blood vessels. First, we intended to use high molar concentrations of potassium chloride (KCl) which acts to stimulate contraction by changing the resting membrane potential of the smooth muscle cell. This leads to the opening of voltage dependent calcium channels in the cell membrane, the influx of calcium, and contraction (Murphy, 1988). Secondly, we intended to use norepinephrine hydrochloride which acts through α_1-adrenoceptors on the smooth muscle cell membrane. This stimulates the release of intracellular calcium through inositol trisphosphate production

and the changing of calcium fluxes by the activation of protein kinase C stimulated by the production of diacylglycerol (Flattery, 1989). Thirdly, we used electrical field stimulation to activate the release of neurotransmitters in the varicosities of the sympathetic dendrites surrounding the blood vessel wall. These neurotransmitters then act on their smooth muscle cell receptors to stimulate contraction. This selection of agonists allowed us to determine if differences in contractile response are seen with direct smooth muscle stimulation through KCl, or only seen through receptor mediated mechanisms, or are the result of different levels of stimulation imparted by the para-arterial nerves in the different strains.

Apoptosis in Blood Vessels Formation

Apoptosis, which is defined as programmed cell death as opposed to cell necrosis due to injury, plays an important role in the development of many structures within the body (Wyllie, 1997). Apoptosis has been shown to be an important process in the structural development of blood vessels during the rearrangement of the vascular system with the change from fetal to adult circulation (Cho et al.,1995). Blood vessel structural changes in the SHR occur very early in their development. At 4 weeks of age muscular arteries of SHR have been shown to have greater wall and media cross-sectional area then age matched WKY (Lee, 1985). This being the case, and with the knowledge that differences in the incidence of apoptosis during artery development would result in structural differences of the blood vessels, we have chosen to quantify the incidence of apoptosis in SHR and age matched WKY vessels at 1-2 weeks of age. We have chosen to employ two methods to measure the incidence of apoptosis, DNA laddering which has been applied to measure smooth muscle cell apoptosis in the SHR (DeBlois et al.,1997), and a newer technique DNA fragment end

labeling (Peng et al.,1997). DNA laddering is a global approach allowing the measurement of apoptosis in a group of vessels from which the DNA is extracted for analysis by gel electrophoresis. The use of end labeling allows the identification of individual apoptotic cells within the various tissue layers of the artery.

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CHAPTER 2

BLOOD PRESSURE AND HEART RATE DEVELOPMENT IN YOUNG SPONTANEOUSLY HYPERTENSIVE RATS

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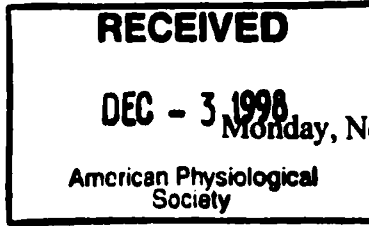

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Introduction

Essential hypertension results from the culmination of a series of pathological changes in the body that lead to a sustained elevation of blood pressure (BP). The spontaneously hypertensive rat (SHR) is a suitable model to study hypertension development because it is similar to humans with essential hypertension. These similarities include: a genetic predisposition to high BP without specific etiology, increased total peripheral resistance without volume expansion, and similar responses to drug treatment (Frohlich, 1986).

A precise knowledge of early BP development is essential to understand hypertension as a disease process. To ascribe a causal role to a defect, this defect should occur at the initiation of BP elevation. Defects that occur only after large BP elevations should be considered secondary to the disease process (Korner et al.,1991). Previous studies of young SHR BP development have yielded conflicting results. Some observers found that SHR and WKY had similar BP at or before four weeks of age (Lee, 1985; Tsuji et al.,1989; Rioux et al.,1977), whereas others found a significant difference in BP at three weeks of age (Lais et al.,1977), or at birth (Gray, 1984a; Gray, 1984b). The reason for these contradictory findings is unclear. However, in most of these studies, the sample size was small ($n \leq 10$), and distinctions between inbreeding lines within the strains were not made.

With inbred animals such as SHR and WKY, the genetic variation of the population typically found continuously between individuals becomes partitioned between inbreeding lines (Falconer, 1961a). Between-line differences within a strain can occur by chance and may be unrelated to the genetic differences responsible for hypertension in SHR. However,

these genetic differences may result in significantly different BP between given inbreeding lines of animals. Therefore, BP differences found between young SHR and WKY in some studies may be due to the effect of random genetic drift within the strains acting on the specific inbreeding lines tested. The question then is whether the early, small, but statistically significant difference in BP between the tested lines of SHR and WKY would affect the eventual outcome of hypertension. Further, it is not known whether the differences among the inbreeding lines tested hold for the population as a whole if a greater number of lines were tested. These questions were examined in this study.

Changes in heart rate (HR) during SHR development may also be important in the pathogenesis of hypertension. Prehypertensive tachycardia has been reported in SHR (Tucker et al.,1984; Rioux et al.,1977). An increased HR would suggest an early elevation in cardiac output if stroke volume remains constant, or is increased with HR. A previous study found that stroke volume was maintained constant with increasing HR in six weeks old SHR. Cardiac output increased linearly with HR from 350 beats per minute (bpm) at rest to 460 bpm under stress (Lundin et al.,1980). SHR also have shown an increased cardiac index at an early age, which later returned to normal leaving an increased mean arterial pressure and total peripheral resistance (Smith et al.,1979).

Body weight change is another aspect of SHR development that has received little attention. Differences in body weight have been found between young SHR and WKY. Young SHR were lighter than age-matched WKY (Swislocki et al.,1993; Buchanan et al.,1992). The reason for this is unclear but this trait co-segregated with hypertension in the F2 progeny of crossbred SHR and WKY (Myers, 1991). Such a difference could be a sign

of some underlying metabolic irregularity in SHR leading to hypertension.

The main purpose of this study was to describe in detail the course of physiological development in SHR as compared to its normotensive control WKY, with emphasis on how this development relates to the pathogenesis of hypertension. This was accomplished by measuring the developmental changes in systolic BP, HR, and body weight between inbreeding lines of SHR and WKY and then comparing these parameters to the severity of hypertension in the adult. Differences in developmental parameters between inbreeding lines that resulted in differences in disease outcome were considered significant in hypertension development. The underlying cause of the prehypertensive tachycardia found in SHR was also explored by analyzing the baroreflex, the level of autonomic drive influencing HR, and the measurement of plasma levels of epinephrine and norepinephrine.

Methods

Developmental study. Male SHR and WKY at two, three, four and six weeks of age were used for BP, HR, and body weight measurements. The number of inbreeding lines and total number of individuals for the respective stains are indicated in Table 2.1. The animals were obtained from colonies maintained at McMaster University's Animal Quarter. These colonies were originally derived from the Charles River strains, and we have maintained them in our institute by continuous full sibling inbreeding for more than 10 years (over 20 generations). The rats were weaned at three weeks of age and were housed with their litter mates, two to four animals per cage, where food and water were available *ad libitum*. The care of these animals was in accordance with the guidelines of the Canadian Council on

Animal Care.

Rats were weighed, and their systolic BP measured using the indirect method of tail-cuff occlusion in conscious animals. HR were calculated from the physiological tracings obtained during BP measurements. Origins of the rats were noted as individual, inbreeding line and strain, so that results could be analyzed to see if strain or line effects were significant for the parameters measured.

Some inbreeding lines showing aberrant developmental characteristics for their strain at four weeks of age were followed into adulthood to see if these differences affected BP. SHR lines that showed significant differences in systolic BP at four weeks of age were maintained until hypertension developed to find the effect of early BP differences on disease outcome. Similarly, WKY lines that showed significant elevation in HR were maintained to find the effect on BP outcome.

Acute study in 4-week-old rats. In addition to the indirect measurement of systolic BP and HR, direct measurements of systolic, diastolic, mean arterial BP, and HR were made at four weeks of age as follows. The ages of SHR and WKY used for these experiments varied from 27 to 31 days, and were chosen in a way that resulted in equal means between the groups. Initial experiments were carried out to standardize the anaesthesia protocol, in order to avoid cardiovascular depressant effects of anaesthesia. These experiments consisted of comparing results of systolic BP measurements using the indirect method in conscious rats to the direct method in rats under anaesthesia. A combined anaesthesia consisting of ketamine as a pre-anaesthetic and urethane as an inducer was used. Ketamine was chosen as a pre-anaesthetic since it produces deep sedation and thus reduces the dose of the general

anaesthetic required (Flecknell, 1987). Urethane was chosen as an inducer since previous studies have shown that it had little cardiovascular depressive effect at low doses (De Wildt et al.,1983; Maggi et al.,1986). The dosages which did not affect the systolic BP of the rats were found to be 75 mg/kg ketamine (IP) with 0.25 g/kg urethane (SC) for the WKY (n = 9), and 50 mg/kg ketamine (IP) with 0.50 g/kg urethane (SC) for the SHR (n = 10). SHR and WKY were therefore anaesthetized using these different doses for femoral artery cannulation to measure HR, systolic, diastolic, and mean arterial BP directly. The results were recorded on a Digi-Med BP analyzer (Micro-Med, Louisville, KY.) attached to a microcomputer for continuous digital data storage allowing the sampling of mean BP and HR at 3 kHz. The values were averaged over five second intervals to record the incremental changes.

Investigation of tachycardia. Prehypertensive tachycardia found in SHR was investigated pharmacologically to detect if it was due to excessive sympathetic excitation, a lack of parasympathetic tone, an exaggerated baroreflex, or other hormonal factors influencing the intrinsic HR. SHR and WKY were anaesthetized with ketamine/urethane. The left femoral artery was cannulated and recordings were made on the Digi-Med BP analyzer. HR was determined during sympathetic blockade with the cardiac selective β_1-receptor antagonist atenolol (0.5 mg/kg), parasympathetic blockade with the muscarinic receptor antagonist atropine sulfate (0.4 mg/kg), and complete autonomic blockade by combining atenolol (0.5 mg/kg) and atropine sulfate (0.4 mg/kg). The baroreflex response was measured by recording HR responses to sudden changes in BP stimulated by intravenous infusion of increasing doses of sodium nitroprusside (6.2, 12.5, 25 μg/kg), or phenylephrine

hydrochloride (1.5, 3.1, 6.2, 12.5, 25 μg/kg). The values of BP reached and changes in HR produced were averaged for each of the given doses in each of the strains.

To investigate further the sympathetic influence on HR, blood samples were collected from 4-week-old rats for the measurement of catecholamine content. Samples were collected in sodium citrate and centrifuged to obtain plasma for analysis by high-performance liquid chromatography (HPLC). EDTA was added as an antioxidant and alumina/acetic acid extraction performed on samples before loading on an HPLC column (Lee et al., 1991a). The HPLC was equipped with an electrochemical detector and peak integration preformed semiautomatically on a microcomputer.

Statistical analysis. Values given are means ± SEM. Data analysis was performed with a computer-based statistical package, SAS® (SAS® Institute Inc.). Mixed model analysis of variance (ANOVA), with fixed effects as strain type (SHR versus WKY), and random effects as inbreeding lines nested inside strain type was used to test the hypotheses of unequal means for the developmental parameters measured at two, three, four, and six weeks of age for strains or inbreeding lines. Line effects were treated as random effects since the division of additive genetic variance into inbreeding lines could produce an infinite number of such inbreeding lines and so the inbreeding lines actually tested comprised only a sample of the population of such lines. Animals within inbreeding lines were treated as independent observations regardless of litter of origin, so differences among these animals comprise the random error when line differences were tested in the F-statistic. Means were compared with Fisher's protected least significant differences (LSD) through multiple comparison tests.

An ANCOVA was performed in SHR and WKY with systolic BP at six or 10 weeks as

the dependent measure, inbreeding line as the independent measure, and HR at three or four weeks as the covariant to test the hypothesis that differences existed between inbreeding line mean systolic BP, and that HR was a predictor of this phenomenon. Means were compared with Fisher's protected LSD multiple comparison test.

All other comparisons were performed by unpaired t-test for significant differences between the strains. Significance was recognized at $P < 0.05$.

Results

Developmental study. Body weight of SHR was less than WKY at two and three weeks of age. At four weeks, body weight was similar between SHR and WKY, and at six weeks SHR had become heavier than WKY (Table 2.1). However, differences between inbreeding lines within the strains contained most of variation in this parameter over all age groups (Table 2.2). This indicates that there was a greater difference within the strains for body weight than between strains. None of the body weight difference between the strains reached statistical significance, when between-line differences were taken into account (Table 2.2).

Indirect BP measurements. Systolic BP was similar between SHR and WKY at two and three weeks of age. Differences were small at four weeks, however, they were statistically significant ($P = 0.02$) and by six weeks systolic BP in SHR had become significantly elevated as compared with WKY ($P = 0.005$) (Table 2.1). At two weeks, most of the variation in systolic BP was found between individuals. At three and four weeks, differences between inbreeding lines accounted for most of the variation in systolic BP, with differences between strains smaller than those between the lines. However, by six weeks differences

between strains overshadowed the differences between inbreeding lines (Table 2.2).

Heart rate was significantly higher in SHR as compared with WKY at two ($P = 0.02$), three ($P = 0.0001$) and four weeks ($P = 0.0001$) of age but this difference was absent by the sixth week (Table 2.1). At two weeks most of the variation in HR was between individuals. At three weeks strain differences dominated and by four weeks differences between the strains and inbreeding lines were both significant. By six weeks of age individual differences again accounted for most of the variation in HR (Table 2.2).

A small increase in HR was noted in the WKY at four weeks over the three week level (Table 2.1). Fisher's protected LSD test showed that the WKY population consisted of two groups that accounted for the significant differences found between inbreeding lines. One group consisting of three lines showed HR approaching the SHR level and produced the slight elevation in HR seen in the WKY strain at four weeks. The other group consisted of seven lines and showed no elevation in HR over the three week level.

The overall pattern of systolic BP and HR development is shown in Figure 2.1. The period of prehypertensive tachycardia for SHR was found between two and four weeks. The subsidence of tachycardia and an increase in systolic BP of the SHR as compared with WKY became apparent in rats older than four weeks.

Direct BP measurements. Direct measurements of hemodynamic parameters were carried out in four-week-old rats. In this instance, systolic BP was not significantly different between the strains with the mean value of 127 ± 6 mmHg in SHR ($n = 5$) versus 117 ± 6 mmHg for WKY ($n = 9$). Similarly diastolic BP was not significantly different with the mean value of 84 ± 14 mmHg in the SHR ($n = 5$) versus 86 ± 12 mmHg for WKY ($n = 7$).

However, pulse pressure was significantly greater in SHR (43 ± 4 mmHg, n = 5) than WKY (28 ± 3 mmHg, n = 7, P = 0.02). Heart rate was also significantly higher in SHR (431 ± 31 bpm, n = 5) than WKY (389 ± 17 bpm, n = 7, P = 0.03).

Effect of between-line differences on the outcome of hypertension. At four weeks of age statistically significant differences existed for inbreeding lines from SHR for systolic BP, and WKY for HR (Table 2.2). These line differences were isolated by Fisher's LSD test allowing the testing of these line differences on hypertension outcome. SHR from inbreeding lines 7 and 12 differed significantly in systolic BP (P = 0.006). Line 7 showed lower-than-average systolic BP (103 ± 1, n = 3) versus the average systolic BP found for the entire population represented by line 12 (127 ± 4 mmHg, n = 4). Data collected from the same animals at eight weeks of age revealed no difference in BP although both lines displayed BP that were clearly hypertensive. Average systolic BP in line 7 was 172 ± 4 mmHg (n = 3), and BP in line 12 was 170 ± 6 mmHg (n = 4).

WKY lines 1, 3, and 7 showed a significant elevation of HR (combined mean 429 ± 10 bpm, n = 10, P = 0.001) over the combined mean for the remainder of the WKY lines (394 ± 5 bpm, n = 24). These tachycardiac lines also showed significant BP elevations at 10 weeks (145 ± 7 mmHg) as compared to age matched WKY rats with no history of HR elevation (127 ± 2 mmHg, P = 0.01). This elevated BP remained at 20 weeks of age (144 ± 5 mmHg, P = 0.005).

ANCOVA was performed within the SHR and WKY strains with systolic BP as a dependent measure, inbreeding line as the independent measure, and HR as a covariant to assess the value of early elevated HR as a predictor of elevated BP. When HR at three

weeks was used as a covariant for systolic BP at six weeks in SHR, no significant differences between the systolic BP for inbreeding lines of SHR were found. However, when HR at four weeks was used as a covariant for systolic BP in WKY at 10 weeks, significant line differences were found. The regression analysis showed that line differences alone accounted for 38% of the variation in the dependent measure (systolic BP). The addition of the covariant (four week HR) increased the variation accounted for in the dependent to 79%. Therefore the covariant HR accounted for 41% of the variation in systolic BP.

To test in a mixed population of hypertensives and normotensive if HR elevation at three weeks is a predictor of hypertension, correlation analysis was performed between HR at three weeks and systolic BP at six weeks combining the individuals of both strains. A positive correlation was found ($P = 0.037$, $n = 26$). The correlation was linear with even distribution of residuals around the predicted line ($r = 0.41$).

Investigation of prehypertensive tachycardia. Baroreflex bradycardia caused by phenylephrine-induced BP increase was similar between the two strains (Figure 2.2). However, baroreflex tachycardia caused by sodium nitroprusside-induced BP decrease was significantly less in SHR than WKY (Figure 2.2). Complete autonomic blockade by atropine sulfate and atenolol resulted in a significantly higher mean intrinsic HR in SHR (411 bpm) than WKY (365 bpm, Figure 2.3). We used the difference between the basal HR (Table 2.1) and the intrinsic HR (Figure 2.3) as the net autonomic tone in these animals. The sympathetic tone as tested by atenolol infusion was higher in the SHR than WKY when expressed as either an absolute number (SHR = 95 and WKY = 74 bpm), or as a percentage of intrinsic rate (SHR = 23% and WKY = 20%). However, this difference did not reach the

level of statistical significance. The sympathetic component of the autonomic tone in both strains at this age increased the intrinsic HR an average of 16 bpm in WKY and 31 bpm in SHR. After removal of all autonomic tone, SHR still showed a significantly higher intrinsic HR than WKY (Figure 2.3). The parasympathetic component of the autonomic tone was tested by atropine sulfate infusion so the increase in HR over the basal rate showed the magnitude of the parasympathetic tone which is expressed as a negative value. The parasympathetic tone expressed as either an absolute number (SHR = −64 and WKY = −62 bpm), or as a percentage of the intrinsic rate (SHR = −16% and WKY = −17%) showed little difference between the strains.

Analysis of plasma catecholamines from four-week-old rats showed the levels of epinephrine (0.73 ± 0.3 in SHR and 1.4 ± 0.3 ng/ml in WKY, $n = 7$) and norepinephrine (1.9 ± 0.2 in SHR and 1.6 ± 0.2 ng/ml in WKY, $n = 7$) did not significantly differ between the strains.

Discussion

The major findings of this study are as follows. A significantly higher HR was present in SHR as compared with WKY before any difference in BP could be detected. BP began to diverge between the strains at four weeks of age. A small but statistically significant difference in BP between inbreeding lines of SHR (24 mmHg at four weeks of age) had no effect on the eventual outcome of hypertension (both group attaining approximately 170 mmHg by eight weeks). However, WKY inbreeding lines which exhibited a significant elevation in HR at four weeks developed a significantly higher BP than those without

previous HR elevation. HR at four weeks when used as a covariant accounted for 41% of the variation in systolic BP of WKY at 10 weeks. An overall positive correlation between HR at three weeks of age and the level of BP at six weeks was also found indicating the predicative value of elevated HR for BP development in the animal population studied.

Earlier studies which described a significant difference in BP between WKY and SHR at three weeks of age (Lais et al.,1977), or at birth (Gray, 1984a; Gray, 1984b) were based on the measurement of a few animals. Lais et. al. (1977) studied six litter mates consisting of both males and females at three weeks of age. The animals from the SHR strain had undergone 22 to 26 generation of full sibling inbreeding (Lais et al.,1977). At this level of inbreeding, the probability that any genetic locus was fixed is greater than 95% (Falconer, 1961b). The genetic variance (V_g) usually found continuously between individuals becomes partitioned between inbreeding lines and the genetic variance of the whole population had approximately doubled, according to the following calculation:

Equation 2.1: Partitioning of genetic variance due to additive genes in inbreeding population

Genetic variance between inbreeding lines	$= 2 \times F \times V_g$	$= 1.972V_g$
Genetic variance between individuals	$= (1-F) \times V_g$	$= 0.014V_g$
Total population genetic variance	$= (1+F) \times V_g$	$= 1.986V_g$

where F = the coefficient of inbreeding; V_g = a symbol representing the variance in the base population before inbreeding. F = 0.986 after 20 generations of full sibling inbreeding (Falconer, 1961c).

In this situation, genetic differences between inbreeding lines within the same strain are exacerbated. However, whether these differences affect the eventual outcome of hypertension is unknown. To test for between strains differences in developmental parameters such as BP, several lines of the same strain must be sampled to estimate the variance within the whole population. A conservative estimate of the number of degrees of freedom in a model would be the number of inbreeding lines tested, not the number of individuals (Cohen et al.,1988). Since this was not done by Lais et. al. for three-week-old rats (Lais et al.,1977), the differences found may simply represent significant differences between the inbreeding lines tested, not differences between the hypertensive and normotensive populations as a whole. Further, in this study, we found the major component of variation in systolic BP was between inbreeding lines at this age and not between the SHR and WKY strains. Differences in BP put forth by Gray at birth fall into a similar category since the number of inbreeding lines in each strain tested were not stated (Gray, 1984b; Gray, 1984a).

The parameters we have measured (BP, HR, body weight, and pulse pressure) are not merely the product of genetic variance between strains, lines, and individuals. These parameters are also affected by environmental variance occurring in the colony and contain some random error components due to inaccuracies of measurement. Since the majority of genetic loci in the tested lines should have been fixed after more than 20 generation of full sibling inbreeding (Falconer, 1961b), the parameters measured with high individual variance represent characteristics not strongly genetically determined. At three and four weeks of age the percentage of individual variation is lowest for HR, followed by body weight and systolic

BP.

Systolic BP is the least genetically determinate parameter at two, three and four weeks of age. However, by six weeks, it becomes the parameter that is most genetically determined (Table 2.2). The fact that significant differences in systolic BP between SHR inbreeding lines (25 mmHg at four weeks), had no effect on the eventual severity of hypertension in these animals confirms the lack of genetic determinance of this characteristic at an early age and suggests that the hypertension disease process occurring in SHR has not yet become fully manifested. This assertion is supported by the finding of structural changes in SHR resistance vessels at this age which may lead to an increased total peripheral resistance but are accompanied by a delayed functional maturity of the arteries (Dickhout et al.,1997).

Body weight was moderately dependent upon changes in environmental factors occurring in the colony at different times. This dependance decreased with age. Body weight was less in SHR than WKY at two and three weeks of age, became similar at four weeks, and exceeded WKY at six weeks. Therefore the rate of body growth was significantly greater in SHR than WKY over this period. This may represent an important trend in the development of hypertension in SHR. The age of maximum weight gain in SHR and WKY rats has been shown to coincide with the period of maximum BP rise in these rats (Schork et al.,1994). This seems to indicate that the surge in BP that occurs in hypertensive and normotensive rats over this time was in response to weight gain (Schork et al.,1994). Therefore the greater rate of weight gain in SHR may be responsible for the greater surge seen in systolic BP as compared with WKY.

The most genetically determined parameter in the SHR and WKY at three and four

weeks of age was HR. This aspect was reflected by the difference between the strains with SHR having elevated HR, and among the different lines within WKY. Once hypertension had become established in SHR, HR had returned to WKY levels. Significant HR differences among WKY inbreeding lines were also significantly related to systolic BP outcome. Inbreeding lines of WKY with higher HR displayed significantly elevated systolic BP at 10 and 20 weeks of age. Since elevated HR in SHR occurred before BP elevation, elevated HR may be considered a primary process in the development of hypertension in SHR by the criteria put forth by Korner and Swales (Korner et al.,1991). Other studies have also noted elevated HR in SHR prior to BP elevation, and when hypertension had become established, SHR HR had returned to WKY levels (Lee et al.,1983). Thyroidectomy at four weeks which decreased HR in SHR to WKY levels, also reduced the BP increase in SHR to the borderline hypertensive range (150 mmHg) (Rioux et al.,1977). These results point to HR as an important process in the development of hypertension in the SHR. In this case, either the HR itself, or other hormonal changes that it represents, may cause the changes that eventually produce the sustained elevation of BP found in SHR.

The mechanism behind elevated HR affecting hypertension development is unknown. The increased HR and/or increased pulse pressure we found in four-week-old SHR may act as a direct mechanical stimulus to vascular growth, which could result in the increased volume of medial smooth muscle found in the resistance blood vessels of young SHR (Dickhout et al.,1997). In the pial arterioles from Sprague-Dawley rats, vascular hypertrophy was produced by an elevation of pulse pressure alone when mean arterial BP was not significantly different between sham and clipped rats (Baumbach, 1996). Other

studies have also found that in the SHR, a reduction in the pulse pressure and HR during antihypertensive therapy may be important in preventing the development of abnormal small artery structure in hypertension (Christensen, 1991).

Increased HR may also lead to increased cardiac output as it did in stressed SHR (Lundin et al.,1980). An increased cardiac index in the early stages of hypertension development (i.e. 34 to 41 days of age) has been noted in the SHR (Smith et al.,1979), and SHR displayed a rightward shift in pressure-flow curves as compared to WKY at 6 and 9 weeks of age. This suggests that some structurally based long-term autoregulatory mechanism was present in the vasculature of SHR to normalize flow with increased pressure (Alson et al.,1985). In the whole animal, however, reducing HR with the non-selective β-blocker nadolol by dosing from birth to 28 weeks did not prevent hypertension, nor did it reduce cardiac or resistance vessel hypertrophy (Lee et al.,1992). Therefore, it seems unlikely that tachycardia alone could bring about hypertension since the correction of this abnormality did not correct the hypertension. Therefore this defect did not satisfy all the postulates for causality put forth by Korner and Swales (Korner et al.,1991). Other factors, such as changes in the vascular growth factors or metabolic changes may be involved.

The surge in HR found in young SHR could represent a surge in sympathetic output since our previous study using sympathectomy and adrenalectomy were found to abolish hypertension in the SHR (Lee et al.,1991b). However, the use of pharmacological blockade in our current study indicates that prehypertensive tachycardia in the SHR is mostly due to an increase in intrinsic HR. Furthermore, plasma levels of norepinephrine and epinephrine were not elevated in these rats at four weeks. This points to other factors such as thyroxine

which may be responsible for the prehypertensive tachycardia found in SHR.

Our findings that tachycardia in prehypertensive rats is a good predictor of eventual BP outcome, is quite similar to the situation in humans. In humans, several studies have shown that faster resting HR is associated with higher BP levels, and that this association is observed across the whole range of BP in the general population and is present at any age (Palatini et al.,1997). An increased HR is typical for young patients with borderline hypertension, and a faster HR has also been found in the offsprings of hypertensive families, suggesting that the increase in HR in hypertension is a marker of an underlying disorder that affects the BP and HR in a parallel fashion (Palatini et al.,1997). More importantly, several epidemiologic studies have confirmed the predictive role of the HR in the development of hypertension (Palatini et al.,1997). The mechanism associated with the increase in HR in humans is not known. Sympathetic over activity in some of these patients (about 25% of subjects with borderline hypertension), based on the presence of elevated norepinephrine levels, may be one of the contributing factors (Palatini et al.,1997). Our results on the prehypertensive SHR suggest that intrinsic HR increase is a major cause of tachycardia in these animals.

In this study, we found that young SHR at four weeks of age showed a depressed baroreflex response to BP reduction. This is similar to previous studies on young adult SHR (8 weeks old) (Huang et al.,1994). However, these results differed from those in six-week-old SHR, where SHR were found to exhibit an increased baroreflex slope in the mean arterial pressure-HR plot as compared with WKY, but the maximum and minimum HR were similar between the strains (Head et al.,1992). The reduced gain in HR we have observed in the

four-week-old SHR in response to pressure drop may be a result of the preexisting elevated HR. This may limit the amount of sympathetic drive that can further increase HR since increasing HR beyond certain levels would result in a decrease in stroke volume and negatively impact on cardiac output.

In conclusion, we have found that HR elevation precedes BP elevation in SHR, and prehypertensive tachycardia may be an important first step during hypertension development in the SHR.

Acknowledgement

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Figure 2.1. Systolic blood pressure and heart rate profiles of SHR (●) and WKY (○) during early development. The number of inbreeding lines and total number of individuals for SHR and WKY at the different age groups are listed in Table 2.1.

Figure 2.1

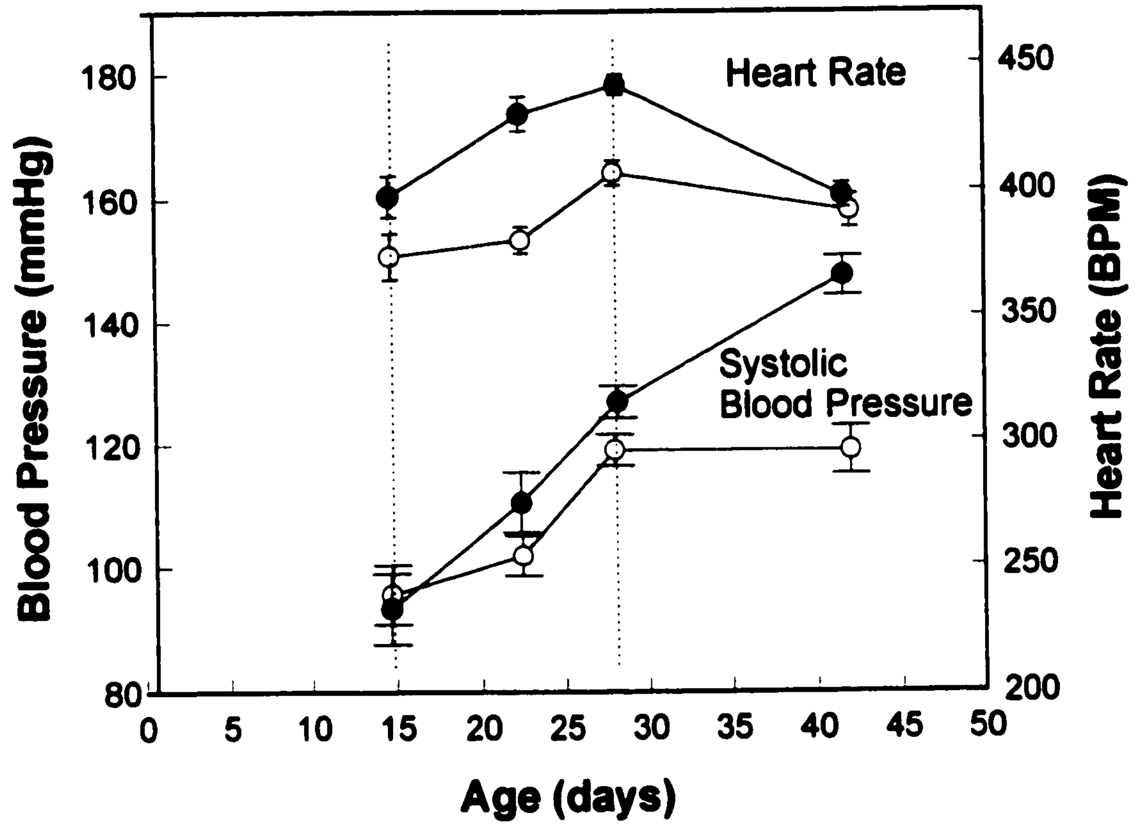

Figure 2.2. Baroreflex responses of anaesthetized four-week-old SHR (●) ($n = 5$) and WKY (○) ($n = 7$) when challenged with sodium nitroprusside or phenylephrine hydrochloride.

Figure 2.2

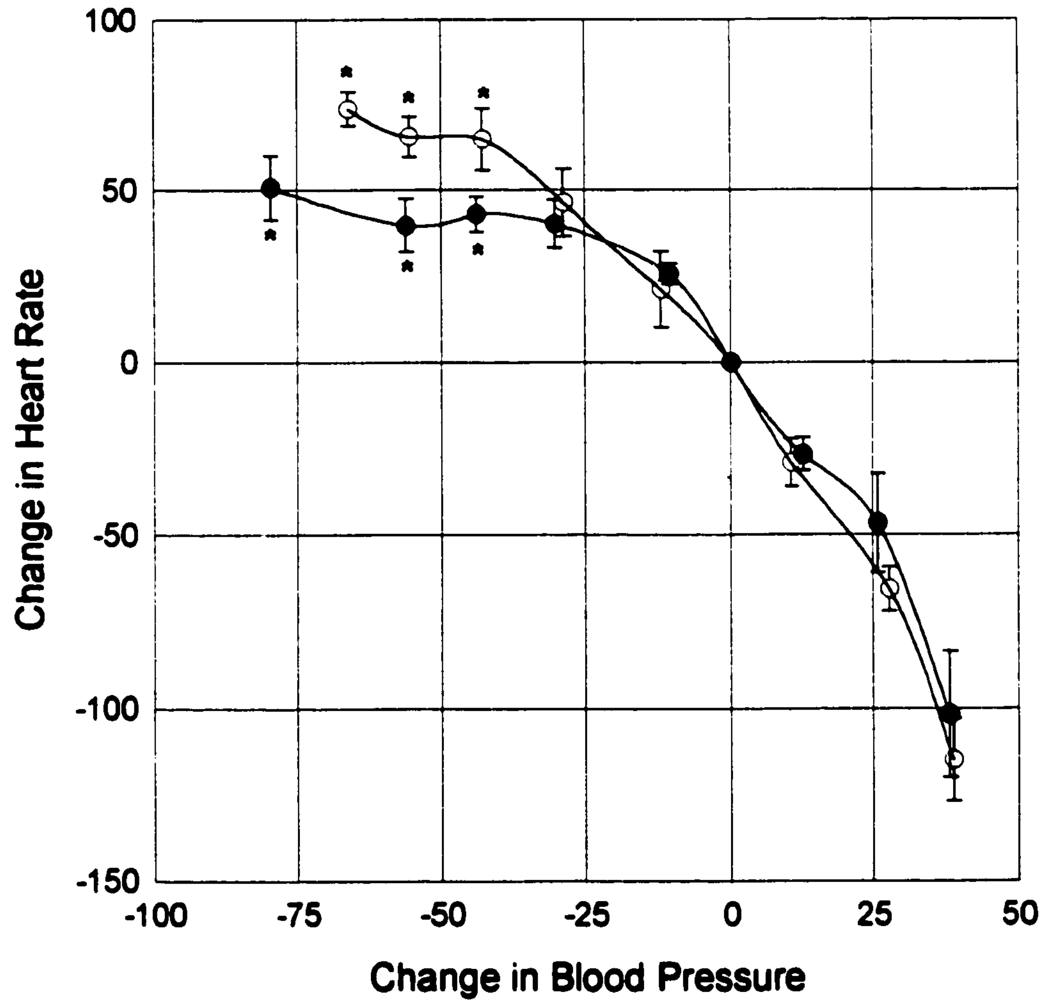

Figure 2.3. Intrinsic heart rate (IHR), sympathetic (IHR+S) and parasympathetic (IHR+P) components of basal heart rate in four-week-old SHR (●) (n = 7) and WKY (○) (n = 5).

Figure 2.3

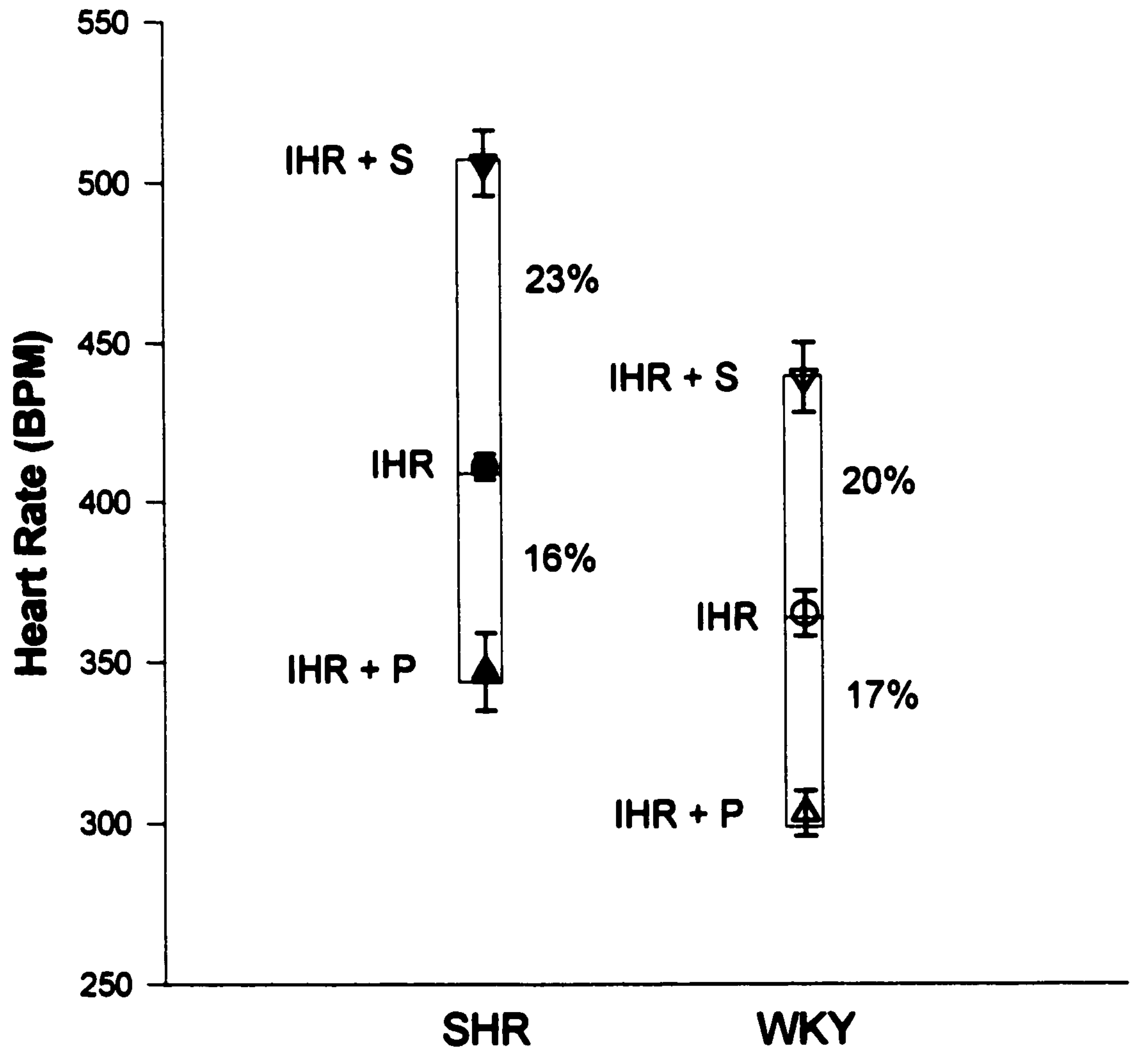

Table 2.1. Developmental parameters for SHR and WKY at 2 ,3, 4, and 6 weeks of age

	2 weeks	3 weeks	4 weeks	6 weeks
Body weight				
SHR	22 ± 1 (5,15)	39 ± 2 (10,25)	63 ± 2 (20,40)	133 ± 4 (7,20)
WKY	26 ± 1 (5,15)	44 ± 1 (10,25)	63 ± 2 (20,40)	120 ± 5 (7,20)
Systolic BP				
SHR	93 ± 6 (5,15)	110 ± 5 (10,25)	127 ± 3* (20,40)	148 ± 3* (7,20)
WKY	96 ± 5 (5,15)	103 ± 3 (10,25)	118 ± 3 (20,40)	120 ± 5 (7,20)
Heart Rate				
SHR	397 ± 8* (5,15)	430 ± 7* (10,25)	441 ± 4* (20,40)	398 ± 5 (7,20)
WKY	373 ± 9 (5,15)	380 ± 5 (10,25)	407 ± 5 (20,40)	392 ± 7 (7,20)

Values are mean ± SEM. The number of inbreeding lines and total number of individuals are given in parentheses for the respective strains (inbreeding lines, individuals). * significantly different from age-matched WKY ($P < 0.05$)

Table 2.2. *Analysis of developmental parameters for SHR and WKY at 2,3,4, and 6 weeks of age*

		2 weeks	3 weeks	4 weeks	6 weeks
Body weight	Strain	21%	10%	0%	9%
	Line	41%*	69%*	70%*	76%*
	Individual	38%	21%	30%	15%
Systolic BP	Strain	0.4%	4%	6%*	48%*
	Line	22%	62%*	63%*	36%*
	Individual	78%	33%	31%	16%
Heart Rate	Strain	11%*	50%*	35%*	9%
	Line	10%	34%*	39%*	35%
	Individual	79%	16%	26%	46%

Values are the percentage of variation in the measured parameter found between different animal groups. Strain = SHR versus WKY; Line = variation among inbreeding lines of SHR and WKY; Individual = individual variation within inbreeding lines. The number of inbreeding lines and total number of individuals are the same as those in Table 2.1 at each of the age groups. * significantly differences were present ($P < 0.05$)

CHAPTER 3

STRUCTURAL AND FUNCTIONAL ANALYSIS OF SMALL ARTERIES FROM YOUNG SPONTANEOUSLY HYPERTENSIVE RAT

JEFFREY G. DICKHOUT and ROBERT M.K.W. LEE

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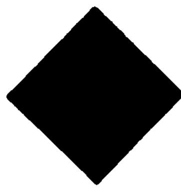

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Introduction

Structural and functional modifications of the resistance vasculature may be important causative factors in the development of essential hypertension. In the spontaneously hypertensive rat (SHR), a sustained elevation of blood pressure (BP) as compared to the normotensive Wistar-Kyoto (WKY) control rat was found at 11, and 17 weeks of age accompanied by an increase in total peripheral resistance, but there was no increase in cardiac output (Smith et al.,1979). Since BP is the product of total peripheral resistance and cardiac output, increased total peripheral resistance may be responsible for the sustained elevation of BP observed in SHR at these ages.

Thickening of the walls of the resistance blood vessels has been associated with an increase in total peripheral resistance in several animal models of human essential hypertension including Dahl salt-sensitive hypertensive rats, DOC-NaCl rats, and SHR (Lee, 1987), as well as in humans with essential hypertension (Aalkjaer et al.,1987). The finding of this type of structural change in the resistance vasculature is frequently concurrent with an elevation of BP. This was the case in the small muscular arteries of SHR at 12, and 28 weeks of age where Lee et al. (Lee et al.,1983) found an increase in the medial and vessel wall cross-sectional area while the BP was approximately 50 mmHg higher in SHR than WKY. The concurrence of BP elevation and medial thickening makes it impossible to assess whether the elevation of BP is the cause, or the consequence of vessel wall hypertrophy.

It is possible that an increased intramural pressure in the hypertensive animal may cause an adaptive response in the blood vessels resulting in wall thickening. Studies on

the hypertrophic response of smooth muscle cells to increased intramural pressure in urinary bladder, small intestine, and ureter have found that hypertrophy of the smooth muscle layer was produced by increased intramural pressure (Gabella, 1990). Similarly, thickening of the vessel wall due to smooth muscle hypertrophy was found in rat portal vein (Malmqvist et al.,1988) and aorta (Olivetti et al.,1980) after coarctation. Some authors have suggested that increased intramural pressure resulting from elevated cardiac output due to volume expansion in young SHR may lead to vessel wall hypertrophy found in these animals (Evenwel et al.,1983). The effect of wall hypertrophy increasing total peripheral resistance could then cause the sustained elevation of BP seen in SHR. If this were the case, BP in SHR would be expected to be elevated due to increased cardiac output before any thickening of the vessel wall had occurred.

At 4 weeks of age, SHR and WKY rats display little difference in BP (Lee, 1985; Tsuji et al.,1989; Rioux et al.,1977). It is also at this age that the BP begins to diverge between these two strains, so that a comparison of artery structure between SHR and WKY at this age should indicate if some components of medial thickening in SHR is pressure independent. Differences in the structure of small muscular arteries from SHR and WKY rats ranging from 3 to 5 weeks of age have been found. In maximally relaxed arteries fixed by perfusion at a perfusion pressure of approximately 20 mmHg, a significant increase in the cross-sectional area of the medial layer, increased media to lumen ratio, and an increase in the number of smooth muscle layers in SHR over WKY rats were found (Lee, 1985). However, the lumenal area of the arteries from SHR and WKY rats were the same (Lee, 1985). This seems to indicate that an increase in the volume of the small

muscular artery wall has already occurred in SHR but does not yet encroach upon the lumen of the arteries. However, the lower than physiological pressure of perfusion used during fixation in these experiments calls into question whether the lumen measurements recorded accurately reflect the lumen diameter of the arteries in the living animal.

Studies on the functional responses of small muscular arteries from 4-6 week old SHR and WKY animals have been performed but have focussed on the response of perfusion pressure in isolated mesenteric vascular beds to various agonists (Tsuji et al.,1989; Kong et al.,1991). The exact anatomical site of response, and relationship to arterial structure remain obscure. In four-week-old animals, no difference in the response of perfusion pressure to norepinephrine (NE) was found between SHR and WKY (Tsuji et al.,1989). In 5-6-week-old SHR and WKY, perfusion pressures were found to increase to higher maximum levels in SHR in response to NE, KCl, serotonin, and vasopressin, with a reduced ED_{50} to NE in SHR in the presence of reuptake inhibition by cocaine (Kong et al.,1991).

The purpose of this study, was to assess the relationship between the development of hypertension and changes in the structure and function of small muscular arteries from young SHR. The advent of the confocal microscope makes it possible to produce multiple optical sections separated by a precisely known distance without the distortion generally associated with light microscopy. We have therefore developed a morphometric protocol using the new staining techniques described in this article in order to measure directly the medial volume per unit length of artery fixed at a physiological pressure. Moreover, we also quantified the lumenal area of vessel per unit length at midsection and number of

smooth muscle layers. With this information, we assessed vessel hypertrophy and its relationship to lumen diameter and to BP development. We related the structural parameters to functional responses of the arteries on a pressure myograph apparatus to KCl, NE, and electrical field stimulation.

Methods

Young male SHR and WKY rats were used for structural and functional analyses. The animals were obtained from colonies maintained at McMaster University's Animal Quarter. The SHR colony was started with rats from Ayerst Laboratory (Montreal, Quebec, Canada) in 1976, and the WKY colony was started with rats from Canadian Breeding Farms (Montreal, Quebec, Canada) in 1983. Both colonies were derived from the strain from Charles River Laboratories (Wilmington, Mass), and we have maintained these colonies in our institute by continuous inbreeding. The care of these animals was in accordance with the guidelines of the Canadian Council on Animal Care. The rats were chosen in such a way as to result in equal mean ages between the groups. Origin of the rats were noted in regards to individual, litter of origin, and strain of origin so that results could be analyzed to see if strain of origin, or litter of origin had any significant effect on the parameters measured.

The rats were weighed, and their systolic BP measured using a tail-cuff occlusion method in conscious animals (model PE300, Narco Bio-Systems, Houston, Texas). For each rat, BP was measured several times in order to obtain a consistent result in these

animals. Arteries were sampled from the third most distal first order branches of the superior mesenteric artery (Figure 3.1). These arteries have been referred to previously as large mesenteric arteries (Lee et al.,1983; Lee, 1985; Lee et al.,1988).

Preparation of the Vessels for Morphometry

In rats anaesthetized with sodium pentobarbitol (65 mg/kg i.p.), mesenteric vessels were cleared of blood by perfusion as follows. An infusion cannula was placed in the abdominal aorta, distal to the origin of the superior mesenteric artery. The aorta was clamped just below the diaphragm, proximal to the origin of the superior mesenteric artery. An exit for the perfusate was cut into the portal vein. This allowed the perfusate, oxygenated Hank's basic salt solution (BSS) containing 1μmol/L sodium nitroprusside, to clear the vasculature in the abdominal viscera. The arteries were perfused at a flow rate of 1 mL/min per 100 g body weight for 5 minutes, resulting in maximal relaxation of the arteries.

During sampling of the arteries, we tried to maintain the *in vivo* length of the arteries in the following way. Arteries to be sampled were tied using 10-0 suture ties at either end. A suture was placed along the artery between these two ties to mark the original length of the artery. The arteries were removed by cutting between the ties at either end (Figure 3.2). They were placed in a petri dish containing oxygenated Hank's BSS and 1μmol/L sodium nitroprusside. The surrounding fat tissue on the arteries was removed by fine dissection under a dissecting microscope. The arteries were then transferred to an organ bath containing oxygenated Hank's BSS. The suture tied at one end of the artery was loosened to slip the artery on a micropipette where it was resecured, and the artery was pressurized. It was found that a pressure of approximately 70 mmHg was required to expand the arteries

to such an extent as to make the filament connecting the two ties regain its original length. These pressurized arteries were fixed and prepared for morphometric measurements using confocal microscopy.

Confocal Microscopy

It has been shown that a combination of the fluorescent DNA specific dyes ethidium bromide, which is membrane impermeant, and Hoechst 33342, which is membrane permeant, were useful in elucidating artery structure and cell vitality in florescence microscopy (Daly et al.,1992). However, the limited range of laser excitation available (wavelength=488, 512, or 543 nm) in most commercial confocal microscopes, including our own in this facility, precludes the use of the Hoechst dye (max_{ex}=346 nm). We have therefore carried out experiments with different concentrations of ethidium bromide (max_{ex}=510 nm) in order to find a suitable condition to produce a membrane permeant molecule for the elucidation of artery structure.

It was observed that trace amounts of sodium borhydride, when left in the organ bath after washing, were capable of reducing ethidium bromide. Reduction of the ethidium dye produced a membrane permeant molecule allowing the elucidation of artery structure so that the intimal, medial, and adventitial layers of the arteries could be distinguished (Figure 3.3a and 3.3b). This was the necessary prerequisite for medial volume quantification under the confocal microscope. The nuclei of smooth muscle cells in the artery wall were also clearly visible which aided in the counting of the number of smooth muscle layers in either group (Figure 3.3c).

The fixative generally used in our laboratory contains 2.5% glutaraldehyde (Lee et al.,1983), which produces a large amount of non-specific fluorescence in tissue. We have found that a fixative containing 3.5% formaldehyde and 0.75% glutaraldehyde in 0.05 mol/L phosphate buffer, allowed preservation of artery structure with little non-specific fluorescence. We therefore used the following method to prepare arteries for confocal microscopy. Arteries were fixed for 1 hour with a fixative containing 3.5% formaldehyde, 0.75% glutaraldehyde, in 0.05 mol/L phosphate buffer, at pH 7.4. The fixative was washed from the organ bath by repeated changes with Hank's BSS (pH 7.4). Free aldehyde groups remaining after fixation were reduced by the addition of 1 mg/mL of sodium borhydride to the bath for 5 minutes. The tissue was washed with Hank's BSS with 0.1% Triton-X at a pH of 8.0. Reduced ethidium bromide (75μg/mL) was then added to the bath. Ethidium bromide reduction was indicated by the loss of deep orange colour from ethidium bromide. Arteries were stained for 20 minutes and removed from the organ bath for washing in Hank's BSS at pH 7.4. After washing, arteries were mounted in 1:1 glycerol/Hank's BSS (pH 7.4). Exposure to ultraviolet light for 10 minutes redeveloped the orange fluorescence characteristic of ethidium bromide in the nuclei of the tissue. At this stage the arteries were ready for viewing under the confocal microscope.

Arteries in 1:1 glycerol/Hank's BSS (pH 7.4) were placed on concave microscope slides for viewing. A Carl Zeiss LSM 10 system (Carl Zeiss Canada, Don Mills, Ontario) was used for confocal microscopy. The optical sections obtained from this system were approximately 0.2 μm thick. The system was equipped with an external argon laser with emission lines at 488 nm and 514 nm. The spectral line at 488 nm produced the best

excitation of the ethidium dye with the least non-specific fluorescence, resulting in an optimal signal-to-noise ratio. An 8 second per frame dwell time, and 16 line averaging were used to improve the image quality. Images were saved to electronic media, and transferred to the Image 1 system for morphometric analysis. Micrographs were obtained from images with a 35 mm camera attached to video frame printer.

Morphometric Analysis

The medial volume was calculated by using a Cavalierian estimator of volume (Equation 3.1). This estimator of volume does not rely on any assumptions about tissue or cell shape, and is independent of tissue orientation (Gundersen et al.,1988). Multiple optical sections were taken longitudinally through the arteries. The 20x objective with an electronic zoom factor of 20 times was used to produce the images with a total magnification of 400X. Sectioning began at a random location more than 10 microns above the artery. Sections were separated by 10 micron intervals, and sectioning continued until the artery was completely traversed. This produced from 20 to 25 optical sections per artery. The sections covered approximately a 500 micron length of artery. Ethidium bromide staining allowed the medial layer to be clearly distinguished from the adventitial layer, and endothelium + internal elastic lamina of the arteries. The area of the medial layer in each optical section was determined by a computer-aided tracing method allowing the calculation of medial volume as in equation 3.1.

Cavalierian Estimator of Volume

$$Volume = \sum (\text{Area}) \times \delta T \qquad (3.1)$$

Where
Volume = the calculated volume of the medial layer
Area = the area of the medial layer found on each optical section.
δT = the distance between each optical section.

The optical section at the centre of the stack was used to determine mid-section lumen area per length of artery. An electronic zoom factor of 40 times was used with the 20X objective (800X), so that the number of smooth muscle layers present in the media of the artery could be counted.

Functional Analysis

A pressurized myograph apparatus similar to that described by Halpern (Halpern et al.,1994), was used to study the functional response of the mesenteric arteries from the 4-week-old SHR and WKY rats. The apparatus consisted of an arterial myograph system in a heated organ bath where temperature was maintained at 37°C. One end of the arteries was tied off with a 10-0 suture. The other end was secured to the tip of a micropipette, which was connected to a pressure reservoir, so that arteries can be inflated with physiological solution at different pressures. A microscope with a video camera attached, was mounted on the bath allowing the recording of external diameter, and lumen diameter of arteries. Arteries were taken directly from living animals anaesthetized with ketamine (75 mg/kg i.p.) and urethane (0.25 g/kg s.c.), and mounted on the micropipette in the bath. Arteries in the bath were maintained in a Krebs solution consisting of (mmol/L): NaCl 116, $NaHCO_3$ 9.3, D-glucose 11, KCl 4.6, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.5, pH 7.4 and aerated with 95% O_2/5% CO_2.

Periarterial nerve stimulation was provided by a Grass model S4 stimulator (Grass Medical Instruments, Quincy, Mass) with an output of monophasic pulses at 150 V of 0.85 ms duration and a train 10 seconds. Arteries from 3-, 4-, and 6-week old rats were stimulated at 20 Hz over a range of pressure from 30 to 250 mmHg to assess both the contractile response, and the maturation of innervation. Tetrodotoxin (TTX) was add to the bath to determine the component of contraction due to periarterial nerve stimulation, and that due to direct muscle stimulation. Dose response curves to NE were performed over the concentration range of 2×10^{-7} to 1×10^{-3} mol/L with beta blockade (1.5×10^{-6} mol/L propranolol). Dose response to KCl was performed over a concentration range of 2×10^{-3} mol/L to 2.4×10^{-1} mol/L. Yielding pressure in arteries maximally contracted by KCl was determined over a pressure range of 50 to 250 mmHg in both SHR and WKY rats.

Statistical Analysis

Values given are mean ± SEM. Analysis of the data was performed with a computer-based statistical package SAS® (SAS® Institute Inc.). Analysis of variance (ANOVA) was used to determine if differences existed for the BP, medial volume, lumen area, and the number of smooth muscle cell layers. Change in lumen size in response to contractile agonists NE and KCl, contraction produced by electrical stimulation, and sensitivity to NE stimulation were compared by Bonferroni's corrected Student's unpaired t-tests at each point. Significance was recognized at the 95% level. Pearson's correlation was performed between medial volume, and number of smooth muscle cell layers. Power analysis of F-tests were performed to determine the probability that the parameters being measured were equal.

Results

Blood Pressure at 4 Weeks

BP was similar between SHR and WKY at 4 weeks of age. The mean values for the SHR group was 127±3 mmHg (n=42), and for the WKY group was 119±3 mmHg (n=38) (Figure 3.4). Mixed model ANOVA with fixed effects as strain type, and random effects as litter type nested inside of strain type showed that the 8-mmHg difference in systolic BP found between the strains was significant (p=0.02). Power analysis of the F-test revealed that there was an 80% chance that BP differed by less than 20 mmHg between the strains. The ANOVA also showed that litters within the strains differed significantly in BP (p=0.007). The model describing the variation in BP as the result of differences in strains and litters accounted for 69% of the variation in BP measured. Of that variation, 6% was found between the strains, and 63% between the litters within the strains, 31% of the variation in BP remained unexplained by the model. BP of the smaller subsets of animals used for morphometry and functional studies showed similar means as those displayed in the general population (Figure 3.4). Mean values were 115±5 mmHg (n=10) for the SHR, and 110±4 mmHg (n=10) for the WKY.

Volume of Medial Layer and Lumenal Area

Two optical section taken longitudinally at the midpoint of the artery, one from SHR and one from WKY (Figure 3.5), were displayed to illustrate the thickness of the medial layer in either group. The mean value for medial volume in the SHR group was 16,790 $\mu m^3/\mu m$ length of artery (n=10), and for the WKY group was 11,250 $\mu m^3/\mu m$ length (n=10, Figure 3.6). ANOVA showed a significant difference between SHR and WKY in regard to

medial volume ($p=0.0002$). The difference between the means represents a 49% greater medial volume in the SHR than the WKY.

Lumen area was not significantly different between SHR and WKY ($p=0.84$, ANOVA). Mean values of 95,060 μm^2 in SHR, as compared to 95,460 μm^2 in WKY for a 500 μm length of artery were recorded (Figure 3.6). Power analysis revealed that there was a 87% chance that the lumen areas differed between the strains by less than 5%.

Number of Smooth Muscle layer and Relationship to Medial Volume

The number of smooth muscle cell layers present in the media differed significantly between the strains ($p=0.0009$, ANOVA). Mean values for the number of smooth muscle layer were 4.1 in SHR and 2.7 in WKY ($n=8$ in each group, Figure 3.6). Pearson's correlation performed between the values obtained for medial volume, and the number of smooth muscle cell layers in the media showed a highly significant result at $p=0.0001$. This analysis showed that the increase in smooth muscle cell layers accounted for 81% of the increase in medial volume.

Functional Analysis of Hypertrophied Vessels

Unstimulated SHR and WKY arteries had similar lumen diameters. Dose-response curves to KCl showed that the area under the curve (AUC) was significantly greater for WKY than SHR arteries ($n=6$ each, $p<0.05$), indicating that over the entire range of KCl concentrations, SHR arteries had smaller lumen diameter than WKY. Moreover, the maximum contractile response for the SHR arteries was significantly greater than that derived from the WKY artery ($p<0.01$) (Figure 3.7a). The pressure at which arteries maximally contracted with KCl could no longer hold their contraction was lower in WKY

than SHR. In the WKY, lumen diameter increased linearly in response to pressure, whereas SHR arteries were able to maintain their maximally contracted lumen diameter until a pressure of approximately 150 mmHg was exceeded. When curves were fitted between the lumen diameter response and increasing pressure, it was found that the inflection point of the fit (i.e. yielding point) was 154 mmHg for the WKY and 184 mmHg for the SHR arteries (n=6 each, $p<0.05$) (Figure 3.7b). Norepinephrine dose-response curves when normalized as percent of maximum contraction due to KCl showed no significant difference in the reactivity of the arteries to NE stimulation (Figure 3.8a). However, when the absolute diameter of the lumen was used to express the response to a dose of NE, the fitted curves again showed a significantly greater AUC for WKY than SHR arteries, indicating that over the entire range of concentrations of NE, SHR arteries had smaller lumen diameters (n=6 each, $p<0.01$, Figure 3.8b). These results suggest that the increased muscle mass on the SHR arteries had a functional manifestation as an increased contractile response to KCl and NE.

Nerve Stimulated Contractile Response

Mesenteric arteries responsive to electrical field stimulation were present in 0 of 10 SHR and 3 of 10 WKY arteries at 3-weeks of age. At 4-weeks, 4 of 15 SHR arteries and 8 of 15 WKY arteries responded to electrical field stimulation. These results were analyzed by a log linear analysis with three factors: strain (SHR versus WKY), age (3 versus 4 weeks) and responsiveness (responders versus nonresponders). The two-factor interaction between stain and responsiveness was significant (χ^2=5.20, $P<.05$), indicating that there was a different response in the two strains. The two-factor interaction between age and responsiveness was also significant (χ^2=4.19, $P<.05$) indicating an overall greater

responsiveness at 4 weeks than at 3 weeks. Of the non-responding arteries all were found to contract to stimulation with NE (10^{-3} mol/L) after unsuccessful field stimulation. Finally at 6-weeks, all the arteries from 6 each of SHR and WKY responded to electrical field stimulation.

When SHR arteries that were responsive to electrical field stimulation were compared to those from WKY over a pressure range of 30 to 250 mmHg, SHR arteries were found to contract more than WKY arteries. This was true for arteries from both 4-week (P=.08, Figure 3.9a) and 6-week (P<.001, Figure 3.9b) groups. AUC for SHR arteries was significantly greater than WKY arteries, indicating that over the entire pressure range, SHR arteries contracted to produce smaller lumen diameters. These responses were blocked by tetrodotoxin indicating that these responses were due to nerve stimulation.

Discussion

The major findings of this study were as follows. SHR arteries displayed significant structural alteration as compared to WKY controls at a time when only small differences in BP existed. These alteration included an increase in the volume of the media layer, and increase in the number of smooth muscle cell layer, but no change in lumen diameter in the relaxed state. Moreover, these structural alterations had a functional manifestation. SHR arteries underwent increased amounts of contraction that resulted in significantly smaller lumen size in response to stimulation by KCl or NE. SHR arteries precontracted with KCl were also able to maintain a smaller lumen size than WKY arteries at comparable intramural pressures. These changes were due to the presence of an increase medial volume, and an

increase in the number of smooth muscle cell layers. However, the number of arteries exhibiting TTX-sensitive contractile response to electrical field stimulation was less frequent in SHR than WKY at 3- and 4-weeks of age. This difference may provide the explanation for the similar BP in these animals at these ages, in spite of the presence of significant vascular structural changes in the mesenteric arteries, as discussed below.

Structural and Functional Observations

The finding that hypertrophy of the medial layer had already taken place before any large difference in BP existed between SHR and WKY, indicates that the thickening of the medial layer in SHR is a primary change in the development of hypertension (Korner et al.,1991). This is in agreement with the results from previous studies (Lee, 1985; Miller et al.,1987; Mulvany et al.,1980; Stephens et al.,1991; Kong et al.,1991; Rizzoni et al.,1994). Therefore, it appears that some unknown process occurring before the elevation of BP in SHR is causing medial thickening. Studies on cultured smooth muscle cells from SHR have shown a greater growth response than cells derived from WKY (Saltis et al.,1992; Hamet et al.,1991; Hadrava et al.,1991). The increased vascular smooth muscle mass in mature SHR may then produce the higher BP observed in these animals by increasing total peripheral resistance. In young SHR, however, the BP of these animals was similar to age-matched WKY, in spite of the presence of an hypertrophied medial wall, and an increase in the media to lumen ratio in the mesenteric arteries and arterioles (Lee, 1985; Stephens et al.,1991; Kong et al.,1991; Rizzoni et al.,1994). It was suggested that BP increase in the SHR may lag behind the thickening of the muscle layer due to the late maturation of neuro-muscular coupling in young SHR, preventing the hypertrophied muscle layer from fully expressing

itself functionally (Lee, 1985). Results from the present study have now provided evidence to this effect. In young SHR, despite the presence of a high innervation density in the mesenteric arteries as compared to WKY (Lee, 1985), a TTX-sensitive response to electrical field stimulation was absent at 3 weeks and less frequently found at 4 weeks of age in the SHR than in WKY arteries. Such a temporal difference in the rate of functional maturation of innervation in the mesenteric arteries from SHR as compared with WKY, may explain why some young SHR did not express their genetic pre-disposition to increased total peripheral resistance by exhihiting a higher BP than WKY. These results are also consistent with a previous study where functional maturation of the nerve terminal-smooth muscle complex (levator palpebrae in the eyelid) was retarded in the SHR relative to WKY during the second and third postnatal weeks (Smith et al.,1982).

At four weeks of age when some arteries from both SHR and WKY displayed a TTX sensitive contractile response, a comparison of percent contraction of the responding arteries could be made. Here it was found that arteries from SHR which responded to electrical stimulation contracted to a greater degree than WKY arteries. This indicates that when functional innervation is present in SHR, the genetic pre-disposition to higher total peripheral resistance is expressed as a greater contraction of small resistance vessels. At 6-weeks when all the arteries in both SHR and WKY responded to field stimulation, the same pattern of a greater degree of contraction in SHR arteries than WKY was again present, therefore showing the important role of vascular structural changes in the control of total peripheral resistance.

Previous studies using the McMregor perfusion of isolated mesenteric vascular bed in 4-week-old SHR and WKY (Tsuji et al.,1989), and isolated perfusion of entire mesenteric vascular bed with the intestinal tract intact (Kong et al.,1991), found that frequency response curves to periarterial nerve stimulation, measured as changes in perfusion pressure, produced a greater response in SHR than WKY despite the lack of a significant difference in systolic BP measured at this age in these animals. This is in agreement with our comparison of responding vessels at 4-weeks of age where SHR vessels contract to a greater degree than WKY. However, it is not known whether all the vascular beds examined from SHR at 4 weeks of age were responsive to electrical stimulation. At 5 weeks of age and using the wire myograph technique, electrical stimulation of small mesenteric arteries from SHR and WKY elicited similar pressure responses in these arteries (Stephens et al.,1991). Differences in the study method may account for the discrepancy between the results obtained in this study and those with the isolated perfusion method, in contrast with the wire myograph method. There are marked differences in the vasoconstrictor responses of the arteries between wire-mounted and pressurized preparations (Heagerty et al.,1995). The other possibility is that the arteries we used in this study were larger than those used by Stephens et al. (Stephens et al.,1991). Nevertheless, by 12 weeks of age, mesenteric arteries from SHR did show a greater response to electrical stimulation than WKY arteries (Stephens et al.,1991).

Lumen diameter in the maximally relaxed pressurized arteries from SHR and WKY were the same. This is consistent with the results obtained from morphometric measurement of mesenteric arteries fixed *in situ* (Lee, 1985), and those calculated from the wire-myographic measurements (Stephens et al.,1991; Rizzoni et al.,1994). This indicates that

the hypertrophied media of the SHR arteries had not encroached upon the lumen to reduce diameter. Large changes in medial thickness may be necessary to reduce lumen diameter due to the elasticity of the artery wall. Small amounts of wall thickening may not decrease lumen diameter *in vivo* since the resulting decrease in flow would increase pressure expanding the non-rigid vessel restoring lumen diameter. Effective reduction in lumen diameter may only occur with a change in vessel wall compliance in the relaxed state, or an increase in the ability of the muscle mass to generate active tension in the contracted state. Immaturity of innervation in the arteries from young SHR may prevent the full expression of this active reduction in lumen diameter in the animal, so that BP were similar between WKY and SHR at 3 and 4-weeks of age.

However, the fact that the mesenteric arteries from these young SHR were able to produce smaller lumen diameters than WKY arteries in response to stimulation by NE and KCl, indicates the functional significance of a larger medial mass in these arteries. Moreover, a comparison of those arteries responding to field stimulation at 4-weeks of age also indicated a greater percent-contraction in vessels from SHR. Therefore, with the complete functional maturation of the small muscular arteries in older SHR it is inevitable that hypertension would develop due to an increase in total peripheral resistance because of the hypertrophied medial wall's ability to generate more active tension. Previous studies using the wire myograph method involving small mesenteric arteries (Stephens et al.,1991; Rizzoni et al.,1994) and isolated perfusion of mesenteric vascular beds (Kong et al.,1991) also found that young SHR (4 or 5 weeks old) exhibited an enhanced contractile response to NE than arteries or vascular beds from WKY

Consideration of the Methods

Confocal Microscopy

Confocal microscopy represents an important advancement in light microscopy because it effectively removes the need for sectioning and eliminates the inherent distortion associated with embedding and sectioning for the viewing of biological materials. The fact that the confocal microscope is able to produce very thin optical sections of the same order of magnitude as thin sections used for electron microscopy (0.2 μm), makes it ideal for stereological techniques based on the use of 2 or more parallel sections, because the distance between optical sections is known precisely (Gundersen et al.,1988). This enabled us to use a Cavalierian estimator of volume (Gundersen et al.,1988), involving the summation of 20-25 parallel sections over 200-250 μm depth of tissue. Such a procedure would be very difficult to perform with ultramicrotomy.

Tissue Preparation

Fixation of arteries by perfusion involves considerable difficulty in matching the hemodynamic parameters present in the living organism during fixation. Fixation with a low viscosity fluid at a physiological flow rate results in low perfusion pressures as were measured in the aorta (Lee, 1985). Increasing the flow rate by many folds (5-10x) greater than physiological tissue flow rates produced only small increases in pressure (<2 fold) in the large vessels, but these high flow rate may damage the walls of the small vessels, especially the endothelial cells. Moreover, the use of large molecular weight fractions to increase fixative viscosity without affecting tonicity, such as dextran, remains problematic because of the non-Newtonian properties of blood, where viscosity is a function of tube

diameter as in all suspensions and decreases in smaller arteries (McDonald, 1974b). This being the case, titration of viscosity to physiological pressures in larger vessels for a fixative solution with Newtonian properties, such as a fixative containing dextran, would produce a pressure too high in smaller vessels, thereby causing some indeterminate damage to the vessels. These problems in establishing ideal perfusion fixation conditions that reflect the hemodynamic parameters of flow and pressure in the living animal brings the data collected for lumenal sizes in perfusion fixed arteries into question. To address these problems, the small muscular arteries used in this experiment were removed from the body and fixed *in vitro* at a physiological pressure without flow.

In vitro fixation also involves potential pitfalls since arteries are elastic tissues and *in vivo* are under considerable longitudinal tension. As such, once they are removed from their tethering connective tissue they shorten. This shorting was shown to be up to 40% in larger elastic vessels such as the iliac arteries, and increased as vessel size decreased (McDonald, 1974a). We therefore used the pressurized myograph method to analysis the functional responses, and also to fix the arteries under pressure for structural analysis. We took care to restore the *in vivo* length of the arteries during fixation, since medial volume, and lumen area were being quantified over certain known length of arteries for comparison between groups. In this way more accurate results than those previously produced were obtainable.

In summary, using a new morphometric protocol involving confocal microscopy, we have shown that structural changes of small muscular arteries have already taken place in young SHR when BP is similar to WKY. There is also a temporal delay in the functional

maturation of innervation in arteries from SHR as compared with WKY. We therefore conclude that structural changes of the small muscular arteries, due to an increase in the medial volume, and increased number of smooth muscle cell layers, are primary changes which contribute to the development of hypertension in the SHR, because these changes are present at the age when BP was similar between SHR and WKY.

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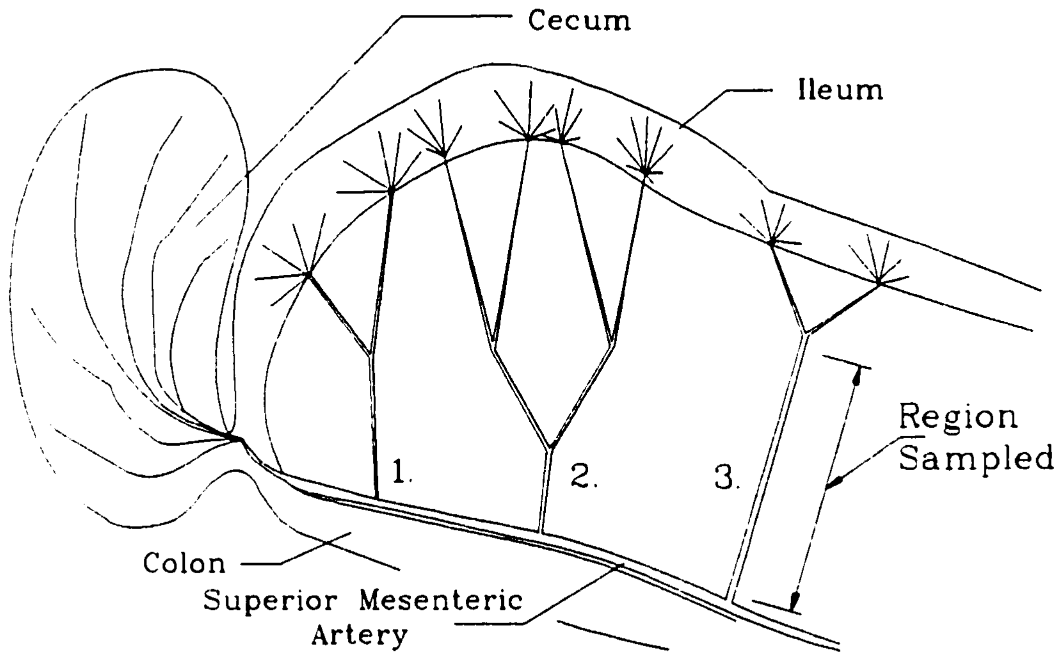


Figure 3.1. Line drawing which illustrates the gross anatomy of the vasculature in the region of sampling. Sampling was done consistently from the same site. The artery third-most distal to the junction between the caecal and superior mesenteric artery was sampled.

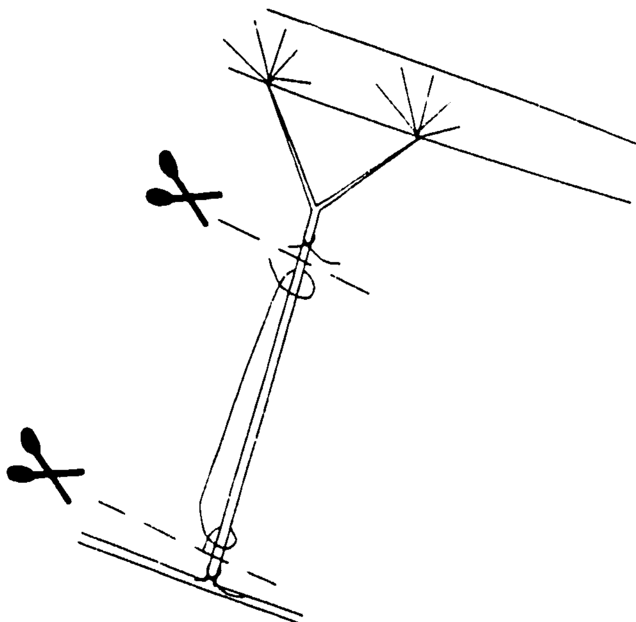


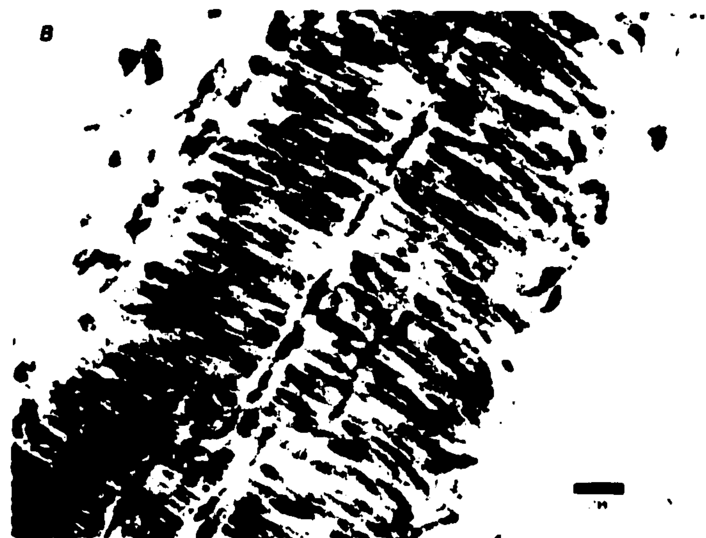
Figure 3.2. Illustration of the technique used to maintain artery *in vivo* length during sampling.

Figure 3.3. (a) Optical section taken longitudinally through an ileal artery from a 4-week-old SHR, showing tunica adventitia (A), and tunica media (M). Note circularly arranged smooth muscle cells in the media. Arrowheads point to the endothelial cells in the intima. Magnification bar = 30 μm.

(b) A higher magnification of Fig 3.3a, showing the resolving power of the technique to distinguish cell shape and vascular boundaries. Magnification bar = 20 μm.

(c) Optical section taken longitudinally through the mid-point of an artery wall showing nuclear profiles in the intima (small arrows), media (M), and adventitia (large arrows). Magnification bar = 20 μm.

A


B

M
M

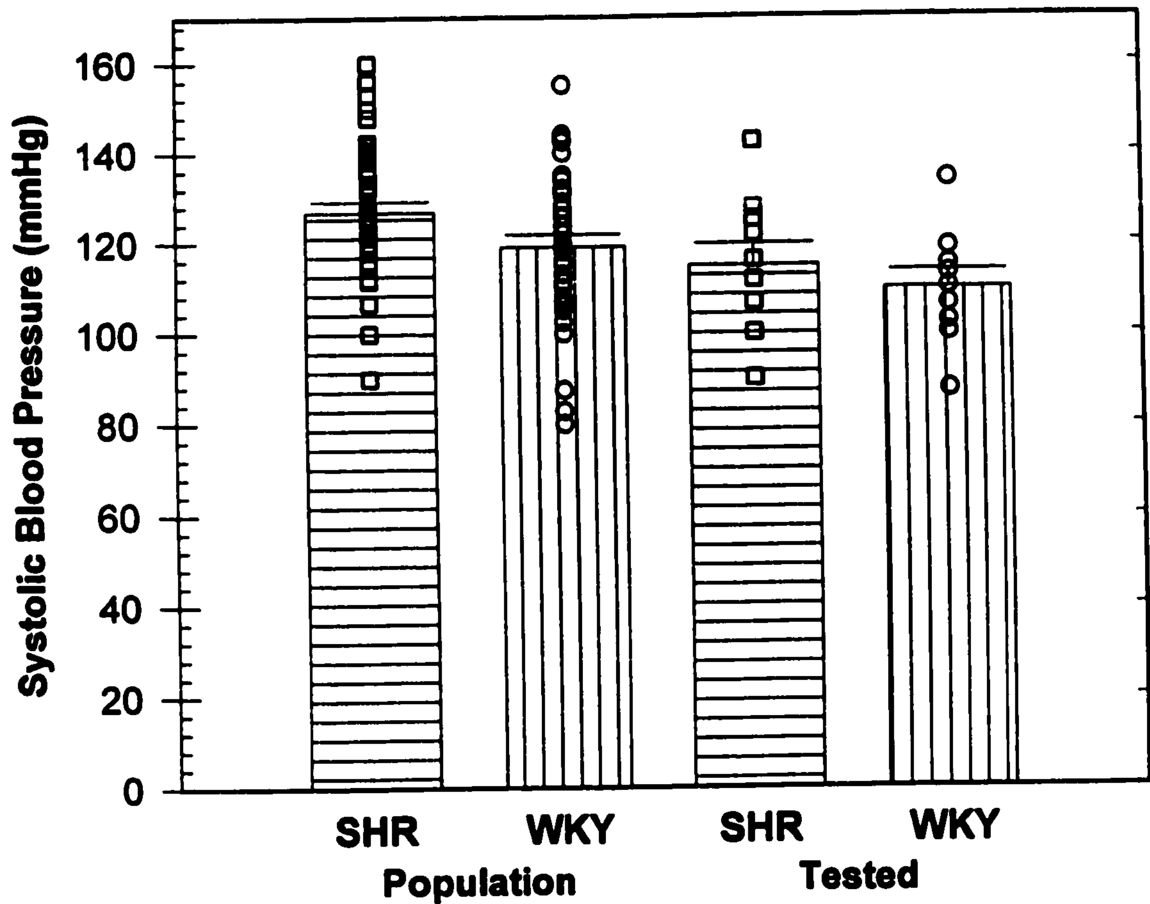
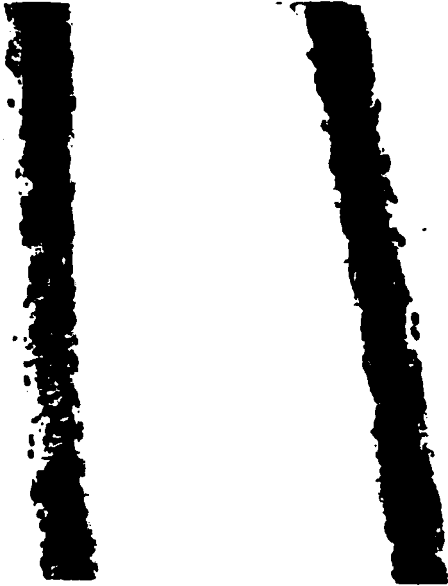


Figure 3.4. Bar graph showing systolic blood pressures measured in different population of SHR and WKY at 4 weeks of age, and the subset of animals used for morphometric and functional studies, measured by the tail cuff occlusion method. Each symbol represents measurement from one animal.

Figure 3.5. Optical section taken longitudinally at the midpoint of the artery from a 4-week-old SHR (A) and WKY (B) rat. Note the thicker mediai layer in the SHR as compared to the WKY artery. Magnification bar = 30 μm.

A



B

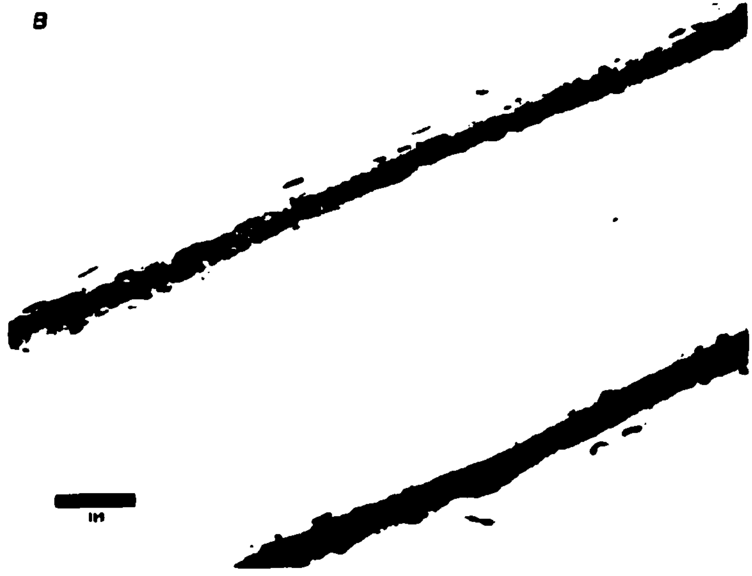


Figure 3.6. Bar graphs illustrating lumen cross-sectional area (A), number of smooth muscle layers (B), and medial volume (C) in SHR versus WKY.

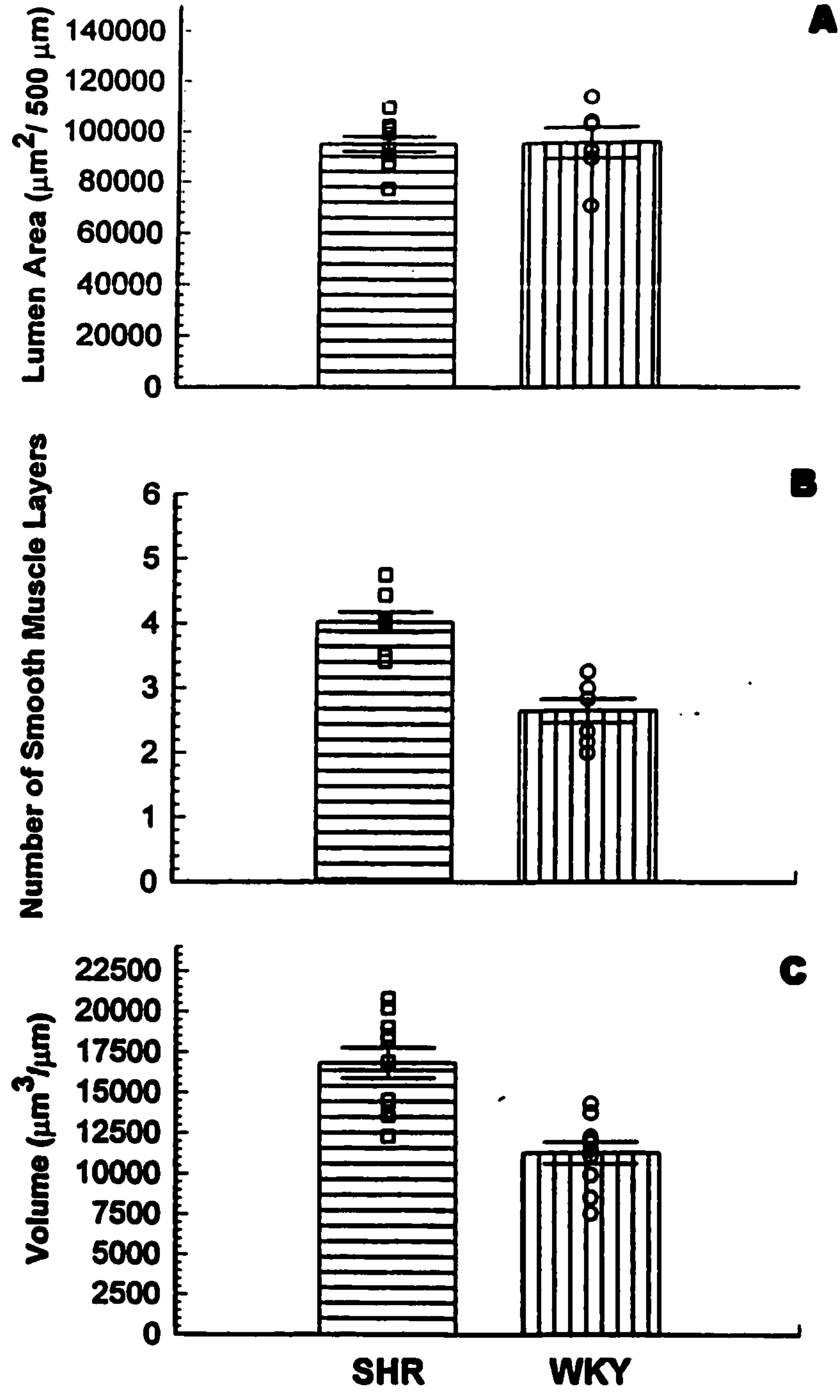
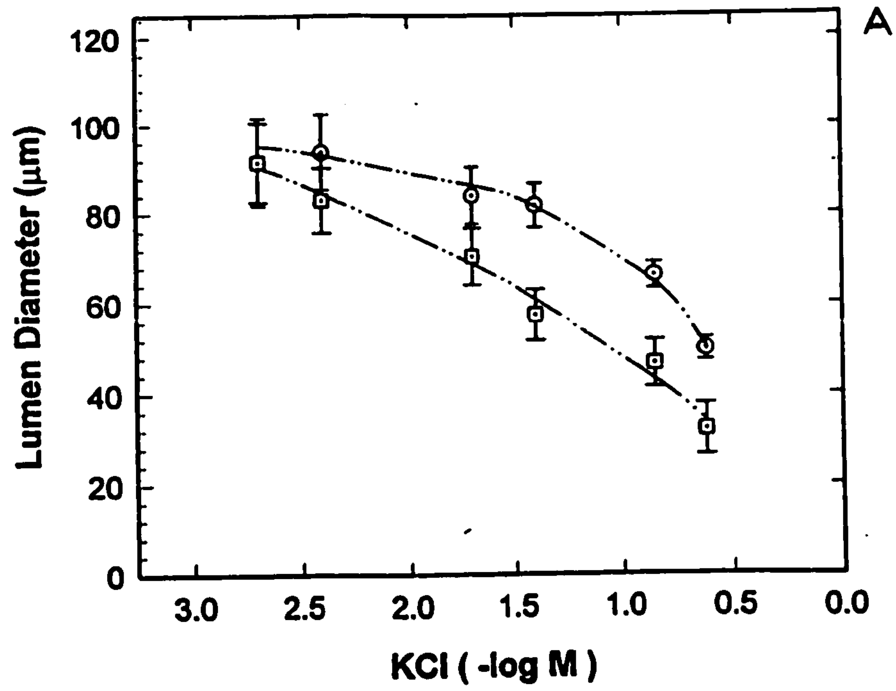

A
B
C
Lumen Area (μm²/ 500 μm)
140000
120000
100000
80000
60000
40000
20000
0
Number of Smooth Muscle Layers
6
5
4
3
2
1
0
Volume (μm³/μm)
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
SHR
WKY

Figure 3.7. (a) Fitted dose-response curves for the response of SHR (□) and WKY (○) arteries to KCl stimulation showing a significantly larger area under the curve (AUC) for WKY than SHR arteries.

(b) Fitted yielding point curves showing lumen diameter of SHR (□) and WKY (○) arteries in response to increasing inflation pressure in arteries maximally contracted to KCl.


A
Lumen Diameter (µm)
120
100
80
60
40
20
0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
KCl (-log M)

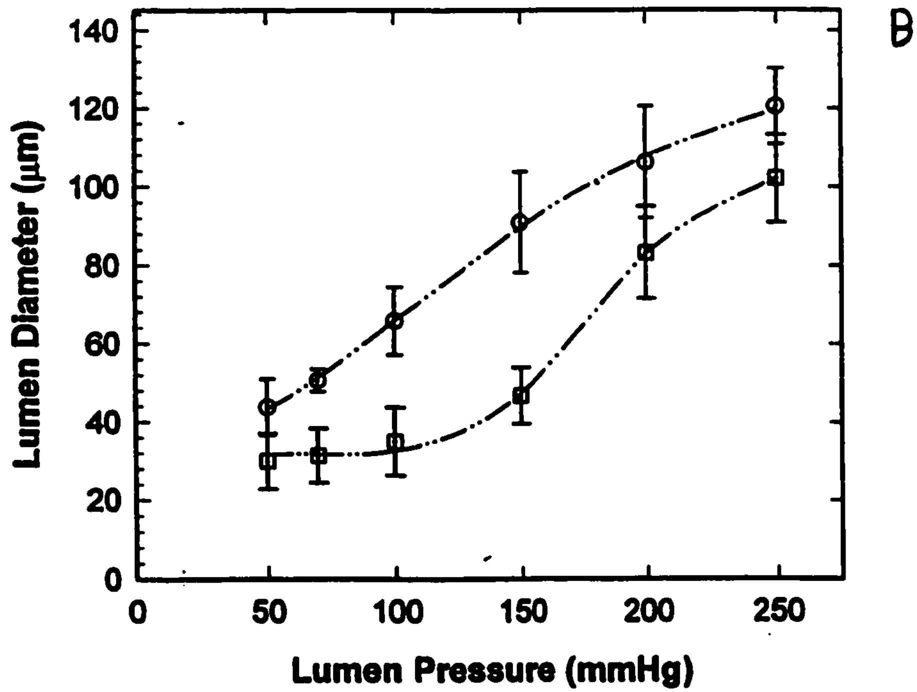
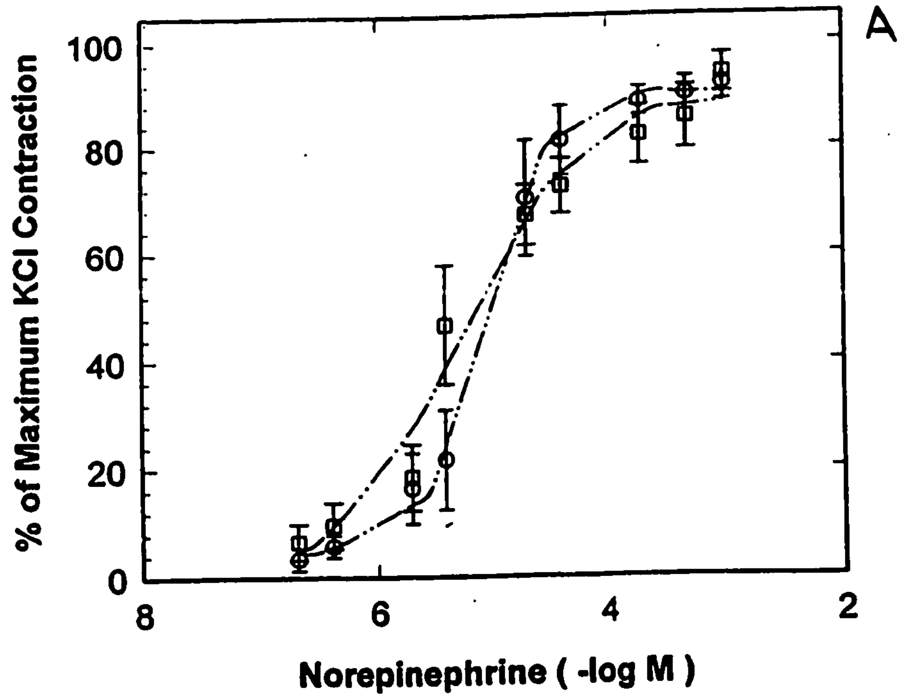

B
Lumen Diameter (µm)
140
120
100
80
60
40
20
0
0
50
100
150
200
250
Lumen Pressure (mmHg)

Figure 3.8 (a) Fitted dose-response curves for arteries from SHR (□) and WKY (○) to norepinephrine expressed as percent of maximum KCl contraction. No significant difference was found between the curves.

(b) Fitted dose-response curves for arteries from SHR (□) and WKY (○) to norepinephrine expressed as absolute lumen diameter as measured in the myograph system.


A
% of Maximum KCl Contraction
100
80
60
40
20
0
8
6
4
2
Norepinephrine (-log M)

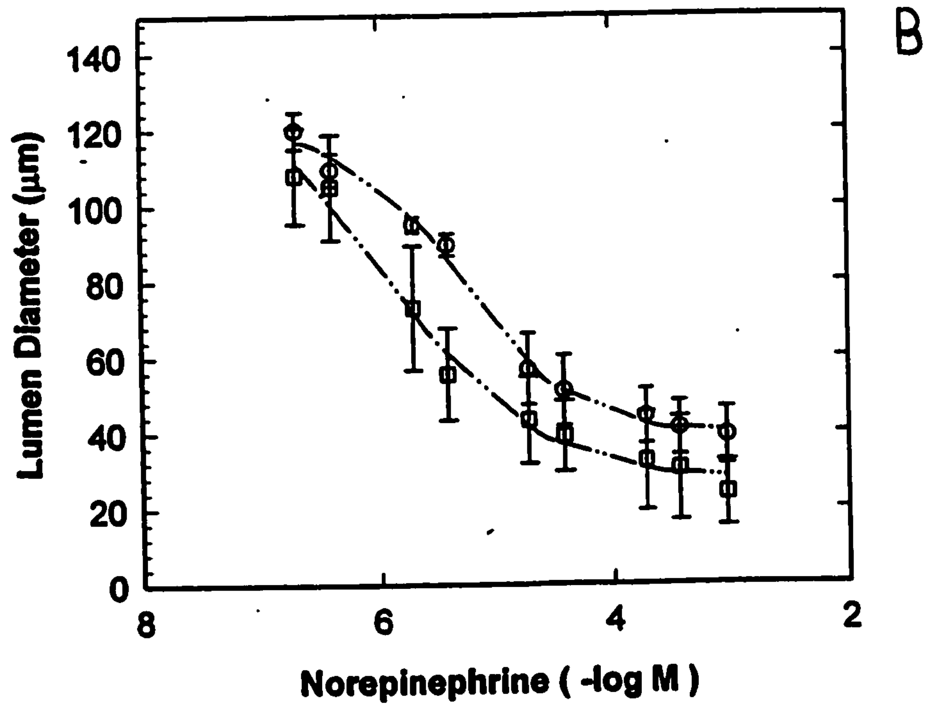
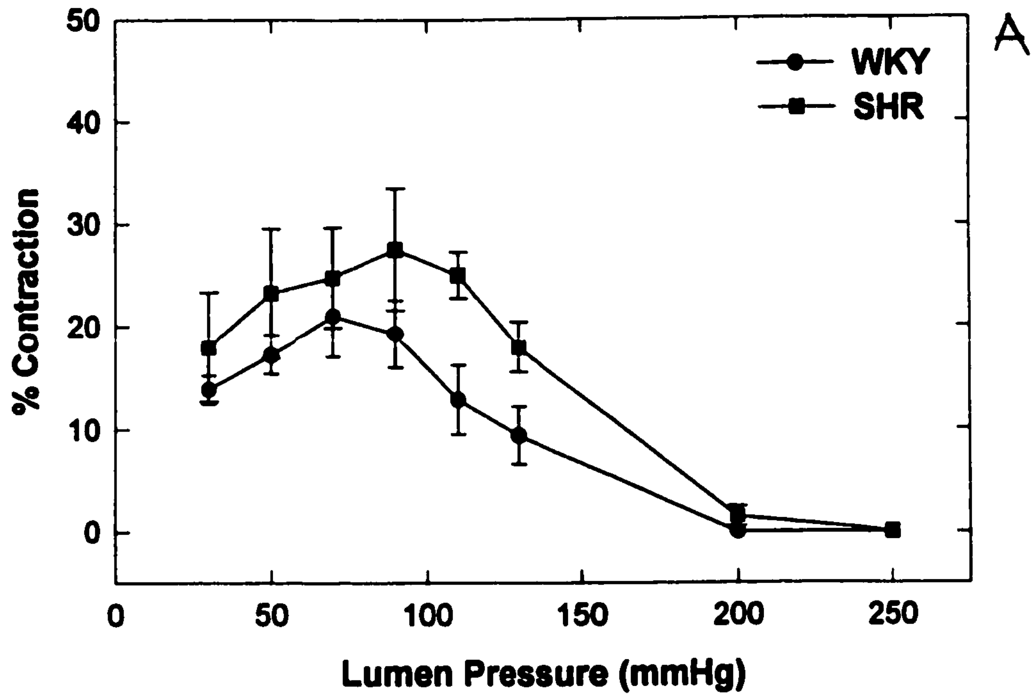
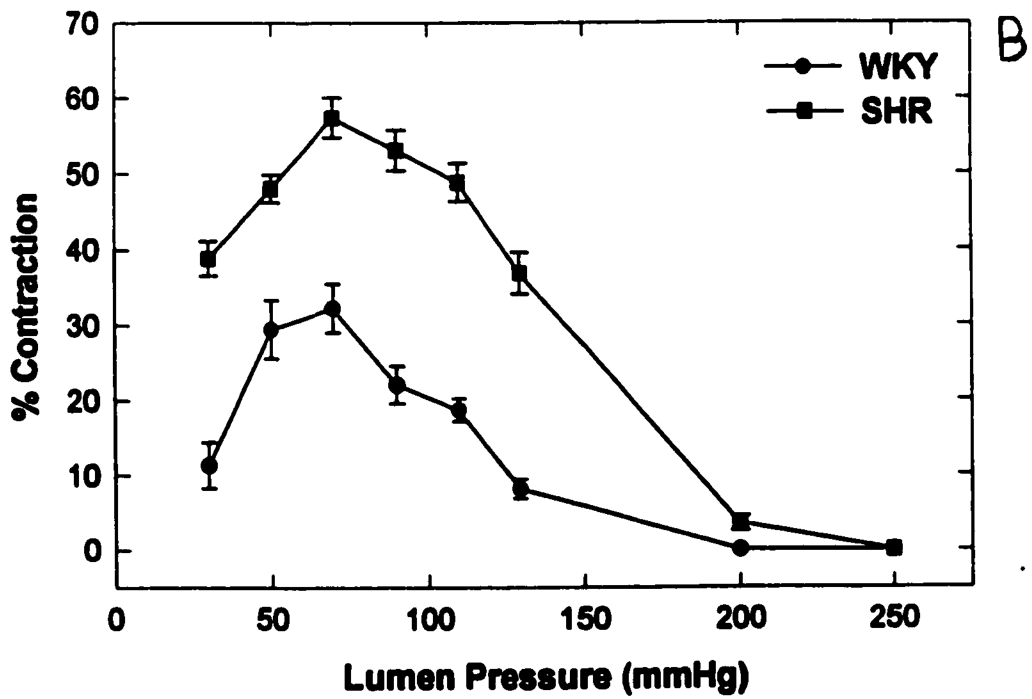

B
Lumen Diameter (µm)
140
120
100
80
60
40
20
0
8
6
4
2
Norepinephrine (-log M)

Figure 3.9. (a) Percent contraction of responding arteries to periarterial nerve stimulation at varying pressures for SHR versus WKY at 4 weeks of age.

(b) Percent contraction of arteries to periarterial nerve stimulation at varying pressures for SHR versus WKY at 6 weeks of age


A
WKY
SHR
% Contraction
Lumen Pressure (mmHg)
0
10
20
30
40
50
0
50
100
150
200
250


B
WKY
SHR
% Contraction
Lumen Pressure (mmHg)
0
10
20
30
40
50
60
70
0
50
100
150
200
250

CHAPTER 4

APOPTOSIS IN THE MUSCULAR ARTERIES FROM YOUNG SPONTANEOUSLY HYPERTENSIVE RATS

JEFFREY G. DICKHOUT and ROBERT M.K.W. LEE

Paper submitted to Journal of Hypertension, 1999

Introduction

In the spontaneously hypertensive rat (SHR), the structures of the superior and large mesenteric arteries at birth are similar to these arteries in its normotensive control the Wistar-Kyoto rat (WKY) (Lee et al.,1988). However, at 3 to 4 weeks of age before a significant elevation of the blood pressure occurs in the SHR strain (Dickhout et al.,1998), large mesenteric arteries from the SHR possessed a larger medial volume and a higher number of smooth muscle cells layers (Lee, 1985; Dickhout et al.,1997). These changes were accompanied by greater contractile responses of SHR arteries to stimulation (Dickhout et al.,1997). Therefore, structural change in the small muscular arteries of SHR is a primary process occurring during the development of hypertension. Moreover, the fact that this caliber of blood vessel is a major site of peripheral resistance in the SHR (Christensen et al.,1993), and that the greater contractile response seen in these structurally modified vessels of the SHR (Dickhout et al.,1997) would lead to greater peripheral resistance, is the basis for our hypothesis that structural change in these blood vessels is playing an important role in the genesis of hypertension in the SHR.

One possible contributing factor to structural change in the SHR resistance blood vessels is an enhanced rate of vascular smooth muscle growth. This was found in the mesenteric arteries of the SHR at 1 week of age as compared to the WKY by [^{3}H]thymidine uptake labeling of synthetic nuclei (Yang et al.,1989). In view of the recent finding that in newborn SHR, apoptosis was markedly decreased in the heart and kidneys as compared with WKY, it is possible that apoptosis may also play a role in contributing to the structural change of SHR arteries (Moreau et al.,1997).

The term apoptosis was introduced by Kerr et al. in 1972 (Kerr et al.,1972) to describe a process of controlled or programmed cell death, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell population. Four distinct characteristics are involved in apoptosis. These are cell volume reduction, chromatin condensation, cell surface change attractive to recognition of these cells by phagocytes, and the dependence of apoptosis on protein synthesis (Wyllie, 1987). Programmed cell death is also associated with DNA fragmentation. In fact, measurement of DNA fragmentation is a popular method of apoptosis detection and quantification (Cho et al.,1995; Moreau et al.,1997; DeBlois et al.,1997; Perlman et al.,1997; Diez et al.,1997b). This is achieved both by DNA laddering, the separation of DNA fragments on a gel; and by end labeling, through the incorporation of some marker on the 3`-OH end of the DNA fragment. However, in some cases, only end labeling can detect DNA fragmentation (Zakeri et al.,1994). It appears that in some systems DNA fragmentation is a late stage in apoptosis and may be the result of lysosomal activity (Zakeri et al.,1994). End labeling may also label some cells which have undergone necrosis as opposed to apoptosis. For this reason, it is preferable to use several techniques to assess apoptosis.

Apoptosis of vascular smooth muscle cells and endothelial cells is involved in vascular remodeling during perinatal arterial development in the lamb (Cho et al.,1995). In the SHR, apoptosis was noted in the heart, kidneys, brain and cultured aortic smooth muscle cells (Hamet et al.,1995; Diez et al.,1997b). Some antihypertensive treatments (e.g. losartan, enalapril, and nifedipine) stimulated apoptosis of smooth muscle cells in the aorta before reducing BP, whereas other drugs (e.g. hydralazine, propranolol or hydrochlorothiazide) did

not cause apoptosis of medial smooth muscle cells (DeBlois et al.,1997). However, whether apoptosis is involved in vascular remodeling in muscular arteries and resistance vessels in hypertension is unknown.

The primary purpose of this study, was to examine the incidence of apoptotic cells in the muscular arteries of SHR and WKY at 1-2 weeks of age using gel electrophoresis and confocal microscopy. Our hypothesis was that differences in the incidence of apoptosis contribute to the vascular structural changes in these arteries. We chose to use end labeling as an additional method to detect DNA fragmentation since DNA laddering may fail to show DNA fragmentation in some cases (Zakeri et al.,1994). Moreover, the end labeling method allowed the quantification of apoptosis with relatively small amounts of tissue (critical for small vessel types) and the precise identification of the cell and tissue origin of the apoptotic cells.

Methods

Large mesenteric arteries were sampled from 1-to-2-week-old male SHR and WKY. These animals were obtained from colonies maintained at McMaster University's Animal Quarter. The SHR colony was started with rats from Ayerst Laboratory (Montreal, Quebec, Canada) in 1976, and the WKY colony was started with rats from Canadian Breeding Farms (Montreal, Quebec, Canada) in 1983. Both colonies were derived from the Charles River strains, and we have maintained these colonies in our institute by continuous inbreeding. The age of SHR and WKY rats used for these experiments varied from 9 to 13 days, and were chosen in such a way as to result in equal mean ages between the groups.

The animals were weighed and arteries were sampled from the first order branches of the superior mesenteric artery. These arteries have been referred to previously as large mesenteric arteries (Lee et al.,1983). The rats were anaesthetized with ketamine hydrochloride (75mg/kg i.p.). The mesenteric vessels were cleared of blood by perfusion as follows. An infusion cannula was placed in the abdominal aorta distal to the origin of the superior mesenteric artery. The aorta was clamped just below the diaphragm. An exit for the perfusate was cut into the portal vein. This allowed the perfusate, oxygenated Hank's basic salt solution (BSS) to clear the vasculature of the abdominal viscera of blood. The arteries were perfused at a flow rate of 1 mL/min/100g of body weight for 15 minutes resulting in maximal relaxation of the arteries. Then the arteries were fixed by perfusion with 4% neutral buffered formaldehyde for one hour at room temperature.

For DNA laddering with gel electrophoresis larger quantities of tissue were required. The entire mesenteric arterial bed starting with the superior mesenteric artery to the quaternary branches adjacent to the intestine was excised after initial perfusion with oxygenated Hank's BSS to clear the blood. These vessels were then frozen in liquid nitrogen until phenol/chloroform DNA extraction could be performed.

Confocal Microscopy

The arteries were washed in Hank's basic salt solution (BSS), cleared of loose connective tissue and cut into two millimeter segments for processing. All arteries were washed in 0.1% glycine for 30 minutes at room temperature after fixation to quench aldehyde groups introduced by fixation. The arteries were then washed in Hank's BSS for five minutes and permeabilized in 3% Triton X-100 for 30 minutes at 37°C with agitation.

Washing was again performed four times for 5 minutes each in Hank's BSS to remove the detergent. DNase I digestion was used to produce positive control arteries for the apoptosis assays by the induction of DNA fragmentation. Negative control arteries were produced by sham staining with the omission of terminal deoxynucleotidyl transferase (TdT) from the labeling procedure. DNase I treatment was performed with 60 Kunitz units/mL of FPLCpure™ DNase I (Pharmacia, Westervile, OH, USA) in 40 mM Tris-HCl buffer at a pH 7.5, in the presence of 6 mM $MgCl_2$ added for enzyme activation. Arteries were then washed and immunohistochemical staining performed with the ApopTag™ *in situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD, USA). After washing, arteries were then counter stained with ethidium bromide (20μg/ml) to allow full morphometry to be preformed on the vessels as in our previous study (Dickhout et al.,1997). The arteries were mounted in 100% glycerol containing 2.5% DABCO (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) as an anti-fade agent (Johnson et al.,1982) and placed on microscope slides for viewing.

A Carl Zeiss LSM 10 system (Carl Zeiss Canada, Don Mills, ON, Canada) was used for confocal microscopy and fluorescence microscopy. The spectral line at 488 nm produced the best excitation of the fluorescein marker for black-and-white imaging. Images were saved to electronic media, and micrographs were obtained from images with a 35-mm camera attached to a video frame printer.

Morphometry and Apoptotic Cell Quantification

Our dual labelling technique allowed both the determination of artery structure using the ethidium dye and apoptotic cell quantification using the fluorescein probe on the same arteries. Apoptotic cell quantification proceeded by optically traversing through the entire

depth of the artery while collecting signal at the 520 nm wavelength, the emittance of the fluorescein probe. Planes within the artery with nuclei emitting in this wavelength were then optically sectioned and the number of emitting nuclei counted. Morphometric measurement of medial, adventitial and lumen volume was carried out using a Cavalierian estimator of volume similar to procedure described earlier (Dickhout et al.,1997).

DNA Laddering

Genomic DNA was extracted from SHR and WKY arteries to determine DNA fragmentation as a quantitative measure of cell apoptosis. This was accomplished using a modification of the method of deBlois et al (DeBlois et al.,1997). In this procedure phenol/chloroform extraction was used to isolated genomic DNA and RNA after proteinase K digestion of the blood vessels which were stripped of fat, loose connective tissue, and veins. Then RNA was digested with 5μg/ml DNase-free RNase (Boehringer Mannheim GmbH) and the DNA re-isolated. The DNA was then subjected to salt/ethanol precipitation and reconstituted in an appropriate amount of Tris/EDTA buffer for the required dilution. The DNA was then run on 1.7% agarose gels with a potential difference between the electrodes of 100 Volts. Molecular weight markers, GeneRuler™ 100 BP DNA Ladder Plus, and quantity standards were also added to the gel. Gels were stained with 10 ug/ml ethidium bromide and photographed under U.V. light through a Kodak Wratten #23A filter with 3000 ASA black and white Polaroid film. Gels were scanned into a microcomputer at 800 DPI resolution with 16bit grays scale pixelization. These gel images were then transported to the NIH image analysis package for quantification. This was achieved by optical density calibration from the quantity standards loaded onto the gel, 20, 50, 100 ng of 1000bp plasmid

DNA, and integration of optical density above background in the gel lanes. The quantity of fragmented DNA above the 600bp molecular weight marker was used to compare between the strains.

Statistical Analysis

Values are mean ± SEM. A computer-based statistical package (SAS Institute Inc) was used to perform an overall ANOVA testing for volumetric differences between the tissue layers and lumen of the SHR and WKY blood vessels. Student's unpaired t-tests were used to compare fragmented DNA on electrophoretic gels and the incidence of apoptotic cells between the strains for age-matched SHR and WKY. Regression analysis was performed with wet weight of extracted tissue as an independent variable and fragmented DNA a dependent variable on electrophoretic gel data. In all tests $P<0.05$ was considered significant.

Results

The ages and body weights of the animals from either strain are given in Table 4.1. Animals were chosen in such a way as to result in equal mean age between the strains. SHR showed lower body weight at this age than WKY, however, this difference did not reach statistical significance ($P=0.13$).

In the arteries from SHR and WKY, no intimal cells were found to be labeled in either strain. Labeled cells were found both in the medial and adventitial layers of SHR and WKY vessels. These labeled cells were clustered in patches in the artery wall and their typical appearance in SHR and WKY is illustrated in Figure 4.1A and 4.1B. The incidence

of labeled cells was significantly lower in the medial and adventitial layers of the arteries from the SHR than WKY (Table 4.2). In arteries treated with DNase I, all the cells in the adventitial, medial, and intimal layer showed fluorescent labeling (Figure 4.1C). This indicated that the fluorescein probe was able to penetrate fully the blood vessel wall. Our negative control arteries which had the TdT enzyme omitted from the staining procedure showed no nuclear labeling.

The typical structural appearance of the blood vessel from SHR and WKY used for apoptotic cell quantification is illustrated in Figure 4.2A and 4.2B. An overall ANOVA between the strains revealed no significant differences in the volume per unit length of the adventitia, media, or lumen (Figure 4.2C) where the incidence of apoptotic cells per unit length was quantified.

Genomic DNA extracted from the mesenteric vascular bed of WKY showed a significantly greater DNA fragmentation than that taken from the SHR when 1.5 mg of tissue was extracted for loading on the gels (WKY=1910±347, SHR=850±74 ng DNA, n=6, P=0.01). Figure 4.3A illustrates such a gel with the DNA extracted from 1.5 mg of tissue loaded in either lane for SHR and WKY. Regression analysis showed fragmented DNA to increase linearly with the increase in tissue extracted. However, the slope of the regression line was significantly greater in WKY (slope=1320 ng fragmented DNA/mg tissue) than SHR (slope=570 ng fragmented DNA/mg tissue) (Figure 4.3B).

Discussion

The main findings of this study were as follows. A lower incidence of apoptosis was present in the muscular arteries of 1-2-week-old SHR as compared with WKY but the volume of adventitia, media, and lumen per unit length of blood vessels was similar between SHR and WKY. Since this lower incidence of apoptosis in the adventitia and media occurs in SHR before significant blood vessel wall volume expansion and blood pressure elevation had occurred, we suggest that this reduction in programmed cell death during early SHR development may lead to the structural differences previously found in SHR at 4 weeks of age (Lee, 1985; Dickhout et al.,1997).

Previous studies of arterial development had focused mostly on the aorta (see reference (Nakamura,1988) for review). In the aorta of normotensive rats, several layers of smooth muscle cells already exist in the media at birth with well defined myofilaments (Nakamura,1988). The number of smooth muscle cells doubled during the first week after birth (Olivetti et al.,1980), with most of the cell proliferation occurring during the three postnatal weeks (Berry et al.,1972). A majority of the subsequent growth is due to the development of elastic laminae in between the smooth muscle cells and connective tissue matrices.

In the superior mesenteric arteries from the SHR and WKY, there are two layers of differentiated smooth muscle cells in the media at birth, as compared with one layer in the large mesenteric arteries from SHR and WKY (Lee et al.,1988), and Sprague-Dawley rats (Woolgar et al.,1989). Very few changes occur in the large mesenteric arteries from the normotensive rats during the first few days after birth. In contrast, significant changes occur

in the large mesenteric arteries of SHR immediately after birth, since in some SHR, another layer of differentiated smooth muscle cells is present on day 2 after birth (Lee et al.,1988). Furthermore, the number of smooth muscle cells layers is similar between 1- and 4-week-old WKY, whereas there is a steady increase in smooth muscle cells layers between 2 and 4 weeks in SHR (Yang et al.,1989). However, the source of these additional smooth muscle cells layers is not known. Differentiation of fibroblast-like cells in the media is one possible source (Lee et al.,1988). Detailed morphological and functional studies of the mesenteric arteries during the 4 week postnatal period are still lacking. This is important because most of the changes in these arteries which are closely linked to the development of hypertension occur during this period (Lee, 1985). Our present finding of a reduced incidence of apoptosis in the muscular arteries from 1-2-week-old SHR as compared with WKY, indicates that this could be another contributing factor to vascular structural changes in the SHR.

The specific type of vascular changes in the mesenteric arteries of SHR as compared with WKY depends on the vessel type and age (Lee, 1987). DNA synthesis also differs markedly between elastic, muscular, and resistance vessels at 6 weeks of age in the SHR whereas in WKY, results from the different type of vessels were similar (De Mey et al.,1991). Heterogeneity in growth may be due to the presence of different phenotypes of vascular smooth muscle cells in different artery types, or differential exposure to growth promoting factors in these different vessels (Daemen et al.,1995). It is possible that the incidence of apoptosis may also show a similar heterogeneity depending on the vessel types. For this reason, it was important for our study to focus on the vessels types where we had previously found primary structural change in the SHR to address whether differential

incidence of apoptosis contributed to the structure differences found previously (Lee, 1985; Dickhout et al.,1997).

Our finding of reduced apoptosis in the muscular arteries of the SHR as compared to WKY at 1-2 weeks, is consistent with the report of a reduced rate of apoptosis in the heart, kidney, and aorta of newborn SHR (Moreau et al.,1997). However, the shortcoming of this previous study is a lack of knowledge of which cell, or tissue types were responsible for the reduced rate of apoptosis in the SHR and how this related to hypertension development. In young adult SHR, the pattern of apoptosis in the aorta is different from those we have found in the mesenteric arteries of neonatal SHR, in that a higher incidence of apoptotic cells was present in the media of aorta from SHR as compared with WKY, and antihypertensive treatment with enalapril or amlodipine caused a further increase in the aorta of SHR (Sharifi et al.,1998). It is possible that the increase incidence of apoptosis in adult SHR is an adaptive response of the arteries to increased blood pressure.

Our finding that the incidence of apoptotic cells in the adventitia of SHR was lower than that of WKY is interesting, and the cell type most likely involved is the fibroblast, as the cell body of the nerves are located elsewhere. This may result in an increased number of fibroblasts in the adventitia of SHR as compared to WKY, and may contribute to the increased adventitial mass found in this vessel type in the SHR at the prehypertensive, developing hypertensive and established phases of hypertension development, due to an increased rate of collagen synthesis in these vessels (see reference (Lee, 1987) for review).

The reason for a reduced incidence of apoptosis in the SHR blood vessels at the 1-2 week age is unknown. Many factors are known to influence the rate of apoptosis in various

cell types. The Bcl-2 family of proto-oncogenes are critical for the regulation of apoptosis (Diez et al.,1997a). Inhibitors of apoptosis include the Bcl-2 protein and inducers include the Bax protein (Diez et al.,1997a). It was found that intramyocardial arteries of adult SHR (150-300 μm in diameter) showed an increased expression of the Bcl-2 protein (Diez et al.,1997a). This increase in Bcl-2 may explain a reduced incidence of apoptosis in SHR.

Hormonal factors may also differentially influence the rate of apoptosis between SHR and WKY. Treatment of 10-week-old SHR with losartan or enalapril for 4 weeks increased the rate of smooth muscle cell apoptosis which results in medial mass reduction and smooth muscle cell number reduction in SHR aorta (DeBlois et al.,1997). Further, treatment of 16-week-old SHR with quinapril increases the expression of Bax protein, a known apoptosis inducer, in the intramyocardial arteries of SHR (Diez et al.,1997a). These results suggest that angiotensin II has an inhibitory effect on smooth muscle cell apoptosis. Insulin-like growth factor also has an inhibitory effect on apoptosis in the stroke-prone SHR (Tagami et al.,1997b; Tagami et al.,1997a). This fact combined with the presence of hyperinsulimia in the SHR (Aitman et al.,1997; Hulman et al.,1993), may point to apoptosis reduction through the influence of abnormally high level of insulin in these animals.

In summary, we have found that a reduced incidence of apoptosis in the medial smooth muscle cells and adventitial cells was present in the muscular arteries of SHR as compared with WKY at 1-2 weeks of age before the onset of vessel wall hypertrophy. Such an occurrence in conjunction with an increased rate of DNA synthesis found in this vessel type in another study, may lead to vessel wall hypertrophy and a greater contractile response of these arteries found in these animals at 4 weeks of age, thereby contributing to the

development of hypertension in these animals.

Acknowledgments

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Figure 4.1. Typical appearance of apoptotic cells (arrowheads) in the arteries from SHR (A) and WKY (B). L = lumen of the artery. Bar = 50 μm. (C) Artery from SHR rat treated with DNase I (positive control) and subsequently labeled with apoptosis labeling procedure showing fluorescent labeling of all cell nuclei in the adventitial, medial, and intimal layers. f = fibroblast nuclei of the adventitial layer, s = smooth muscle nuclei of the medial layer, e = endothelial cell nuclei of the intimal layer. Bar = 50 μm.

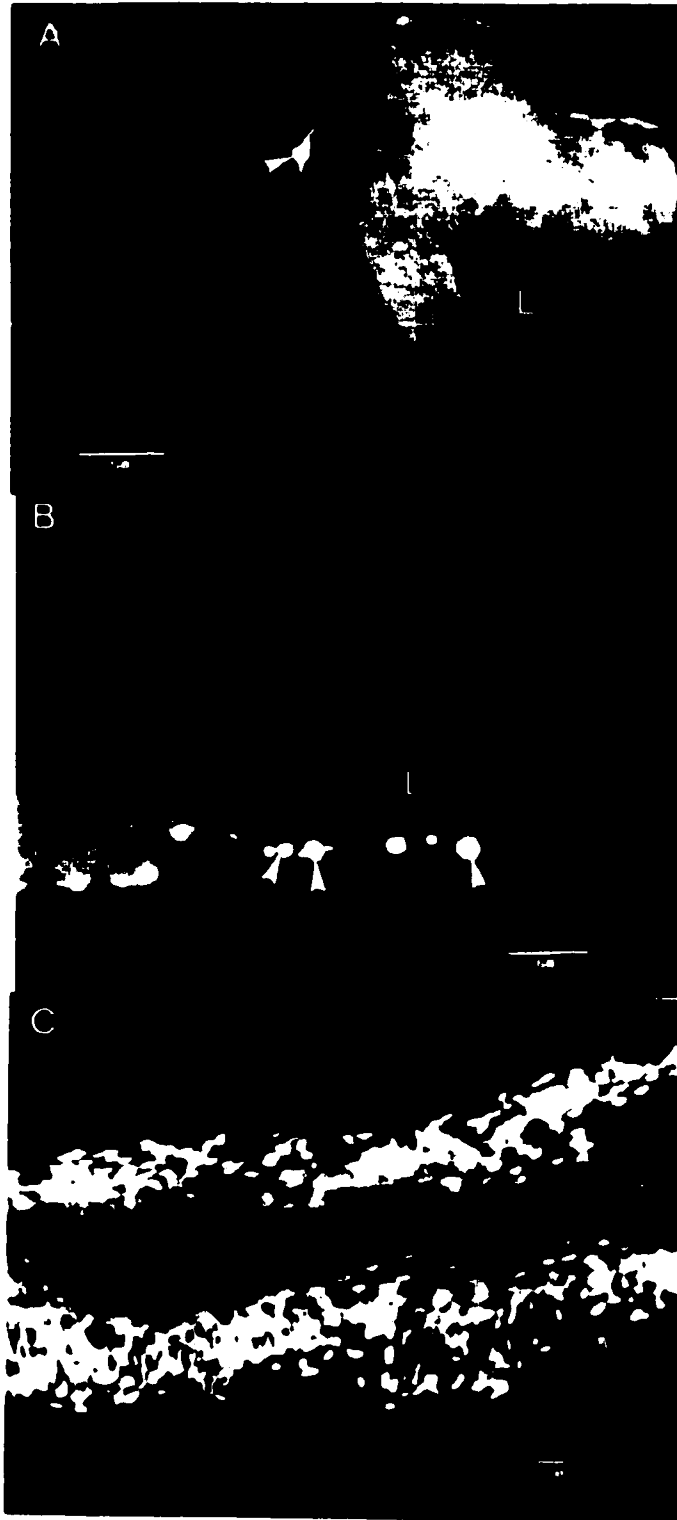
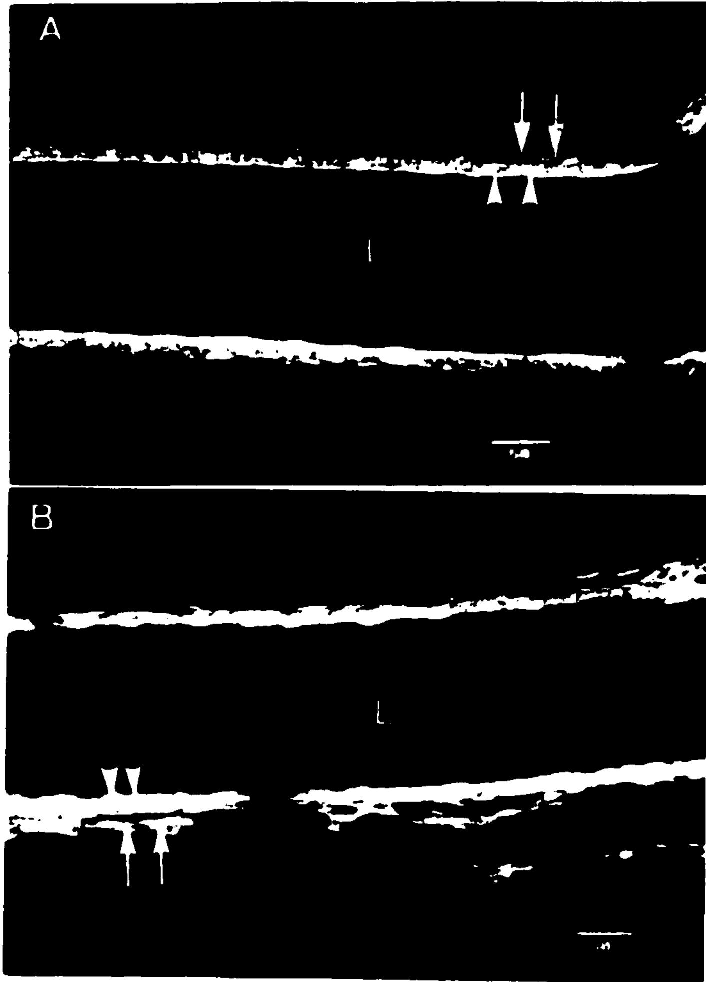

A
L
B
L
C

Figure 4.2. Large mesenteric artery from SHR (A) and WKY (B) in longitudinal optical sections obtained at mid-depth from the arteries used for volume quantification. Arrowheads point to the media, arrows point to the adventitia, and L = lumen of the artery. Bar = 50 μm. (C) Comparison of large mesenteric artery volume between SHR and WKY measured from the adventitial and medial layers as well as the artery lumen with optical sections derived from the signals provided by the ethidium probe. Overall ANOVA revealed no significant differences between the strains for any of the parameters measured.


A
L
B
L

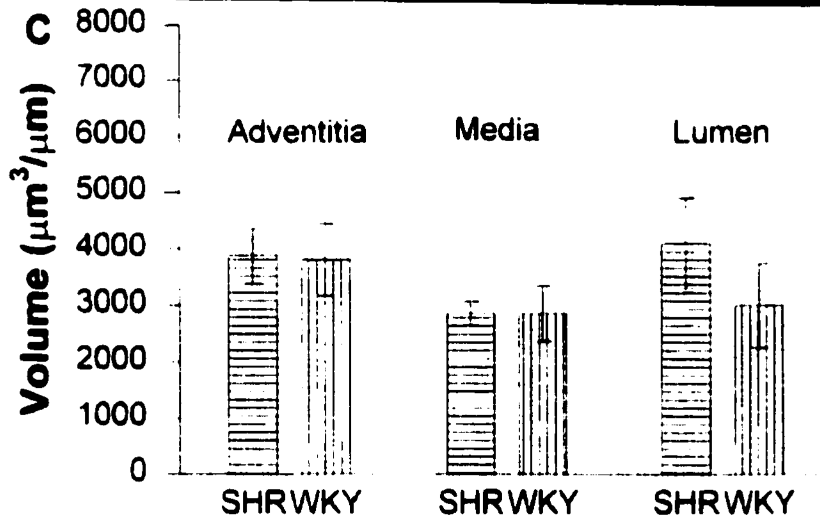
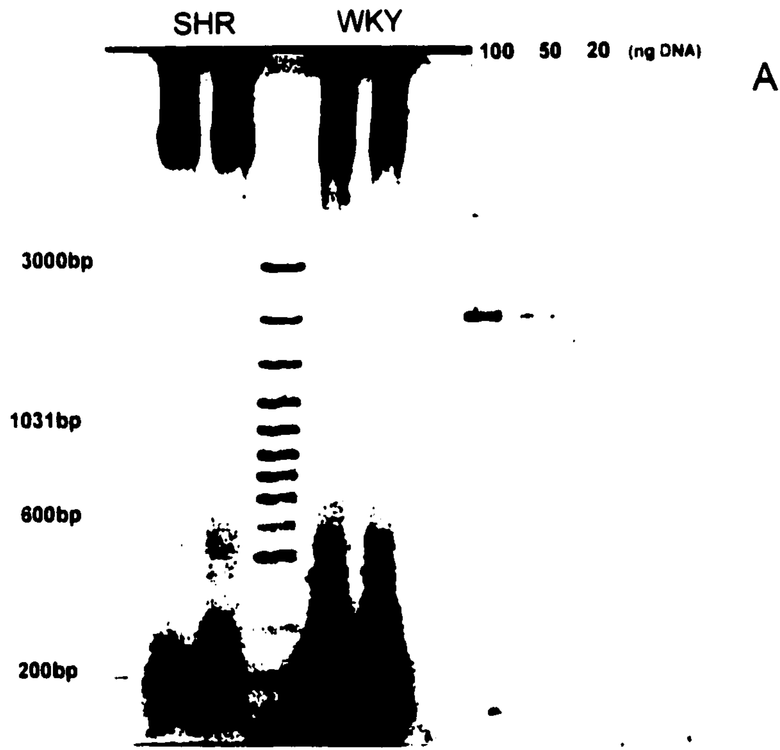

C
8000
7000
6000
5000
4000
3000
2000
1000
0
Volume ($\mu m^3/\mu m$)
Adventitia
Media
Lumen
SHR WKY
SHR WKY
SHR WKY

Figure 4.3. (A) Comparison of fragmented DNA (ng), fragment size < 600bp, measured on 1.7% agarose gels stained with ethidium bromide where DNA quantity standards were used to calibrate the integrated optical density of lanes above the 600bp marker of our GeneRuler™ 100bp DNA Ladder. This was done for 0.375, 0.75, and 1.5 mg of tissue where DNA was extracted from the primary to quaternary branches of the superior mesenteric artery in 1-2 week old SHR and WKY. (B) Regression analysis between the amount of fragmented DNA and wet tissue weight of the arteries used for DNA extraction.


SHR
WKY
100
50
20
(ng DNA)
A
3000bp
1031bp
600bp
200bp

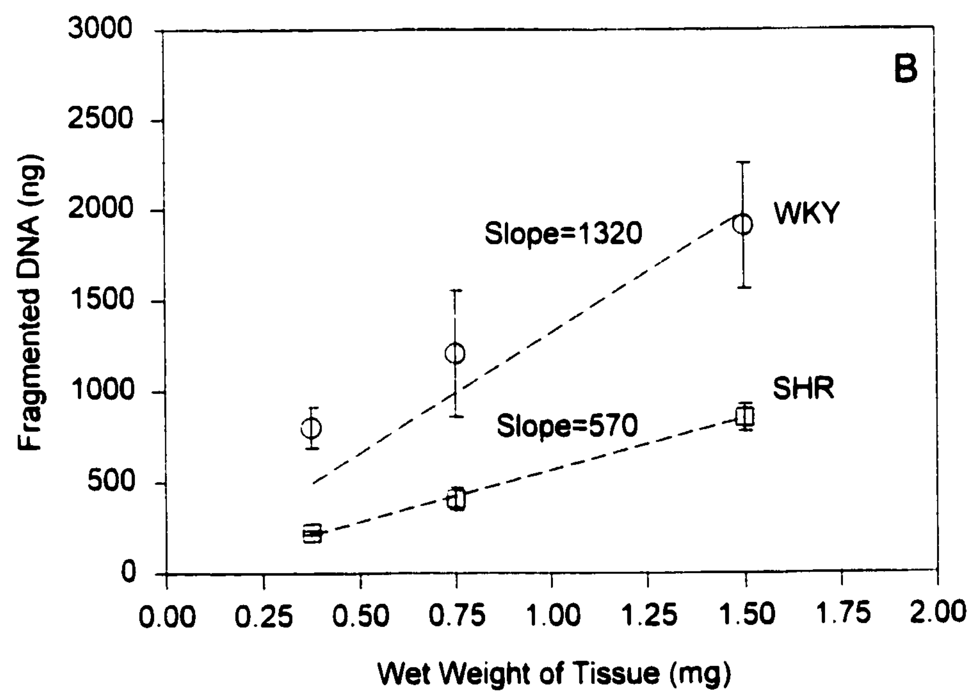

B
Fragmented DNA (ng)
Wet Weight of Tissue (mg)
Slope=1320
Slope=570
WKY
SHR
3000
2500
2000
1500
1000
500
0
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00

TABLE 4.1. Age and body weight of animals used for apoptotic cell quantification and artery morphometry.

Strain	**Age** (days)	**Body Weight** (g)
SHR	10.5 +/- 0.8	18.0 +/- 0.8
WKY	10.5 +/- 1.0	21.5 +/- 1.8

Values are means ± SEM, n=6 animals in each strain. There was no difference between the strains by Student's unpaired t-test.

TABLE 4.2. Incidence of apoptotic cells per 500 μm length of artery in the different tissue layers of large mesenteric arteries from SHR versus WKY rats.

Strain	Number of Apoptotic Cells		
	Adventitia	**Media**	**Intima**
SHR	3.5 +/- 0.4	1.0 +/- 0.6	0
WKY	7.2 +/- 1.1	11.2 +/- 1.7	0
P	0.01	0.0001	NS

Values are means ± SEM, n=6 animals in each strain for apoptosis quantification. Significant differences were determined by Student's unpaired t-test. (NS = Not Significant)

CHAPTER 5

INCREASED MEDIAL SMOOTH MUSCLE LENGTH IS RESPONSIBLE FOR VASCULAR HYPERTROPHY IN YOUNG SPONTANEOUSLY HYPERTENSIVE RATS

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Introduction

Essential hypertension is a disease associated with an increase in total peripheral resistance. This is true in animal models of hypertension such as the spontaneously hypertensive rats (SHR)(Folkow et al.,1970), Dahl salt-sensitive rats (Boegehold, 1993), and in human essential hypertension (Mulvany, 1996). This increase in total peripheral resistance is related to structural changes of the blood vessels in the animals models(Lee et al.,1983; Lee, 1985; Lee et al.,1986)and in humans (Schiffrin, 1996). The exact nature of the structural change may vary between animal models, vessel types, and different stages of the disease (Lee, 1987).

Structural change of the resistance blood vessels may be an important factor in the genesis of hypertension in the SHR. We have found that blood pressure begins to diverge between the SHR and its normotensive control the WKY rat at 4 weeks of age (Dickhout et al.,1998). Thus, 4 weeks is an important age to investigate structural change in the SHR since we can rule out increased blood pressure as a factor in generating the structural change. We have found in 4-week-old SHR an increased medial volume in the small muscular arteries as compared to age-matched WKY (Dickhout et al.,1997). This is accompanied by a greater contractile response of these arteries to various agonist and to electrical field stimulation (Dickhout et al.,1997). These results support our hypothesis that an increased medial smooth muscle volume in small muscular arteries of the SHR is an important causative factor leading to increased total peripheral resistance and hypertension in the SHR.

The main purpose of this study was to test the hypothesis that medial volume hypertrophy in the large mesenteric arteries of the 4-week-old SHR was due to an increase

in smooth muscle cell (SMC) number. To achieve this goal, two fixation methods, *in vitro* and *in situ*, were applied to the mesenteric blood vessels of SHR and their normotensive WKY control. This was done in order to determine if preparation method had any influence on our results. Then the volume of the medial layer and the lumen area at mid-longitudinal section were quantified for either method. The numerical density of SMC nuclei was assessed by a dissector method (Gundersen et al.,1988), as well as by exhaustive serial sectioning. From these results, the number of SMC per unit length of vessels were calculated. The average length of SMC nuclei and SMC length in the *in situ* fixed arteries were also calculated. This information was correlated with the medial volume to determine if the increase in SMC length was responsible for medial volume expansion in the SHR.

Method

Four-week-old male SHR and WKY rats were used for medial SMC quantification and measurement. The animals were obtained from colonies maintained at McMaster University's Animal Quarter. The SHR colony was originally obtained from Ayerst Laboratory (Montreal, Quebec, Canada), and the WKY from Canadian Breeding Farms (Montreal, Quebec, Canada). Both colonies were derived from the Charles River Laboratories strains (Wilmington, Mass), and we have maintained these colonies in our institute by continuous full-sibling inbreeding for over 20 generations. The care of these animals was in accordance with the guidelines of the Canadian Council on Animal Care.

Animals were weighed and their systolic blood pressures measured using a tail cuff occlusion method (model PE300, Narco Bio-Systems, Houston, Texas) in conscious animals.

The rats were anaesthetized with sodium pentobarbitol (65 mg/kg i.p.) to allow fixation of arteries for morphometric studies. Ileal arteries which form the primary branches of the superior mesenteric artery were used, as in the case of our previous study (Dickhout et al.,1997). These mesenteric vessels were cleared of blood by perfusion as follows. An infusion cannula was placed in the abdominal aorta, distal to the origin of the superior mesenteric artery. The aorta was clamped just below the diaphragm proximal to the origin of the superior mesenteric artery. An exit for the perfusate was cut into the portal vein. This allowed the perfusate, oxygenated Hank's basic salt solution (BSS) containing 1 μmol/L sodium nitroprusside, to clear the vasculature in the abdominal viscera. The arteries were perfused at a flow rate of 1 mL/min/100 g body weight for 5 minutes, resulting in maximal relaxation of the arteries.

Fixation of Arteries

Arteries prepared by the *in vitro* fixation method were dissected out, cleared of fat, connected to micropippets and fixed at 70 mmHg pressure for 1 hour as in our pervious study (Dickhout et al.,1997). The fixative consisted of 3.5% formaldehyde and 0.75% glutaraldehyde in 0.05 mol/L phosphate buffer at pH 7.4.

In arteries prepared by the *in situ* fixation method, after perfusion with sodium nitroprusside, the fixative was introduced by perfusion at 1 mL/min/100 g body weight. This allowed the arteries length to be preserved by their attachment to the connective tissues of the mesentery. In this case, any change in perfusion pressure and in artery diameter was monitored throughout fixation to determine if our procedure altered the vessel from its original state. This was accomplished by attaching a pressure transducer to the perfusion

cannula, and the pressure was recorded using the Digi-Med BP analyzer (Micro-Med, Louisville, KY). A video camera attached to a dissection microscope was used to record the change in blood vessel lumen diameter before and during perfusion. Vessels were fixed for 1 hour in the fixative as above and then removed from the mesentery, cleared of fat, and prepared for confocal microscopy.

Confocal Microscopy

Fixed vessels were stained with either reduced ethidium bromide for overall morphomethric measurements, or with acridine orange for high resolution nuclear observations to determine SMC numerical density and nuclear length. Staining first involved reducing free aldehyde groups by addition of 1 mg/mL of sodium borhydride in Hank's BSS. Half the vessels sampled were stained with reduced ethidium bromide as described earlier,(Dickhout et al.,1997) and the remainder were stained with 50 µg/ml acridine orange (Sigma) in Hank's BSS. All arteries were mounted in 100% glycerol containing 2.5% DABCO (Sigma) as an antifade agent.

A Carl Zeiss LSM 10 system (Carl Zeiss Canada, Don Mills, Ontario) was used for confocal microscopy. The system was equipped with an external argon laser with emission lines at 488 nm and 514 nm and an internal HeNe laser with an emission line at 543 nm. The spectral line at 488 nm produced the best excitation for both the ethidium and acridine dyes with the least non-specific fluorescence, resulting in an optimal signal-to-noise ratio. The 515-565 band pass filter was used to receive signal from the acridine dye and the 575-640 band pass filter was used to receive signal from the ethidium dye.

An 8 second per frame dwell time, and 16 line averaging were used to improve the

image quality in specimens stained with the ethidium dye and images were collected with the 20X objective and a 20X zoom factor to yield a total magnification of 400X. Optical sectioning began at a random distance above the artery and sectioning continued at 10 μm intervals until the artery was completely traversed. Figure 5.1A illustrates a 3-dimensional representation of the artery where optical sections are shown to be taken longitudinally through its volume. The volume (Figure 5.1A) is calculated as the sum (Σ) of the measured area on each optical section over the 1 to N optical sections multiplied by the distance between each slice, dT. Figure 1B illustrates a 3-dimensional reconstruction of the optical sections obtained from a WKY artery stained with the ethidium dye where the total volume of the medial layer was integrated as above.

The acridine orange dye was used since it provided better nuclear detail for the quantification of SMC numerical density. It however suffered from faster photobleaching than the ethidium dye. In this case a 2 second per frame dwell time and 4 line averaging was used to reduce the photobleaching effect. Sections were taken with the 40X objective at a zoom factor of 50X to produce an overall magnification of 2,000X. A reference volume comprising a depth of 20 μm above the mid longitudinal section was used to calculate the numerical density of SMC by the disector method (Gundersen et al.,1988). In each artery, for each fixation method, two such reference volumes were randomly chosen providing replicates of the measure. The optical sections taken through these volumes were separated by 5 μm. Figure 5.2A illustrates an optical section obtained with this method through an SHR artery where the SMC nuclei can be clearly seen. This method allowed SMC nuclei to be followed through the medial layer to determined if they were, or were not shared by

adjacent optical sections (Figure 5.2B). SMC nuclei not shared by adjacent optical sections were counted while moving first up, then down, through the stack. This procedure is analogous to counting either the tops or bottoms of SMC nuclei. Since each nucleus of SMC has only one unique top or bottom this count represents the number of SMC nuclei in a given reference volume. Assuming that each SMC has only one nucleus, this method gives us the number of SMC in a given reference volume. This is a reasonable assumption since in mesenteric arteries from SHR and WKY, the incidence of polyploid SMC was found to be very low (2-4%) and there was no difference between SHR and WKY (Black et al.,1988; Owens et al.,1988). Thus, the numerical density of SMC in the medial layer for either strain, for either method of fixation was calculated. Further, in the *in situ* fixed arteries, exhaustive optical sectioning was preformed to recalculate the numerical density of SMC in the medial layer using optical sections separated by 0.5 μm in order to see if our sampling distance, 5 μm, was adequate to provide proper estimates of the numerical density.

Analysis of Confocal Data

Images were saved to electronic media, and transferred to a microcomputer using Scion Corporation's (Frederick, MD, USA) port to the x86™ architecture of the National Institutes of Health's (NIH, Bethesda, Maryland, USA) NIH Image for measurement of medial volumes, mid-section lumen area, and calculation of SMC numerical density. The number of SMC per unit length of artery was obtained as the product of the volume of the medial layer and the SMC numerical density (Figure 5.1A). The average length of the SMC nuclei was calculated from the *in situ* fixed material where exhaustive optical sectioning was performed, using equation 5.1.

$$\text{Mean Nuclei Length} = (N_t / N_d) \times H_d \text{ .... } (5.1)$$

where N_t = total number of nuclei profiles in top section,
N_d = number of nuclei profiles not extending to bottom section of disector
H_d = depth of dissector reference volume

The length of the SMC was calculated from the optical sections obtained for volume integration by following 10 SMC per artery through the blood vessel wall and determining the change in depth (dz), the change in their position within the wall (dx), and the diameter (D) of the arc within the blood vessel wall that they circumscribed. The length of a unique arc (dL) representing the length of the SMC was calculated using equation 5.2.

$$dL = \pi D \times \frac{2\sin^{-1}\left(\frac{\sqrt{(dx^2+dz^2)}}{D}\right)}{360} \text{ .... } (5.2)$$

Micrographs were obtained from electronic images with a 35 mm camera attached to a video frame printer.

Statistical Analysis

Values given are mean ± SEM. Analysis of the data was performed with a computer-based statistical package SAS® (SAS® Institute Inc.). Analysis of variance (ANOVA) was used to determine if differences existed for the body weight, blood pressure; medial volume, lumen area at mid longitudinal section, and the number of SMC nuclei per 100 micron length of artery. The numerical density of SMC, SMC nuclei length, and SMC length were

compared between the strains by mixed model ANOVA where replicated length measures were continuous variables nested within the strain's fixed effects. Regression analysis was performed to determine if the increase in SHR SMC length could explain the increase in SHR medial volume.

Results

The physiological parameters collected from the animals used for morphometric analysis are listed in Table 5.1. There was no significant difference in age, body weight or blood pressure between the strains. Medial volume per unit length of blood vessel for either method of fixation is illustrated in Figure 5.3A and 5.3B for SHR and WKY. ANOVA revealed that the mean medial volume was significantly larger in SHR (*in vitro*: 16,790 ± 945 $\mu m^3/\mu m$, *in situ*: 15,490 ± 1,160 $\mu m^3/\mu m$) than WKY (*in vitro*: 11,250 ± 673 $\mu m^3/\mu m$, *in situ*: 10,190 ± 671 $\mu m^3/\mu m$) for both methods of fixation. There was no difference in the medial volume obtained with the different methods of fixation for SHR or WKY. Figure 5.3C and 5.3D illustrate the mean lumen area at mid longitudinal section for SHR (*in vitro*: 95,060 ± 3,036 $\mu m^2/500\ \mu m$, *in situ*: 102,860 ± 3,686 $\mu m^2/500\ \mu m$) and WKY (*in vitro*: 95,450 ± 6,103 $\mu m^2/500\ \mu m$, *in situ*: 113,660 ± 7,183 $\mu m^2/500\ \mu m$) in vessels prepared by either method of fixation. ANOVA showed no significant difference between the strains, or between the arteries prepared using different fixation methods within each strain.

Figure 5.4A and 5.4B illustrate the changes in perfusion pressure as well as the changes in lumen diameter for the blood vessels being studied during *in situ* fixation for SHR and WKY. We found that lumen diameter increased with perfusion pressure. The initial

perfusion pressure was far below the physiological pressure in these vessels causing a decrease in lumen diameter. However, the addition of fixative began to increase perfusion pressures to physiological levels as the tissue becomes more rigid and presumably the resistance to flow increased. The lumen diameter eventually reached a level close to that found at the beginning of perfusion fixation (85% in SHR, and 91% in WKY of their *in vivo* lumen diameter).

The values of SMC numerical density from arteries of SHR and WKY fixed by either method are listed in Table 5.2. Arteries from SHR had a significantly smaller SMC numerical density than WKY. When this data was combined with the volume data for a 100 micron length of artery, it was found that the number of SMC per unit length of artery did not differ between SHR and WKY regardless of the method of fixation (Table 5.2).

In the *in situ* fixed arteries, exhaustive optical sectioning was performed to test the reliability of our numerical density sampling method which was based on optical sections separated by 5 microns. The exhaustively sectioned volume allowed us to count every nuclear top or bottom in the reference volume. These results were compared to the results obtained by our sampling method and found to differ by less than 5%, thus demonstrating the validity of our method. A 3-dimensional reconstruction from the exhaustive sectioned material over 10 microns of the reference volume is illustrated in Figure 5.5 showing the appearance of the SMC nuclei within the vessel wall from above (Figure 5.5A) and rotated away from the viewer at an angle of 60 degrees (Figure 5.5B). The exhaustive optical sections were also used to measure the mean length of SMC nuclei. It was found that SHR had significantly longer SMC nuclei than WKY (Table 5.2). The SMC length was also

found to be significantly longer in SHR than WKY (Table 5.2). Regression analysis of SMC length on medial volume found a significant relationship ($p = 0.0012$) and showed that increase in SMC length accounted for 80% of the increase in medial volume (Figure 5.5C).

Discussion

The major findings of this study were as follows. At an age where blood pressure did not significantly differ between SHR and WKY, there was a significant increase in medial volume without a decrease in mid-section lumen area. In the SHR, this increase in medial volume was accompanied by a decrease in numerical density of the SMC within that tissue layer as determined by optical sectioning with confocal microscopy and the application of the disector method. The number of SMC per unit length of mesenteric artery did not differ between the arteries from SHR and WKY. These finding were mirrored in both our *in vitro* fixed arteries where the vessels were pressurized on micropipettes at their *in vivo* pressure, and by our *in situ* fixation method were the vessels were perfusion-fixed while remained attached to the connective tissues thus preventing any distortions in our measurements which may be caused by vessel shorting upon excision. Further, in the *in situ* fixed material exhaustive optically sectioning was preformed to verify our numerical density measurements derived from the disector method based on optical section separated by 5 microns. These results confirmed our findings showing that our sampling method produced results that differed from direct measurements by less than 5%.

In our previous studies, we have determined that a significantly greater number of SMC layers were present in the SHR in comparison to WKY at this age (Dickhout et

al.,1997). This result and other analysis we have done with scanning electron microscopy (Krizmanich et al.,1993), precluded the possibility of a general hypertrophy of the SHR SMC, because SMC width was similar between SHR and WKY at this age. We therefore investigated whether SMC lengthening might explain the greater medial volume and the similar number of SMC cells we have observed in these vessels. Our results supported this hypothesis with our regression analysis revealing that SMC lengthening explained 80% of the medial volume expansion in SHR.

Other studies have revealed similar finding in different models of genetic hypertension. In the mesenteric arteries from the genetically hypertensive New Zealand strain of rats (Ledingham et al.,1993), and in stroke-prone SHR (Arribas et al.,1997), media volume increase and a decrease in the numerical density of SMC as compared with that from normotensives were found. Further, no change in the total number of SMC per unit length of blood vessel was found (Arribas et al.,1997). This is similar to our results in this study. These previous studies, however, did not measure the size of the SMC.

The present finding that an increase in length of the SMC is a contributor to medial wall hypertrophy in the mesenteric arteries from the SHR, is different from both our previous study (Lee, 1987), and studies by others (Mulvany et al.,1985), which concluded that hyperplasia of the SMC was responsible for the increase in medial volume. In our previous study, the method used to determine if the increase in medial thickness as observed in SHR was due to hypertrophy or hyperplasia was to calculate the volume to surface ratio of SMC. This measure could be preformed on randomly oriented sections without the necessity of serial reconstruction. It was hypothesized that a general hypertrophy of the SMC would lead

to an increase of the volume to surface ratio. This is true for any equidistant solid when it increases in size, such as a sphere. We also made one important assumption, that there is no change in SMC length, based on a previous report that SMC length was similar between the mesenteric arteries from SHR and WKY (Warshaw et al.,1979). Further modeling however, revealed that the use of volume to surface ratio is not appropriate for elongated solids such as the SMC. In Figure 5.6A, a SMC is closely approximated by two cones of some radius (R) and some height (H). At any constant radius, increasing H past a certain point would no longer result in an increase in volume to surface ratio (Figure 5.6B), showing that volume to surface ratio is not a sensitive measure of SMC length change. On the other hand, at a constant length, volume to surface ratio is very reliable in detecting the increase in the radius of the cells (Figure 5.6C). Since we found no change in volume to surface ratio we concluded that medial SMC could not be increase in size, so they had to be increased in number (Lee, 1987). However, this new analysis reveals that they may also have been increased in length.

Our findings also differ from those of Mulvany et al where it was concluded that SMC hyperplasia was responsible for media volume increase (Mulvany et al.,1985). In this study, tertiary branches of the superior mesenteric artery from 19 week old SHR and WKY were wire mounted with a radially applied force estimated to represent a transmural pressure of 100 mmHg and fixed for morphometric measurements using a disector method. A series of 1 micron thick serial sections were used to produce a reference volume for the disector. These results may differ from our study due to the different age of animals used, the different order of mesenteric artery branch used, or the different methods employed.

Discussion of Methods

Serial sectioning by mechanical means suffers from variable section thickness, lack of precision of measurement over small distances (< 1 μm), and the exhaustive nature of the process. The ability of confocal microscopy to resolve structure more easily in three dimensions may be of particular importance in the analysis of arterial structure as it relates to hypertension (Baumbach et al.,1993). One major criticism, however, of the currently available confocal microscopes, is the lack of a transmission mode of operation (Gundersen et al.,1988). As such, dyes generally used for light microscopy which increase the optical density of certain elements of the cell, usually the nuclei and structural proteins in the cytoplasm, are useless in the confocal microscope. The lack of a fluorescent dye which provides clear general histology has seriously limited the use of this instrument, and forced those willing to take up its use to resort to such devices as non-specific fluorescence induced by glutaraldehyde fixation for image generation (Baumbach et al.,1993). We have found that reduced ethidium bromide is an ideal fluorescent stain to provide nuclear contrast in confocal microscopy (Dickhout et al.,1997). It is excited at 510 nm in the range of argon lasers commonly used in confocal microscopes and the chemical reduction of ethidium bromide greatly increased its utility as a nuclear fluorophore by increasing its permeability across the cellular membrane, allowing its use in whole mount tissues. Moreover, the long laser exposures necessary to produce many optical sections were only possible due to the low photobleaching properties of the ethidium dye.

Vessel shortening upon excision is a significant problem faced by any investigator who is attempting to quantify some feature per unit length of blood vessel such as tissue

volume or cell number. This shortening of the vessel makes the calculation of effective 'pressures' in a wire style myograph prone to errors, since this apparatus applies only radial force, whereas true intramural pressure applies forces in all directions. This may produce systemic errors of measurement if the vessels being compared shorten to different degrees on the wire mounted preparation. This type of difference between SHR and WKY vessel measurements in pressurized and wire-mounted apparatus has been previously noted (Lew et al.,1992). The elastic modulus of the artery wall has been shown to be considerably less in the longitudinal directions. In canine femoral artery, it was found to be five times less in the longitudinal direction, resulting in mostly lengthening of artery segments *in vitro* with increasing pressure (McDonald, 1974). The estimates of volume, and numerical density of SMC normalized to specific length of artery segments mounted and fixed on a wire myograph (Mulvany et al.,1985), is prone to large errors due to changes in length from that of the vessel in the animal.

In conclusion, we have demonstrated in this study that medial volume expansion occurs in pre-hypertensive SHR as compared to their normotensive WKY controls. This medial volume expansion, which we have previously shown to be accompanied by a greater contractile response of the SHR arteries to various agonists (Dickhout et al.,1997), is accounted for by a lengthening of the SHR SMC up to the 80% level. These are primary changes in the SHR small muscular arteries which may lead to the eventual development of hypertension in these animals through an increase in total peripheral resistance.

Acknowledgement

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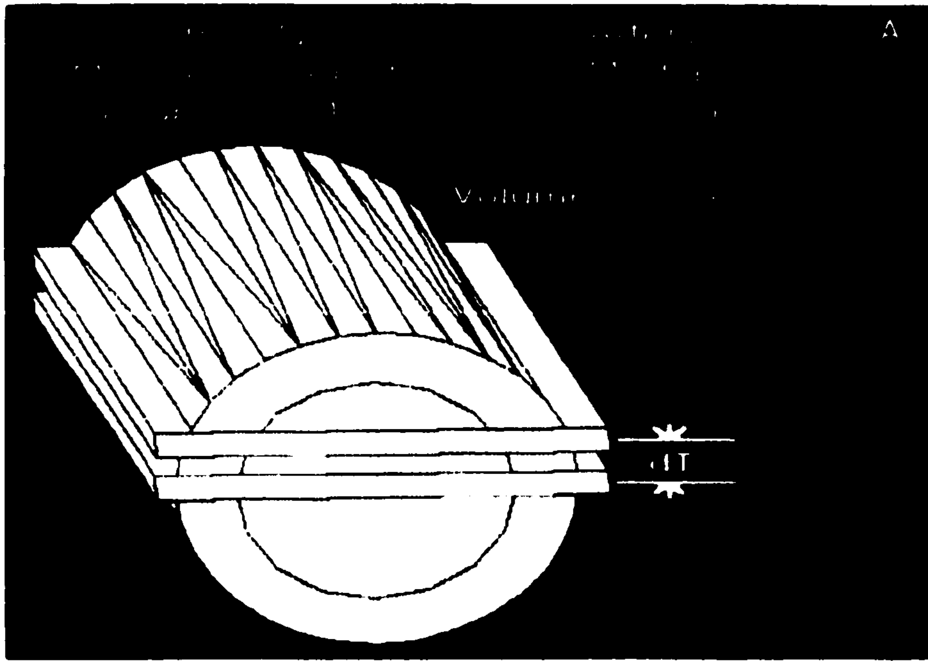
Figure 5.1. A. Model of a blood vessel illustrating how optical sections were cut longitudinally through the arteries and the volume of the media layer and cell number calculated. B illustrates an actual WKY large mesenteric artery which was reconstructed from optical sections used to produce the medial volume measurement.



B

Figure 5.2. Acridine orange labeling of smooth muscle nuclei in medial layer of SHR large mesenteric artery (A). Numerical density of medial smooth muscle cells was determined by the disector method of nuclear counting where optical sections separated by a known distance, in this case 5 μm, were used to form a reference volume. Nuclei shared by both frames were not counted. Unshared nuclei were counted (B). The numerical density of smooth muscle cells was so determined, then multiplied by the volume of the medial layer in a given length of artery to produce the total number of cells in that artery length.

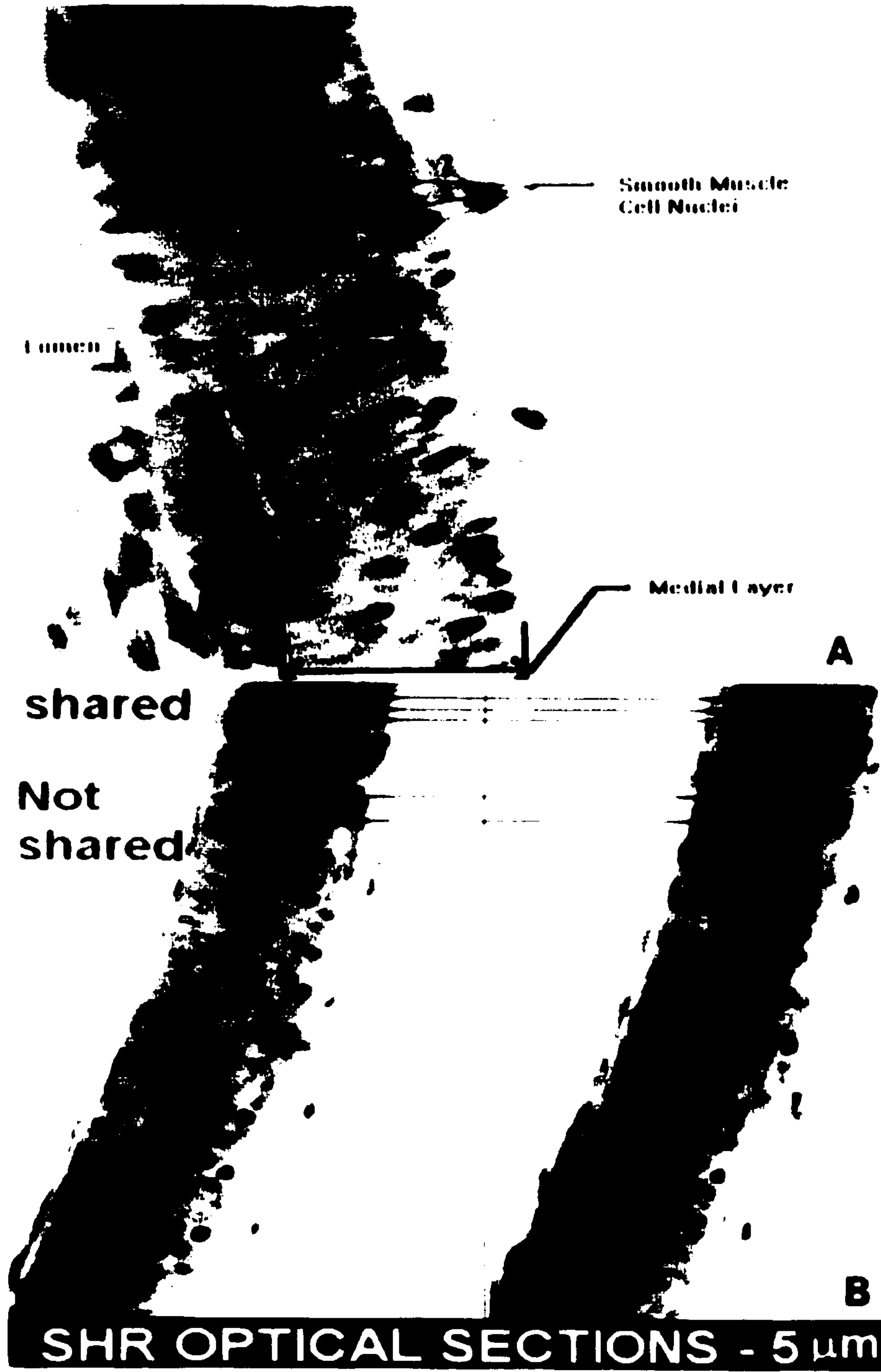
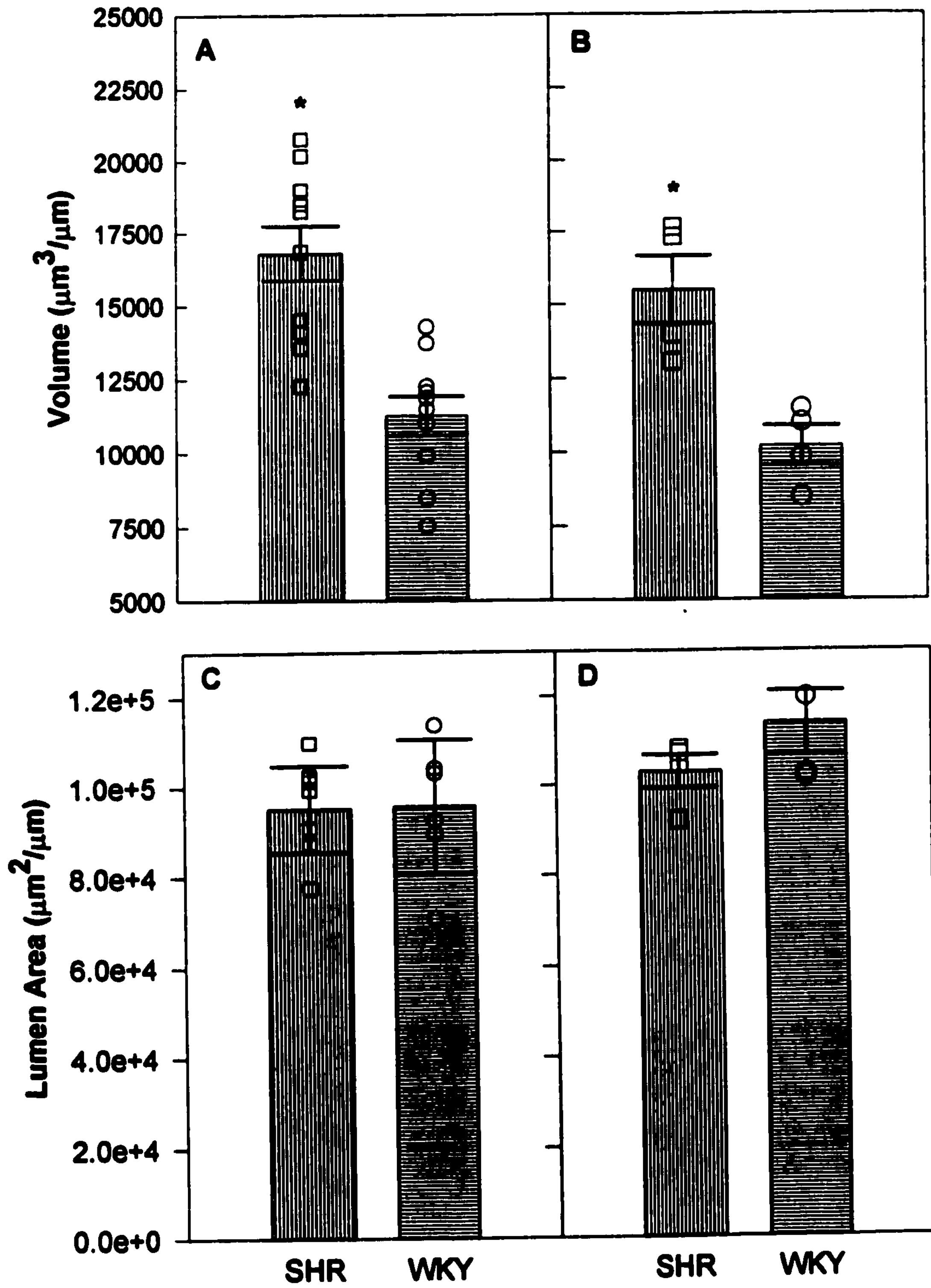

Smooth Muscle
Cell Nuclei
Lumen
Medial Layer
A
shared
Not
shared
B
SHR OPTICAL SECTIONS - 5 μm

Figure 5.3. A comparison between SHR (□) and WKY (○) on medial volume per unit length ($\mu m^3/\mu m$) of SHR for the in vitro (A) and in situ (B) fixation methods, the lumen area per unit length (μm^2/ 500 μm) on the mid-longitudinal optical sections in the in vitro (C) and in situ (D) fixation methods.



A
B
C
D
Volume (μm³/μm)
25000
22500
20000
17500
15000
12500
10000
7500
5000
Lumen Area (μm²/μm)
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0e+0
SHR
WKY
SHR
WKY

Figure 5.4. Changes in perfusion pressure during the course of *in situ* fixation and the corresponding changes in lumen diameter of the blood vessels before and during perfusion fixation.

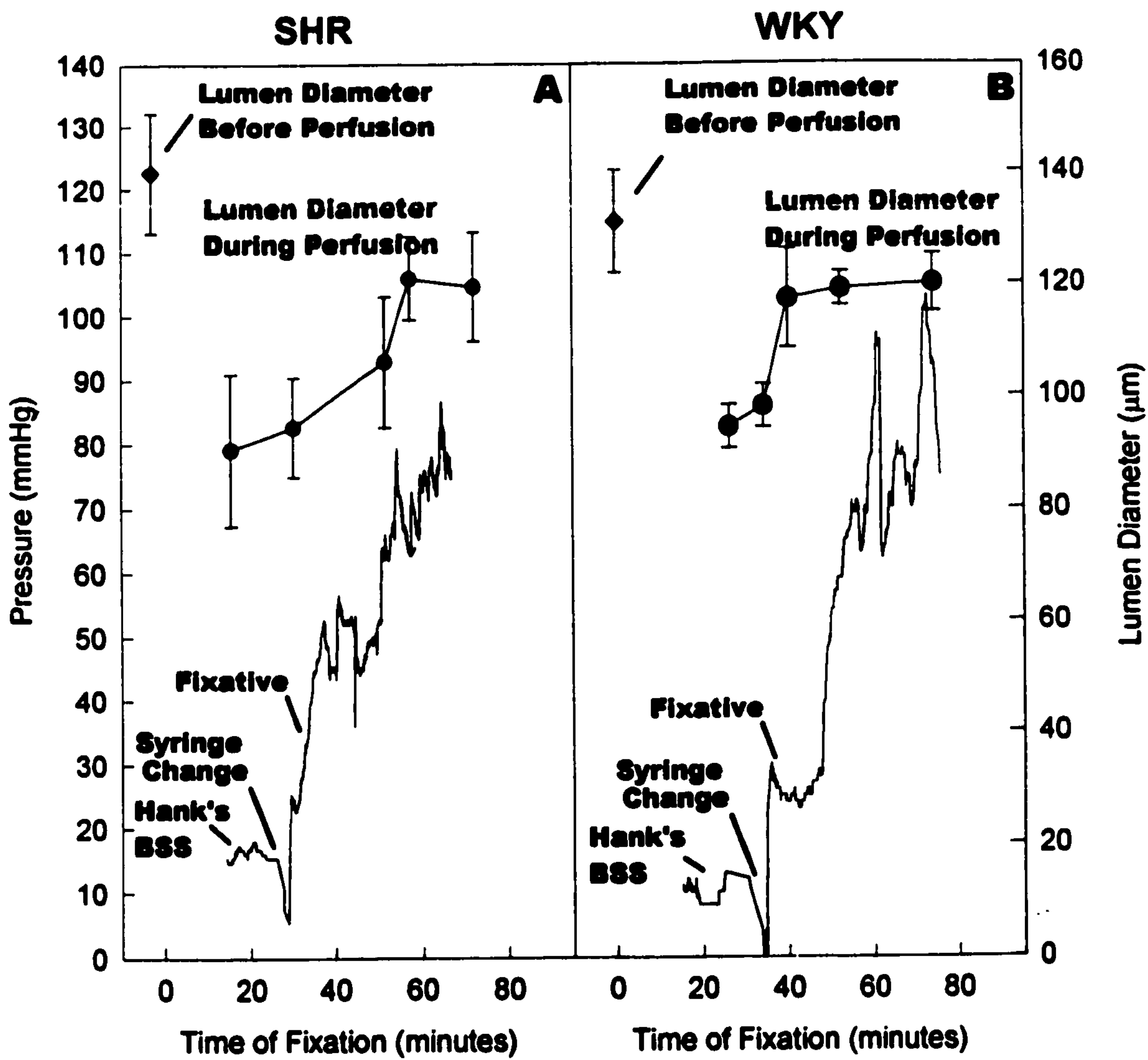
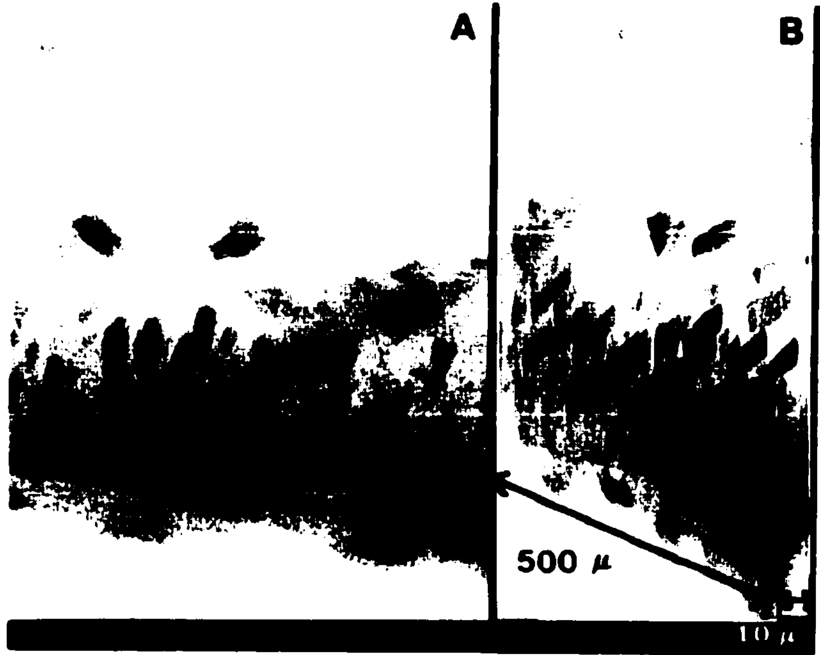

SHR
WKY
A
B
Lumen Diameter Before Perfusion
Lumen Diameter During Perfusion
Fixative
Syringe Change
Hank's BSS
Pressure (mmHg)
Lumen Diameter (µm)
Time of Fixation (minutes)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
160
140
120
100
80
60
40
20
0
0
20
40
60
80

Figure 5.5. 3D-reconstruction of exhaustive optical sections illustrating the reference volume used to determine smooth muscle cell numerical density for a WKY artery seen from above (A), and rotated away from the view at 60° (B). (C) Graph detailing the relationship between smooth muscle cell length and medial volume for the *in situ* fixed vessels.


A
B
500 μ
10 μ

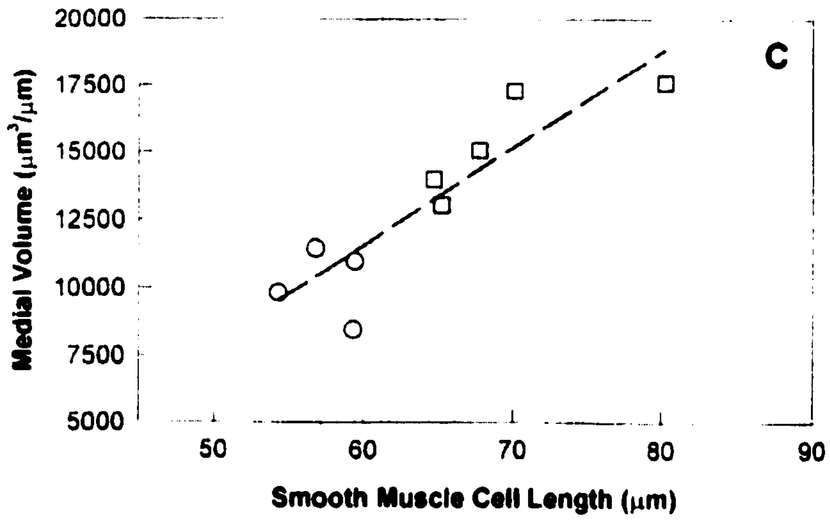
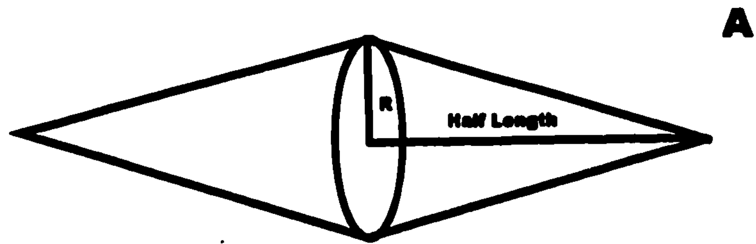
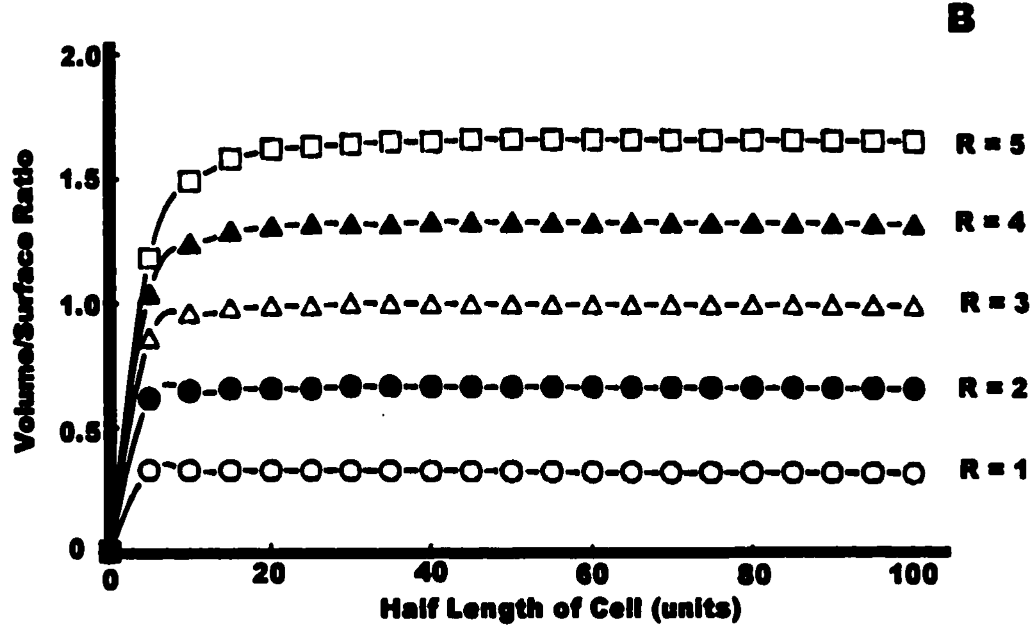

C
20000
17500
15000
12500
10000
7500
5000
Medial Volume (μm³/μm)
50
60
70
80
90
Smooth Muscle Cell Length (μm)

Figure 5.6. Geometric model used to represent medial smooth muscle cells as two regular cones placed base to base where R is the radius of the circle which describes the cone's base, and H is the height of the cone (A). The relationship between cone height and volume to surface ratio at fixed radii of R=1,2,3,4,5 is shown in B, and the relationship between cone radius and volume to surface ratio at a fixed height is shown in C.


A
R
Half Length


B
2.0
1.5
1.0
0.5
0
Volume/Surface Ratio
R = 5
R = 4
R = 3
R = 2
R = 1
0
20
40
60
80
100
Half Length of Cell (units)

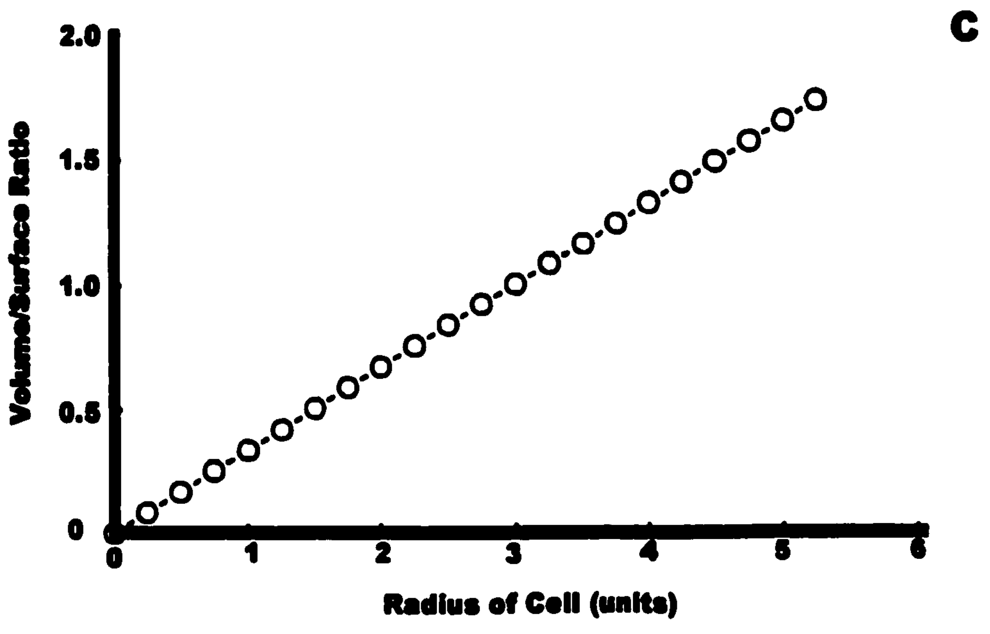

C
2.0
1.5
1.0
0.5
0
Volume/Surface Ratio
0
1
2
3
4
5
6
Radius of Cell (units)

Table 5.1: Physiological Characteristics of Animals used for Study

	In Vitro Fixation			*In Situ* Fixation		
Strain	Age (days)	Body Weight (g)	Blood Pressure (mmHg)	Age (days)	Body Weight (g)	Blood Pressure (mmHg)
SHR	28 ± 0.1 (10)	73.4 ± 5.0 (10)	115 ± 4.8 (10)	29.0 ± 0.2 (6)	61.0 ± 2.5 (6)	140 ± 6.1 (6)
WKY	28 ± 0.2 (7)	66.3 ± 3.4 (7)	110 ± 3.9 (7)	29.0 ± 0.3 (5)	62.0 ± 2.3 (5)	122 ± 6.5 (5)

Values are means ± SEM. The physiological characteristics were compared between the strains by ANOVA. Significance was tested at the 5%-level. There was no significant differences between the strains. Numbers in parentheses are the number of animals in each group.

Table 5.2: Medial Smooth Muscle Cell Number and Characteristics

Strain	SHR		WKY		P-Level SHR vs. WKY	
Fixation Method	*In vitro*	*In situ*	*In vitro*	*In situ*	*In vitro*	*In situ*
Numerical density	0.64 ± 0.08 (10)	0.74 ± 0.06 (6)	1.2 ± 0.2 (7)	1.2 ± 0.1 (5)	ρ<0.01	ρ<0.01
Cell number	1110 ± 120 (10)	1050 ± 110 (6)	1370 ± 120 (7)	1030 ± 80 (5)	NS	NS
Nuclei length		24.8 ± 1.9 (6)		17.6 ± 0.3 (5)		ρ=0.01
Cell length		70 ± 1.1 (5)		57 ± 0.9 (4)		ρ=0.01

Values are means ± SEM. Numerical density is expressed as # of Cells /μm³. Cell number was the product of numerical density and the medial volume of a 100 micron segment of artery producing the number of cells in the 100 micron segment. Nuclei length was determined from equation 1 and cell length from equation 2. All lengths are expressed in microns. Differences between the strains were tested by ANOVA at the 5%-level. NS = not significant. Numbers in parentheses are the number of animals in each group.

Chapter 6. General Discussion and Conclusions

6.1 Introduction

Our use of SHR as an animal model of human essential hypertension has allowed a detailed study of the changes that lead to the development of hypertension. It also allowed us to study muscular artery structure and function during hypertension development and examine cellular processes that may have led to the structural and functional differences of the hypertensive blood vessels. This work combined with extensive evidence in the literature has led us to postulate a structural basis for spontaneous hypertension in the SHR, and to examine data collected from human essential hypertensives to determine if the model accurately reflects the situation in at least some forms of the human disease.

Our first hypothesis was that the structural change in the small muscular arteries of the SHR is a primary process in the development of hypertension in this animal model leading to an increase in TPR and high BP. We have demonstrated that the structural change in the small muscular arteries of the SHR mesentery is a primary process since it predates the elevation of BP in SHR. These modified arteries contract to a greater degree than those from normotensive WKY rats at a given pressure. This greater degree of contraction results in a smaller lumen diameter and therefore increases the resistance of the blood vessels to flow as described in equation 1.2. This increase in resistance to blood flow would contribute to

an increase in peripheral resistance as observed in this model by hindquarter perfusion (Albrecht et al.,1975) as well as observed in human essential hypertensives by forearm resistance measurements (Pedrinelli et al.,1997). Higher BP would then result from the increase in TPR as detailed in equation 1.1. We further hypothesized that structural change involves an increase in the smooth muscle of the medial layer in small muscular arteries and may be the result of hypertrophy or hyperplasia of the smooth muscle cells. The increase in medial smooth muscle volume was demonstrated by our confocal measurements in the SHR as compared to the WKY controls. The reason for this medial volume expansion was found to be primarily the result of smooth muscle cell lengthening and not an increase in cell number. The number of smooth muscle cells in the SHR and WKY arteries remained similar. This smooth muscle cell lengthening increases the degree of arc these cells wrap around the hypertensive vessels in the relaxed state, and may lead to the greater lumen occlusion we see in these vessels under stimulation.

Our last hypothesis was that a different incidence of apoptosis occurs in these blood vessels during their development. This may result in structural differences between hypertensive and normotensive vessels. We did find that apoptosis occurs during the development of these blood vessels in both the SHR and its normotensive control, WKY, at 1-2 weeks of age. Further, there was a differential incidence of apoptosis between SHR and WKY which predates structural modification of the SHR blood vessels and so may be a contributing factor to structural change. These finding will now be discussed in more detail while considering their relevance to the development of hypertension in the SHR and the similarities of this process to human essential hypertension.

6.2 Summary of Results

a) Blood pressure and heart rate development in young SHR

(Refer to Chapter 2.)

In this study, the course of hypertension development in young SHR was examined by the measurement of changes in systolic BP, body weight, and heart rate at two, three, four, and six weeks of age by the comparison of inbreeding lines of SHR and WKY. We found that SHR rats had a lower body weight than WKY rats at 2 and 3 weeks of age. BP began to diverge between SHR and WKY at four weeks of age. Heart rate was elevated in SHR over age-matched WKY at three weeks of age and positively correlated to the level of BP attained by individual animals at six weeks. The prehypertensive tachycardia in SHR was investigated further and found to result from an increased intrinsic HR with a minor sympathetic component.

The lower body weight of SHR at 2 and 3 weeks of age is similar to low birth weight found in the offspring of human hypertensive mothers (Stevenson, 1977). This may be due to the reduction in placental circulation found in hypertensive mothers, and the resultant reduction of nutrient as well as oxygen delivery to the fetus (Stevenson, 1977). The fetus must adapt to this reduction in maternal blood supply and the intrauterine environment of the hypertensive mother, according to Barker and Godfrey (Barker, 1998; Godfrey, 1998), may predispose the fetus to hypertension in adult life.

In this scenario, the hypertensive trait would not be coded for in the genome as a single, or even multiple genetic trait. Hypertension would be passed from mother to offspring and be manifest regardless of external environmental conditions due to the effect

of the *in utero* environment. In a random breeding population this effect may be minimized as hypertensive mothers are less likely to mate with hypertensive males due to the low frequency of the disease in the population. In the development of the SHR population, however, selection of hypertensives to form mating pairs was carried out to produce a uniformly hypertensive population. Then breeding continued by bother/sister mating thus exacerbating the effect of material environment on offspring.

In the case of hypertension resulting from the influence of the maternal environment the patterns of inheritance would not show a Mendelian character. The F2 generation of a cross between hypertensive and normotensive parents would show a continuous gradation of the hypertensive trait from normal to high BP instead of showing increments of hypertension consistent with the number of hypertensive genes the offspring inherited. When hypertensive/normotensive cross studies were done with SHR, it was found that the F2 generation of large crosses shows a continuous gradation of the hypertensive trait (Myers, 1991; Harrap, 1986). Moreover, low birth weight follows the higher BP in the F2 progeny (Myers, 1991).

Another approach to investigate the relationship between the hypertensive *in utero* environment and the outcome of hypertension in the offspring is to normalize the hypertension in the mother and determine if the trait is expressed. The persistent effect of ACE inhibitors even after the withdrawal of treatment makes them an ideal candidate for such a study allowing the reduction of material BP long after any drug would be cleared from the system. Such a study in the SHR demonstrated that mothers treated with the ACE inhibitor captopril showed persistent BP lowering 1 month after withdrawal of the treatment,

mean arterial pressure reduced from 166 ± 3 to 126 ± 3 mmHg (Wu et al.,1993). This BP lowering effect was passed on to the second generation despite the fact they were never exposed to the drug. Mean arterial pressure was significantly lower in second generation, 137 ± 2 mmHg, than in non-treated SHR controls, 166 ± 3 mmHg (Wu et al.,1993). These findings suggest that hypertension in the SHR is at least in part due to the hypertensive *in utero* environment which may produce profound changes in the vascular structure of the developing fetus.

The finding of a divergence of BP at 4 weeks of age between the strains directs our future investigations to test the hypothesis that the structural change in the small muscular arteries of the SHR is a primary process leading to hypertension in the SHR. Since, if structural change is present in the SHR mesenteric arteries at an age that predates BP elevation it can not be the consequence of BP elevation. Instead it may be due to other developmental processes as discussed earlier.

The finding that heart rate elevation predates BP elevation in the SHR as compared to its normotensive control WKY, and that the elevated heart rate phenotype in the young rats co-segregates with the elevated BP phenotype in the adult, suggests that increased cardiac output in the SHR may be an important trigger in the development of hypertension. First order mesenteric vessels which have undergone experimental conditions of increased blood flow develop larger wall and medial cross-sectional areas and display increased contractile properties (Pourageaud et al.,1997). Increased flow in the small muscular arteries of SHR brought about by higher heart rates increasing cardiac output may have an influence on vascular structure and so result in structural differences between SHR and WKY vessels.

b) Structural and Functional Analysis of Small Arteries from Young SHR

(Refer to Chapter 3.)

Based on the previous consideration, structural and functional changes in the small muscular arteries from the mesenteric vascular bed of 3-4-week old SHR and WKY normotensive control rats were examined. As found previously, systolic BP of SHR and WKY rats were similar at 4 weeks (Dickhout et al.,1997). However, significant structural changes in the mesenteric vessels of the SHR were already present. Arteries fixed under pressure *in vitro* from SHR had a larger medial volume, increased number of smooth muscle cell layers, but similar lumen size when compared with WKY in the maximally relaxed condition. These structural changes are primary changes because they are present at an age when BP was similar between SHR and WKY.

In a "Debate on the Role of Resistance Arteries in Hypertension"; five conditions for establishing causality of a defect in the genesis of hypertension were put forward (Korner et al.,1991). They are : (1) the defect should be present at the initiation of hypertension, (2) the defect should cosegregate with high BP in normotensive/hypertensive hybrid crosses, (3) correction of the abnormality should correct the hypertension, (4) correction of the hypertension should not completely correct the defect, (5) there should be a plausible relationship between the defect and hypertension. I will now discuss the findings in the SHR regarding structure of the arteries in reference to these 5 conditions. Our finding of primary structural changes in the SHR small muscular arteries satisfies one of the five conditions for establishing causality of a defect in the genesis of hypertension since the defect is present at the initiation of hypertension. There is also a plausible relationship between the defect and

hypertension. This relationship being that structurally modified vessels lead to greater contractility under agonist stimulation increasing TPR and so BP. However, this leaves three other of the conditions unsatisfied.

Turning to the literature it is yet to be demonstrated that structural change in the small muscular arteries at the initiation of hypertension as we found in the SHR cosegregates with higher BP in normotensive/hypertensive hybrid crosses. Indeed evidence exists that cardiac hypertrophy does not cosegregate with higher BP in SHR and WKY crosses (Grassi de Gende, 1988; Harrap et al.,1997). This lack of evidence leaves open the possibility that the type of structural abnormalities we describe in the SHR's small muscular arteries are merely incidental differences between the SHR and WKY, not differences that led to the development of higher BP. It may also be that structurally modified small muscular arteries are only one of the necessary conditions for the genesis of hypertension in the SHR. Thus, if this trait is maintained in a hypertensive/normotensive cross but the other conditions are not, hypertension will not occur.

Evidence does exist to show that reversal of the structural abnormalities in the small muscular arteries of SHR reverses the hypertension. This is particularly clear with ACE inhibitor treatment where both a permanent reduction in medial hypertrophy and permanent lowering of BP occurs in the SHR after withdrawal of drug treatment (Harrap et al.,1990; Lee et al.,1991; Morishita et al.,1992; Lee et al.,1997). Further, the lowering of BP with other drugs such as the vasodilator hydralazine which does not affect the structure of resistance vessels in the SHR does not reverse the hypertension after treatment withdrawal (Smeda et al.,1991; Soltis, 1993).

One paradoxical finding in my study was that at 3-4 weeks of age structurally modified vessels exist in the SHR without an increased BP. One may put this forward as evidence that such a defect is not sufficient to cause hypertension. Our further finding that TTX-sensitive contractile responses to electrical field stimulation were absent at 2-weeks and less frequent in SHR than WKY at 3- and 4-weeks of age provides an explanation to this apparent contradiction (Dickhout et al.,1997). It is apparent that a lag in the functional maturation of the sympathetic innervation of the SHR arteries prevents the high BP phenotype from becoming fully manifest. Once the SHR animals reached six weeks of age, all arteries responded to electrical field stimulation (Dickhout et al.,1997) and hypertension was manifested (Dickhout et al.,1998a).

The finding of a corresponding increase in smooth muscle cell layers with medial volume expansion (Dickhout et al.,1997) suggests either an increase in smooth muscle cell number, or a greater smooth muscle cell length. It excludes an increase in the width of the smooth muscle cells since wider cells composing a larger volume would not produce a greater number of cell layers. An increase in cell length causing extra wrapping of individual smooth muscle cells around the blood vessel lumen, or an increase in cell number, or a combination of these two factors, would explain the increase in cell layers. Such an increase in smooth muscle cell layers could result in the greater contractile response of the SHR arteries. This larger smooth muscle mass would allow the arteries to generate more force to act against the pressure within the lumen. The cellular aspect of the vessel wall hypertrophy needs to be examined further to ascertain its nature (see 6.2 d, and also Chapter 5).

c) Apoptosis in the Muscular Arteries from Young Spontaneously Hypertensive Rats

(Refer to Chapter 4.)

In this study, we examined the incidence of apoptotic cells in the blood vessel wall of small muscular arteries from the SHR and its normotensive control the WKY rat at 1-2 weeks of age using confocal microscopy. Our hypothesis was that differences in the incidence of apoptosis in early arterial development between the hypertensive and normotensive strains contributes to the structural differences in blood vessels found between these rats at a later stage in the animal's development. In order to measure the incidence of apoptosis, we used a 3'-OH end labeling method where the enzyme terminal deoxynucleotidyl transferase catalyzes the addition of fluorescein-conjugated nucleotides to the cut ends of DNA to detect DNA fragmentation. This method allowed us to quantify apoptosis with relatively small amounts of tissue and precisely identify microscopically the cell and tissue type origin of the apoptotic cells. The blood vessel's structural components were also measured by optical sectioning with the confocal microscope.

We found that arteries from SHR rats had a significantly decreased incidence of cellular apoptosis over WKY in both the adventitia and the media layers, but the volumes of the adventitia, and media layers and the lumen size were similar between the strains in this age range. These findings suggest that the differential incidence of cellular apoptosis found at 1-2 weeks of age may be at least in part responsible for the larger media volumes found in older SHR and thus contributes to the development of structurally modified blood vessels.

The incidence of apoptosis present in the muscular arteries of 1-2-week-old WKY was relatively small when compared with the total cell number. On average this incidence

was 7.2 cells in the adventitia and 11.2 cells in the media per 500 microns of artery length (Dickhout et al.,1999). Although it was not possible to measure directly the total number of cells in these arteries, our work on the 4-week vessels revealed that the total number of smooth muscle cells was approximately 5000 per 500 micron length of artery (Dickhout et al.,1998b). If one assumes approximately equal cell size between the 1-2 week blood vessels and the 4-week blood vessels and takes into account the difference in vessel wall volume found, a result of between 2000 - 3000 cells per 500 micron length of artery is obtained. So the 20 apoptotic cells per 500 micron length found in the WKY artery would result in 1% of the cells in apoptosis at any time. The rate of disappearance of apoptotic cells in this vascular system is unknown. If the time of disappearance of apoptotic cells is long, in the range of 48-72 hours, the difference in the incidence of apoptosis we have found at 1-2 weeks may have little affect on vessel wall hypertrophy in the SHR. However, if the time of disappearance is short (e.g. 1-2 hours) and the difference in incidence persists for days or weeks, apoptosis may have a profound affect on vessel wall hypertrophy in the SHR.

d) Increased medial smooth muscle cells length is responsible for vascular hypertrophy in young SHR. ***(Refer to Chapter 5.)***

Our previous studies have demonstrated that large mesenteric arteries from 3-4-week-old SHR showed medial volume hypertrophy accompanied by an increased contractile response to various agonists before significant BP increase (6.2 b). In this study, we determined the cellular changes which contribute to this vascular hypertrophy. Numerical density of smooth muscle cell nuclei in the medial layer was assessed with a three dimensional disector method under confocal microscopy using the nuclear stain acridine

orange. The numerical density of medial smooth muscle cells was significantly less in SHR than WKY, and the total number of smooth muscle cells per 100 micron length of artery were similar between SHR and WKY. The average nuclear length (24.8 ± 1.9 μm) and smooth muscle cell length (70 ± 1.1 μm) from SHR was significantly longer than WKY nuclear (17.6 ± 0.3 μm, $p = 0.01$) and smooth muscle cell length (57 ± 0.9 μm,$p < 0.01$). Using regression analysis we determined that the increase in smooth muscle cell length explained 80% of the medial volume increase. We therefore concluded that an increase in smooth muscle cell length in SHR at the pre-hypertensive phase of hypertension development is responsible for the increased medial volume, and an increase in the number of smooth muscle cell layers.

A lengthened smooth muscle cell may increase the contractility of the artery by being able to shorten to a greater degree. Smooth muscle cells that have been induced to lengthen in cell culture by serum deprivation have shown greater contractile ability in comparison to freshly isolated cells (Ma et al.,1998). Smooth muscle cells in culture undergoing cell division loose much of their contractile ability (Ma et al.,1998). Skinned myometrial smooth muscle fibers with hypertrophied smooth muscle cells were also able to generate greater maximal force than fibers without cell hypertrophy (Morano et al.,1997). This increased force generation was associated with a 2-fold increased expression of the myosin light chain isoform over normal tissue (Morano et al.,1997). This evidence suggest that both smooth muscle cell hypertrophy and proliferation affect the cell's contractile ability by changing the production of contractile proteins. Cell division decreases contractile protein production and cell hypertrophy increases it.

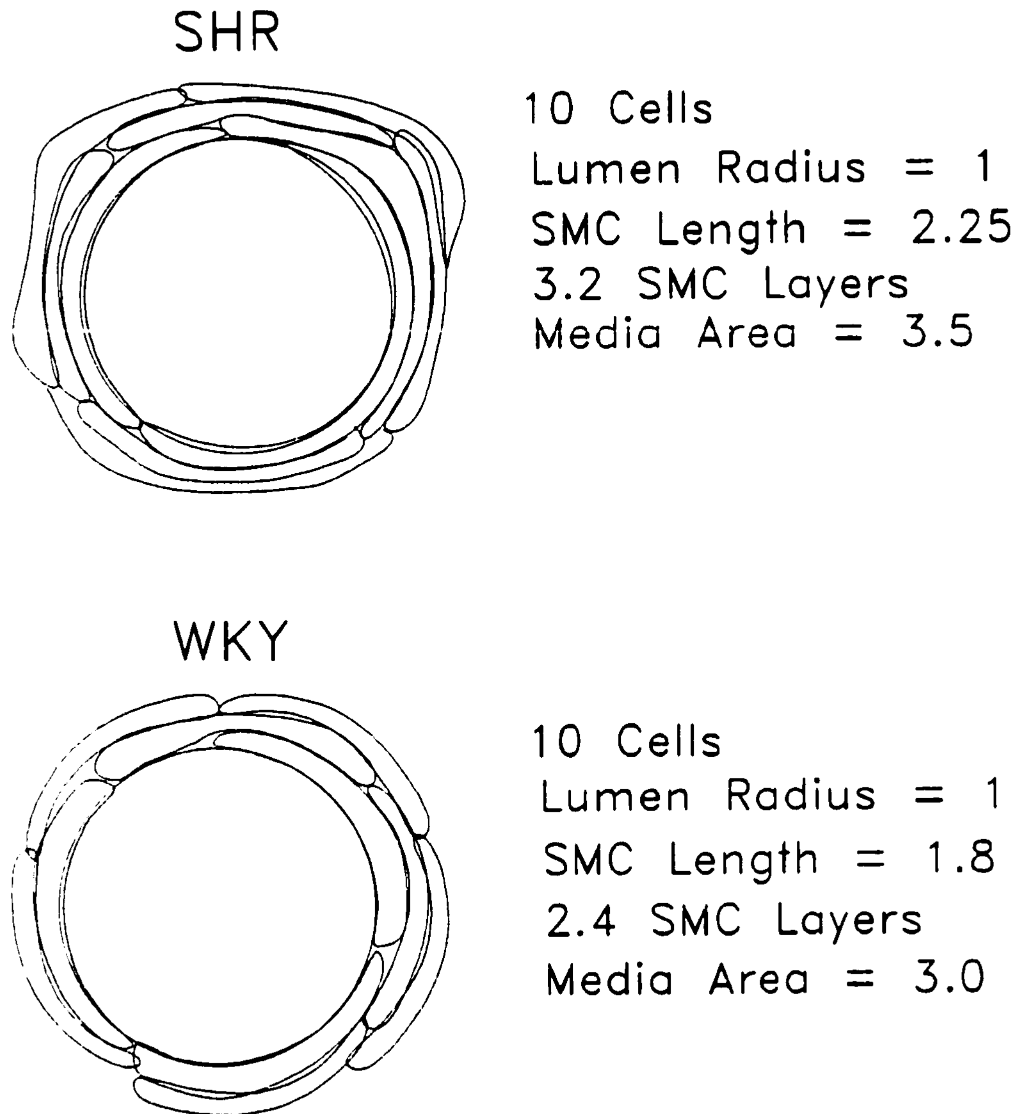


Figure 6.1. Model of SHR structurally modified blood vessels.

This figure illustrates a model of the SHR structurally modified small muscular artery which is compared to the WKY vessels. Both vessels are composed of the same number of smooth muscle cells which wrap around a lumen of the same radius, R=1. However, the SHR smooth muscle cells, SMC, are longer than WKY SMC in the same ratio we have found in the four week animals. This produces a greater number of SMC layers in the SHR vessel (3.2) than the WKY (2.4) and greater medial cross-sectional area.

6.3 Overall model of the hypertensive artery and basis for developing increased BP

The overall model of the hypertensive artery in the SHR we are presenting is a blood vessel with an increased medial volume due to a larger number of smooth muscle cell layers, consisting of longer smooth muscle cells which wrap around the vessel to a greater degree of arc, and cause greater lumen diameter reduction during contraction. A model of this SHR blood vessel is illustrated in figure 6.1 in comparison to the WKY blood vessel. Both vessels have the same lumen sizes and each vessel consists of the same number of cells. However, the smooth muscle cells in the SHR vessel are longer to the same proportions we have found them to be longer in the 4-week rats. As such, they wrap around the similarly sized lumen to a greater degree of arc thus creating a greater number of smooth muscle cell layer.

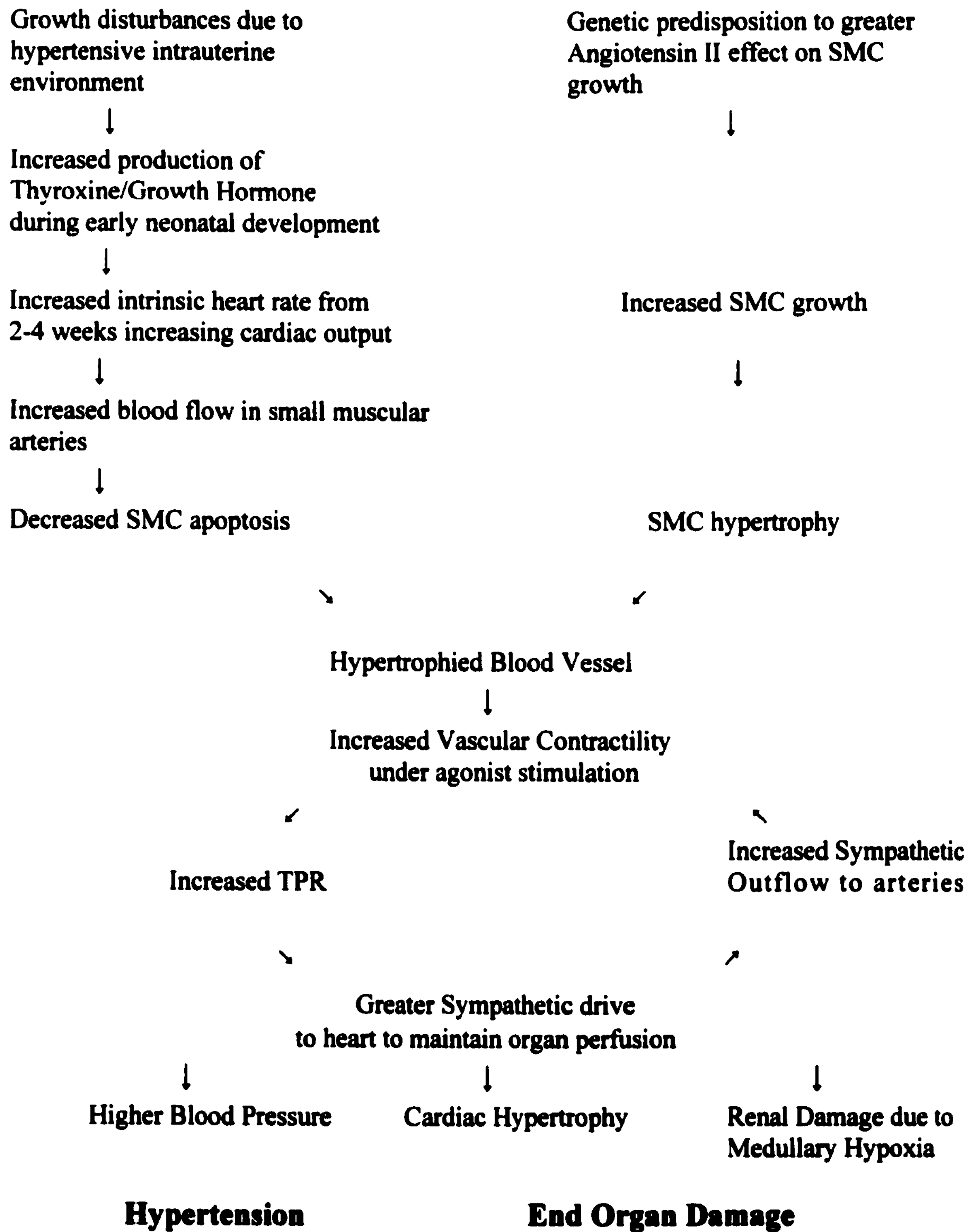
This structural modification of the SHR vessel may produce greater contraction in one or more of the following ways. The greater smooth muscle cell volume in the medial layer of the SHR artery represents a greater amount of contractile apparatus. This greater amount of contractile apparatus would be able to generate more force and so reduce the lumen of the SHR vessel to a greater degree than that of the WKY vessel at a given pressure. It may also be that SHR smooth muscle cells that wrap around the lumen to a greater degree of arc can shorten to a greater extent due to an enhanced length tension relationship at a given pressure. The larger smooth muscle cells may also have a greater number of receptor binding sites to contractile agonists on their cell surface. This may produce a higher level of smooth muscle cell stimulation with agonist exposure and so greater contraction. Any of these mechanisms or some combination of them would result in the greater contractility we have observed in the SHR vessels (Dickhout et al.,1997). This greater small vessel

contractility would result in the higher total peripheral resistance seen in the SHR model and explain the need for these rats to develop higher BP to maintain adequate organ perfusion.

Various hormonal factors may mediate these structural changes in the developing vessels. One potential mediator is angiotensin II. This peptide is a growth factor causing vessel wall hypertrophy (Lever et al.,1992). Inhibition of the production and action of this peptide have both shown strong anti-hypertensive effects in the SHR as well as in human essential hypertensives. The angiotensin converting enzyme inhibitors captopril (Lee et al.,1991) and the AT_1 receptor antagonist losartan (Li et al.,1997) correct BP, even after withdrawal of treatment, and correct differences in vascular structure between hypertensives and normotensives in the SHR model and in human essential hypertension (Schiffrin, 1996). Further, studies in normotensive Sprague Dawley rats demonstrated that angiotensin II infusion for 4 or 12-weeks results in a dose-dependent increase in BP up to a level of 180 mmHg systolic at a dose of 200 ng per kg per min (Simon et al.,1998). This infusion also produces structurally modified blood vessels with greater medial thickness due to smooth muscle cell hypertrophy (Simon et al.,1998). This suggests that angiotensin II may be an important hormonal factor mediating the structural change of hypertensive blood vessels.

An overall model of the genesis of hypertension in the SHR based on the findings of our research and additional information obtained from the literature is illustrated in figure 6.2 (see page 176). This figure details the central role that structurally modified small muscular arteries play in the genesis of hypertension in the SHR. Describing its progression, the lower than normal weight of SHR pups from 2-3-weeks of age that we have found (Dickhout et al.,1998a) could be due to growth disturbances introduced by the hypertensive

Figure 6.2 Proposed Model of the Genesis of Hypertension in the SHR



intrauterine environment and there is evidence of SHR perinatal growth disturbances in the literature (Lewis et al.,1997). The elevation in intrinsic heart rate that we have found in the SHR (Dickhout et al.,1998a) could be due to an increased production of thyroxine. Further, thyroidectomy in young SHR prevents the development of hypertension (Rioux et al.,1977). The increase in cardiac output due to the increase in heart rate would increase blood flow. High rates of blood flow through the fetal abdominal aorta correlates with a reduced incidence of apoptosis in this vessel (Cho et al.,1995). When blood flow decrease in this vessel after birth the incidence of apoptosis increases (Cho et al.,1995). We have found a reduced incidence of apoptosis in SHR mesenteric arteries that later became structurally modified (Dickhout et al.,1999). These may be important events leading to the hypertrophied blood vessels. Other factors, such as angiotensin II, can increase smooth muscle cell growth resulting in the longer smooth muscle cells we found in the SHR vessels (Dickhout et al.,1998b). Although no increase in circulating levels of renin or angiotensin II has been found in young SHR (Morton et al.,1990), retrovirally mediated delivery of the angiotensin II type 1 receptor anti-sense gene has prevented the development of hypertension in the SHR (Martens et al.,1998). This may indicate that although increased levels of angiotensin II are not present in young SHR the normal levels have a greater effect on smooth muscle growth than in WKY. This may result in the hypertrophied vessels we have found in 4-week SHR and their greater contractility (Dickhout et al.,1997).

Once these events have established a hypertrophied blood vessel, the greater contractility would increase TPR in the animals leading to hypertension. This greater TPR would also increase strain on the heart resulting in cardiac hypertrophy and may reduce

oxygen tensions in regions of the kidney with low blood flow such as the medulla. This type of renal ischemia would result in the type of sympathetic nerve dependent renal damage that has been found in the SHR (Gattone et al.,1990). Thus these events lead to the hypertension and typical end organ damage that we see in SHR as well as in human essential hypertensives.

6.4 Similarities of the SHR model to Human Essential Hypertension

We have shown that like human essential hypertensives SHR undergo a period of hypertension development which coincides with the sexual maturation of the animal (Dickhout et al.,1998a). This process, like in humans, need not be induced by any external influence. However, like in humans, it is exasperated by a high salt intake (Wyss et al.,1994). Similar to human hypertensives (Pedrinelli et al.,1997), it involves an increase in the peripheral resistance in the SHR (Albrecht et al.,1975). This increase in peripheral resistance is accompanied by structural change in the small muscular arteries involving hypertrophy of the blood vessels wall. These hypertrophied arteries in both SHR (Dickhout et al.,1997) and human essential hypertensives demonstrate a greater contractile ability (Rosei et al.,1995). The hypertension can be reversed with ACE inhibitor treatment in the SHR as well as in human essential hypertensives. This treatment also decreases the vascular hypertrophy in SHR(Morishita et al.,1992; Lee et al.,1991) and in humans (Schiffrin, 1996). The disease is accompanied by cardiac hypertrophy and renal pathology in both SHR (Karam et al.,1996) and humans (Cutler, 1996).

6.5 Recommendations for future research

Future studies should be done to determine the time course of apoptotic cell generation and disappearance in the SHR model in order to better interpret our results concerning the difference in apoptotic cell incidence between SHR and WKY and its effect of vessel structure.

Most of our work has concerned the mesenteric vascular bed. This is only one

vascular bed in the body and so further work should be conducted to determine if other vascular beds in SHR are similarly modified. Some work has already been done on renal arteries. Additional work is necessary to confirm or infirm the hypothesis that the kidneys play a central role in the long-term regulation of BP through the mechanism of pressure-natriuresis. Perfusion of maximally relaxed kidneys from SHR and WKY normotensive controls revealed that resistance did not significantly differ (Smeda et al.,1988). However, the medial cross sectional area of SHR renal arteries were significantly increased over WKY controls in both 4-5-week-old prehypertensive animals and in adult animals, 21-weeks of age (Smeda et al.,1988). These modified SHR vessels were no more sensitive to agonist stimulation but stimulation elevated renal vascular resistance to a greater degree in SHR than in WKY (Smeda et al.,1988). Further, treatment with the vasodilator hydralazine from birth to 21-weeks reduced BP in the SHR to normotensive levels but did not reverse the structural change in the renal arteries (Smeda et al.,1991). This suggests that the structural change in the renal vessels was not merely a response to elevated BP in the animal but is a primary process that may be playing a role in initiating the hypertension. These findings are very similar to our results in the mesenteric bed.

Another vascular bed in the SHR that may differ considerably from those examined that supply the abdominal viscera are the beds which supply the skeletal muscles. Here it was found that small muscular arteries from adult SHR are structurally modified with thickened media (Korneeva et al.,1997). Also that in the spinotrapezius muscle bed of the SHR there was a reduced partial pressure of oxygen under muscle stimulation as compared to the WKY (Lash, 1995). However, it is not known if these changes occur at the initiation

of hypertension or are merely the result of it. The adipose tissue also represents an extensive vascular bed in the body. The frequent association between hypertension and obesity points to the importance of this vascular bed in hypertension (Hernandez et al.,1998; Gus et al.,1998; Qiao et al.,1998; Garrison et al.,1987). The increase of its size, through obesity, may result in an increase in TPR due to the lengthening of the conduits the heart must perfuse as vascular resistance increases proportionally with vessel length (see equation 1.2). This process, however, is not operating in the SHR or in lean human essential hypertensives. Lean individuals may show small muscular artery hypertrophy and increased vessel contractility in the adipose tissue that led to an increase in TPR. Further work to look at structural change of small muscular arteries in the skeletal muscle and adipose tissue of young SHR would allow us to determine if these vessels are also playing a role in the genesis of hypertension in the SHR.

Another avenue of research which merits pursuit is to determine if angiotensin II will produce the structural modification we found in the SHR vessels. Appendix 1 has been included to detail developmental work we have done on a system to culture whole blood vessels under flow and pressure. This system will allow us to isolate the effect of angiotensin II on the blood vessel, without the effects of other physiological changes induced by angiotensin II infusion in the whole animal. Small resistance vessels from four-week-old WKY rats were grown in this apparatus. It was found that serum containing culture medium transformed smooth muscle cells in these arteries into a proliferative state, and caused them to lose their normal ultrastructural characteristics. However, a serum-free medium containing 1 to 1 DME and Ham's F12 medium supplemented with insulin, transferrin, and

selenious acid maintained these cells without proliferation. When stimulated by a known growth agonist, PDGF BB homodimer at a concentration of 5ng/ml, this medium supported the growth of both SHR and WKY smooth muscle cells. Arteries were able to survive in this medium for periods up to 1 week.

This apparatus provides an ideal opportunity to test a number of hypotheses. (1) Angiotensin II will produce the type of structural change we have seen in the blood vessels of the pre-hypertensive SHR. (2) These structurally modified vessels will show a similar increase in contractile ability to that found in the pre-hypertensive SHR vessels. (3) Angiotensin II will produce a greater medial hypertrophy in the arteries from SHR than WKY.

6.6 Conclusions

To conclude, these studies have allowed us to relate the structural change in small muscular arteries of the young SHR to their contractile responses. We found that the type of structural modifications typically found in the small muscular arteries of SHR and in human essential hypertensives leads to greater contractile responses in these vessels. This may explain the greater TPR found in both SHR and human essential hypertensives. We have also developed new techniques using the confocal microscope which has allowed us to characterize the structural modifications of the SHR blood vessels in three dimensions and to determine the cellular characteristics of structural modification in the SHR blood vessels. Further, we have applied these techniques to quantify the incidence of cellular apoptosis occurring in these vessels during their development and found differences between

hypertensives and normotensives that may lead to the type of structural modification that occurs in these vessels. This data has allowed us to construct a model of the genesis of hypertension in the SHR, and compare it to the process in human essential hypertensives.

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APPENDIX 1

DEVELOPMENT OF A PRESSURIZED ARTERY SYSTEM TO CULTURE BLOOD VESSELS FOR THE STUDY OF VASCULAR GROWTH

JEFFREY G. DICKHOUT and ROBERT M.K.W. LEE

Introduction

The finding that resistance blood vessels have thickened walls, has been associated with an increase in total peripheral resistance in several animal models of hypertension including Dahl salt sensitive hypertensive rats, DOC-NaCl rats, and SHR (Lee, 1987), as well as in human essential hypertension (Aalkjaer et al.,1987). In SHR hypertrophy of the medial layer had already taken place before any large difference in blood pressure (BP) existed between the SHR and its normotensive control WKY (Lee, 1985; Miller et al.,1987; Mulvany et al.,1980). This indicates that the thickening of the medial layer in SHR is a primary change in the development of hypertension (Korner et al.,1991). It appears that some unknown trophic process occurring before the elevation of BP in SHR is causing medial thickening. Studies on cultured SMC from SHR have shown a greater growth response to various agonists than cells derived from WKY (Saltis et al.,1992; Hamet et al.,1991; Hadrava et al.,1991). The increased vascular smooth muscle mass in SHR may produce the higher pressures observed in this strain by increasing total peripheral resistance through the ability to generate greater contractile force in the resistance blood vessels. However, the investigation of growth responses of smooth muscle in tissue culture conditions may not easily translate into growth responses in whole arteries since interactions between the vessels wall components, endothelial cells, SMC, and extracelluar matrix, are absent.

Vascular growth studies in the whole animal studies, on the other hand, demand the manipulation of pressure and hormonal factors without their interaction. Studies which have used organ culture method include Black et. al. (Black et al.,1993), who blocked the

production of angiotensin II (Ag II) with an ACE inhibitor, prindopril, and replaced the endogenous Ag II with either Ag II, or norepinephrine at a dose which produced equal pressures in the whole animal. However, the conclusion that Ag II had a direct growth effect on the vasculature since the Ag II group showed an increased vascular growth (Black et al.,1993), avoids the fact that Ag II infused into the whole animal had effects on other hormonal systems. These include stimulation of the release of aldosterone, anti-diuretic hormone release; binding sites in the hypothalamus and cerebral ventricles, and circumventricular organ (Cohen et al.,1988); as well as influencing sympathetic outflow (Chan et al.,1991). Therefore whole animal studies may introduce confounding factors that can not be accounted for in the analysis. To discern the unique role mechanical and specific hormonal factors may play in medial hypertrophy, an *in vitro* method is required where these factors can be manipulated independently. An organ culture method for arteries from SHR and WKY animals would allow such independent control of mechanical and hormonal factors. The work of Gotlieb and Koo suggested that organ culture of whole vessels has been a useful method to study cell-cell interactions between endothelial and SMC, and cell interactions with the vessel wall matrix (Koo et al.,1992). The presence of such interactions extends this method beyond what could be gained by studying the effects of hormonal factors on SMC grown alone in tissue culture. Moreover, if the whole vessel can be grown under a pulsating pressure, with flow through the lumen, as occurs in the *in vivo* state, the effect of these mechanical factors may be investigated.

The organ culture approach has been applied to the study of various vascular growth factors. These methods include the use of arteries suspended on wires (De Mey et al.,1991;

Sung et al.,1993; Cuspidi et al.,1991), or incubation of arterial segments in culture medium (Koo et al.,1991; Koo et al.,1989; Soltis, 1993). In these methods, the arteries were not grown under pressure, therefore lacking one essential feature of the *in vivo* conditions. A perfusion system was used to grow rat thoracic aorta under pulsatile pressure. After 6 days of culture, there was a reduction in response by 50-60% to all the agonists, including KCl, norepinephrine, angiotensin II and 5-HT (Monopoli et al.,1991). The intimal surface was mostly devoid of endothelial cells. The effect of such a culture system on vascular SMC morphology and number was unknown.

Here we describe the development of an organ culture apparatus for growing arteries, which allowed us to control intramural pressure, rate of flow, and pulse frequency independently. Hormonal factors can be placed in the bathing media to see their effect on artery growth. Experiments were conducted to investigate the effect of the basal growth media on smooth muscle growth, and the long-term effects of such a system on the vitality of the arteries.

Methods

Culture Apparatus

A culture apparatus was developed utilizing tygon tubing, a peristaltic pump (Watson-Marlow Ltd.), a respiration pump (Harvard Apparatus), and movable micropipettes housed inside an organ culture chamber. The organ chamber was designed in such a way as to allow the placement of four lengths of arteries simultaneously (Figure 1). These arteries were secured to the micropipettes in the culture chamber with 10-0 surgical suture. The

pulse pressure generated by the organ culture system was measured directly by artery puncture with a micropipette attached to a volume displacement amplification apparatus for pressure measurement. The servo-nulling pressure system (Model 5, IPM, San Diego) was used. The output of a Statham P23ID pressure transducer attached to the bell of the servo-nulling pressure system was connected to a chart recorder for output. This allowed the calibration of respiration pump tidal volumes, and peristaltic pump flow rates to produce the desired pressures, and pulse amplitudes in the artery lumen (Figure 2).

Effect of Culture Mediums on Smooth Muscle Cells

SMC derived from WKY rats were grown in DME culture medium (Gibco BRL) containing 10% fetal calf serum (FCS, Gibco BRL) at 37°C in an incubator containing an atmosphere of 5% CO_2, 95% air. Monolayers of SMC were grown to confluency, and double-fixed with with 2.5% glutaraldehyde and 1% OsO_4 in cacodylate buffer. Cells were washed and dehydrated with graded ethanol after fixation. Epon embedding was performed directly in culture dishes. Gold sections for transmission electron microscopy were cut from the block face, and stained with uranyl acetate and lead citrate. Sections were viewed under a Phillips 301 transmission electron microscope at 60 KV.

Similar SMC from both WKY, and SHR rats were grown in a defined culture medium containing 1:1 DME:Ham F12 media(Gibco BRL) supplemented with insulin, transferrin, and selenious acid (ITS premix) to see the effect on cell growth of this serum free medium. A known growth factor, PDGF BB homodimer (5 ng/ml), was added to the serum free culture medium to see if this medium would support growth.

Assessment of Artery Vitality

A vital staining method that distinguishes living from dead cells in the whole artery due to membrane permeability was developed to assess the ability of the culture apparatus to maintain the arteries. Ileal arteries were removed from male WKY at four weeks of age, and stained with ethidium bromide (Sigma) and Hoechst 33342 (Sigma) at various concentration. These arteries were examined live by epiflorescence microscopy suspended in culture medium with a Band Pass at 400-440 nm for excitation. It was found that the combination of ethidium bromide (10 ug/ml), with Hoechst 33342 (10 ug/ml) allowed differentiation between live and dead cells by epiflorescence microscopy. Experiments were performed with similar arteries after three hours removal from the body, in oxygenated or non-oxygenated serum free media, and after a week in organ culture, to investigate the type of artery damage which might occur under these conditions.

Results

Culture Apparatus

Pressure recordings obtained from the culture apparatus at the site of the artery indicated that pressure, and pulse frequency, and pulse amplitude could be set independently. Mean pressure was determined by the ratio between input volume of the respiration pump, and the diameter of the gas outlet valve. The pulse frequency was determined by the rate of both the respiration pump (large wave), and the peristaltic pump (small wave) (Figure 3). The pulse amplitude around a given mean pressure could be increased by increasing the total gas flow through the system, i.e. increasing respiration pump volume and gas outlet valve

diameter simultaneously while maintaining mean pressure.

Effect of Culture Media on Smooth Muscle Cells

SMC derived from WKY rats grown in FCS containing culture media proliferated rapidly to confluence, and display ultrastructural characteristic that differed markedly from SMC normally present in the artery wall. The overall appearance of these cells is displayed in Figure 4a. It was clear that cells were in a metabolically active phase since the cytoplasm contained many mitochondria, rough endoplasmic reticulum (RER), and ribosomes (Figure 4b). On closer examination the RER was clearly seen to be dilated, and filled with granular material (Figure 4c). Few myofibril were present except at the periphery of the cell involved in cell/cell attachment (Figure 4d).

SMC derived from SHR, and WKY, when grown in a culture medium free of FCS, maintained cell number at a constant level, but proliferated in response to a known growth agonist, PDGF BB homodimer (5 ng/ml). The proliferation response of SMC from SHR was apparently greater than those cells derived from WKY rats (Figure 5).

Vital Staining

The vital staining method proved to be effective in distinguishing live from dead cells since ethidium bromide made cells with permeabilized membranes appear red, and Hoechst 33342 made cells with intact membranes appear blue/green. This was demonstrated by mechanically damaging an artery before applying the vital staining technique. The site of mechanical damage was clearly differentiated from the surrounding undamaged cells (Figure 6a). Oxygenation of the bathing media was found to be a key factor in preventing early cell death in the adventitial layer of the arteries since cells maintained in a non-oxygenated media

displayed dead cells in the adventitia. However, even arteries held in an oxygenated culture medium for 3 hours after removal from the body displayed some cell death (Figure 6b). Arteries grown in the culture apparatus for 1 week, and examined with the vital staining method appeared to contain only live cells (Figure 6c). Preliminary results showed that density of cell nuclei in these arteries were reduced over normal arteries freshly removed from the animal.

Discussion

The major findings of this study were that small resistance vessels from four week old WKY rats can be grown in an organ culture apparatus with flow, and a pulsatile pressure for periods up to 1 week. Serum containing culture medium seems to transform SMC derived from these arteries into a proliferative state, and causes them to lose their normal ultrastructural characteristics. However, a serum free medium containing 1:1 DME:Ham F12, supplemented with insulin, transferrin, and selenious acid proved to maintain these cells without proliferation. When stimulated by a known growth agonist, PDGF BB homodimer at a concentration of 5ng/ml, this medium supported growth of both SHR and WKY SMC.

The fact that SMC grown in serum-containing medium appeared to undergo a phenotypic change from a contractile phenotype to a proliferative one has been noted before (Stadler et al.,1989; Chamley-Campbell et al.,1981). The use of a nutritionally adequate medium, but one that dose not cause change in the normal response of SMC in the artery wall was the requirement for growth in our organ culture system. The serum-free medium described here proved to be adequate for our requirements.

The organ culture apparatus described here allows the growth of arteries with flow, and a pulsatile pressure, therefore making it suitable for the testing of growth response in the artery to mechanical stimulation. In a pervious study in this laboratory (Dickhout et al.,1998), we have found that an increase in heart rate in SHR may be of great importance in the underlying pathophysiology of the development of hypertension since it occurs just before the BP surge in these animals. The organ culture system is an ideal device to test the hypothesis that an increase in heart rate (frequency of pulse pressure) could lead to structural changes in the resistance vessels. On the other hand, increased heart rate may not act directly on the artery to cause a structural change, but may only be a sign of the increased sympathetic outflow present in the SHR animal. The nature of the increase in heart rate found in SHR with little increase in systolic BP, but increased pulse pressure shows a similar pattern to that produced by the acute infusion of epinephrine in an animal (Kalant et al.,1989). Elevated plasma levels of epinephrine in young SHR may act directly on the SMC of the artery as a growth factor, and produce the medial thickening present in young SHR (Lee, 1985). The organ culture apparatus allows the testing of this hypothesis since epinephrine can be added easily to the serum free medium described, and the arteries grown in this condition analyzed to see if any change in structure results independent of pressure effects.

Another hormonal factor that may be of great importance in stimulating the hypertrophy of the SHR artery wall is angiotensin II, since studies with angiotensin converting enzyme inhibitors in SHR have shown that these drugs prevent the hypertrophy of the vessel wall (Lee et al.,1991; Morishita et al.,1992). The inclusion of angiotensin II in the bathing media would allow testing to see if this substance produces vessel wall

hypertrophy independent of pressure effects.

It is possible that small muscular arteries from SHR grow at a faster rate than arteries from WKY under the same conditions. The results indicating a greater growth response in SHR SMC to PDGF BB than in WKY rats supports this idea. This may be tested in the organ culture system since arteries from SHR, and WKY rats can be grown side by side in the same chamber, and their growth compared.

Small mesenteric arteries undergo remodelling in the hypertensive organisms. This is a reduction in lumen diameter not necessarily involving an increase in medial thickness (Heistad et al.,1991). There is some evidence to suggest that this process, which would involve a rearrangement of the artery wall, occurs in arteries exposed to reduction in rate of flow (Langille, 1991; Langille et al.,1989). It would be possible to produce such reductions in flow in the organ culture apparatus, and then determine if remodelling did occur by analysis of artery structure. Remodelling may involve apoptosis of some cells in the artery wall. Examination of the arteries grown in chronically reduced flow for increased apoptotic cells, by electron microscopy through nuclear morphometry, or immuno histochemistry with label markers of apoptotic cells, would allow us to determine if remodelling is a process involving apoptosis.

In conclusion, the organ culture system described in this paper allowed the growth of small resistance vessels from four week old rats with flow, and a pulsatile pressure. A serum-free medium containing 1:1 DME:Ham F12, supplemented with insulin, transferrin, and selenious acid proved adequate to meet the nutritional requirements of these arteries, and allow growth response of SMCs to a known growth agonist. The combination of this system

and a manipulation of the content in the culture medium affords many opportunities to investigate the fundamental causes of the primary structural alterations occurring in the arteries of the SHR.

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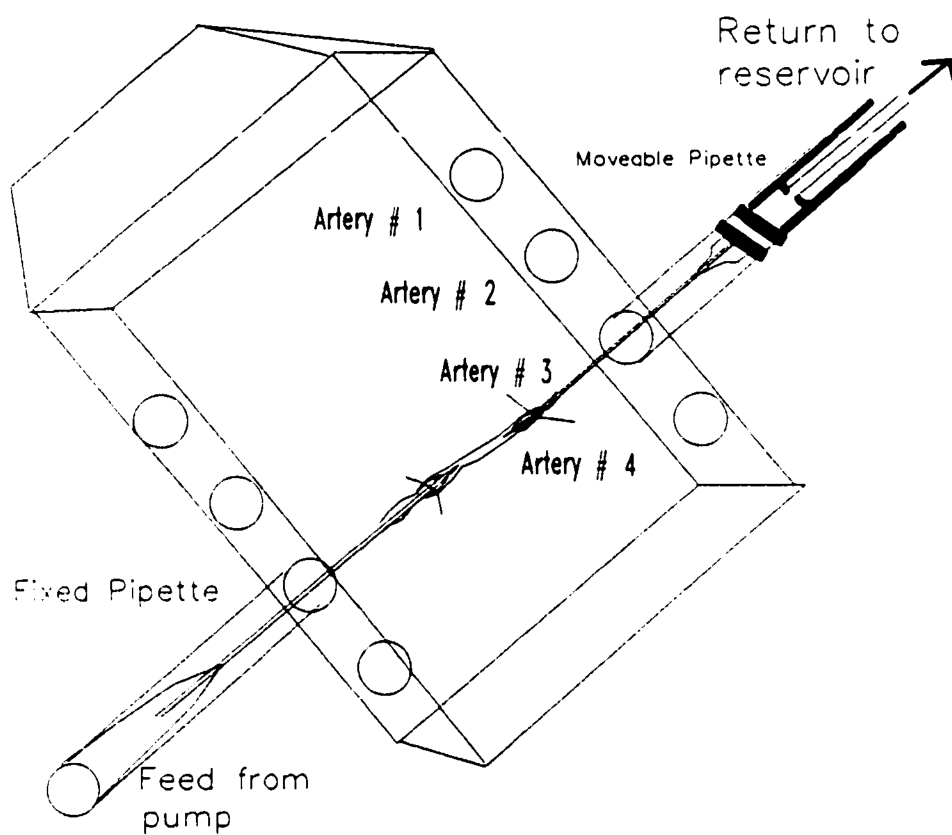

Figure 1. Organ culture chamber illustrating artery placement

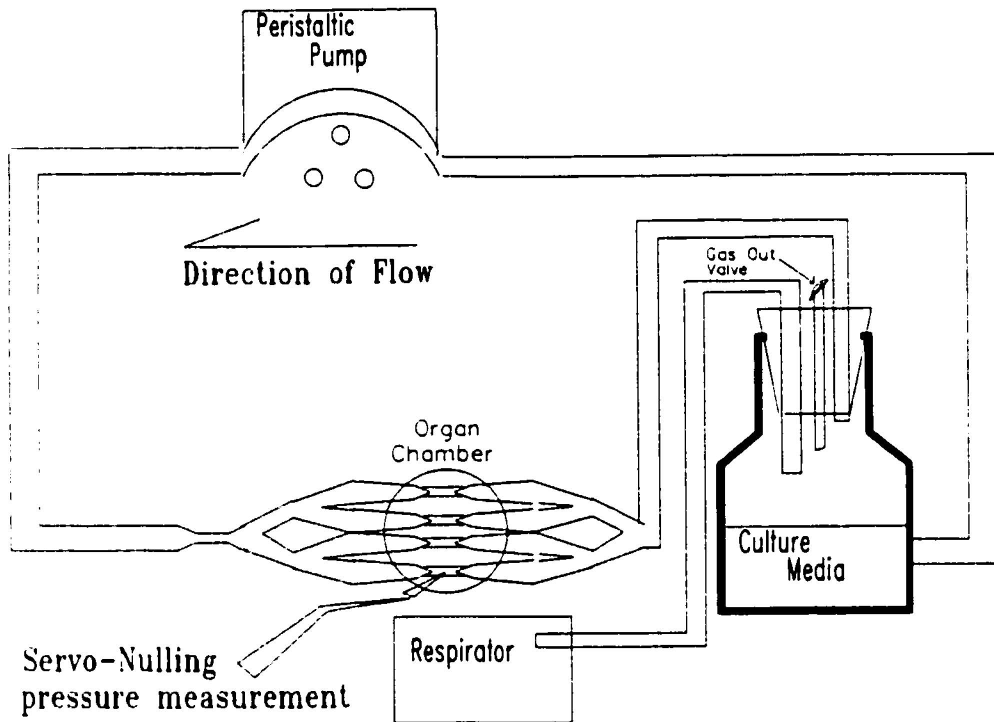

Figure 2. **Organ culture apparatus showing point of pressure measurement with servo-nulling pressure measurement apparatus.**

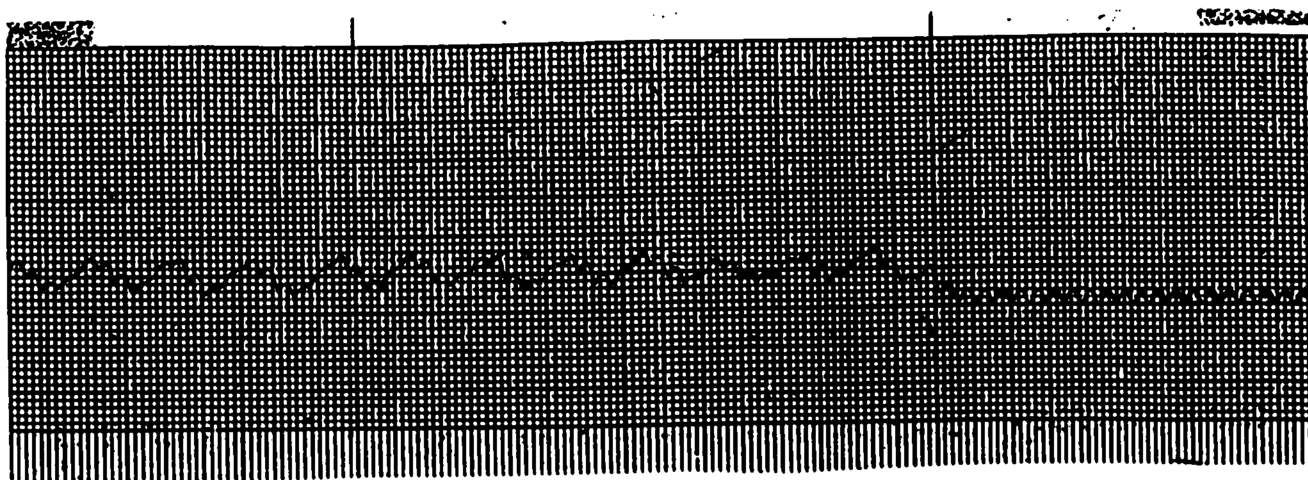

Figure 3. Pressure tracing recording from artery lumen during apparatus operation.

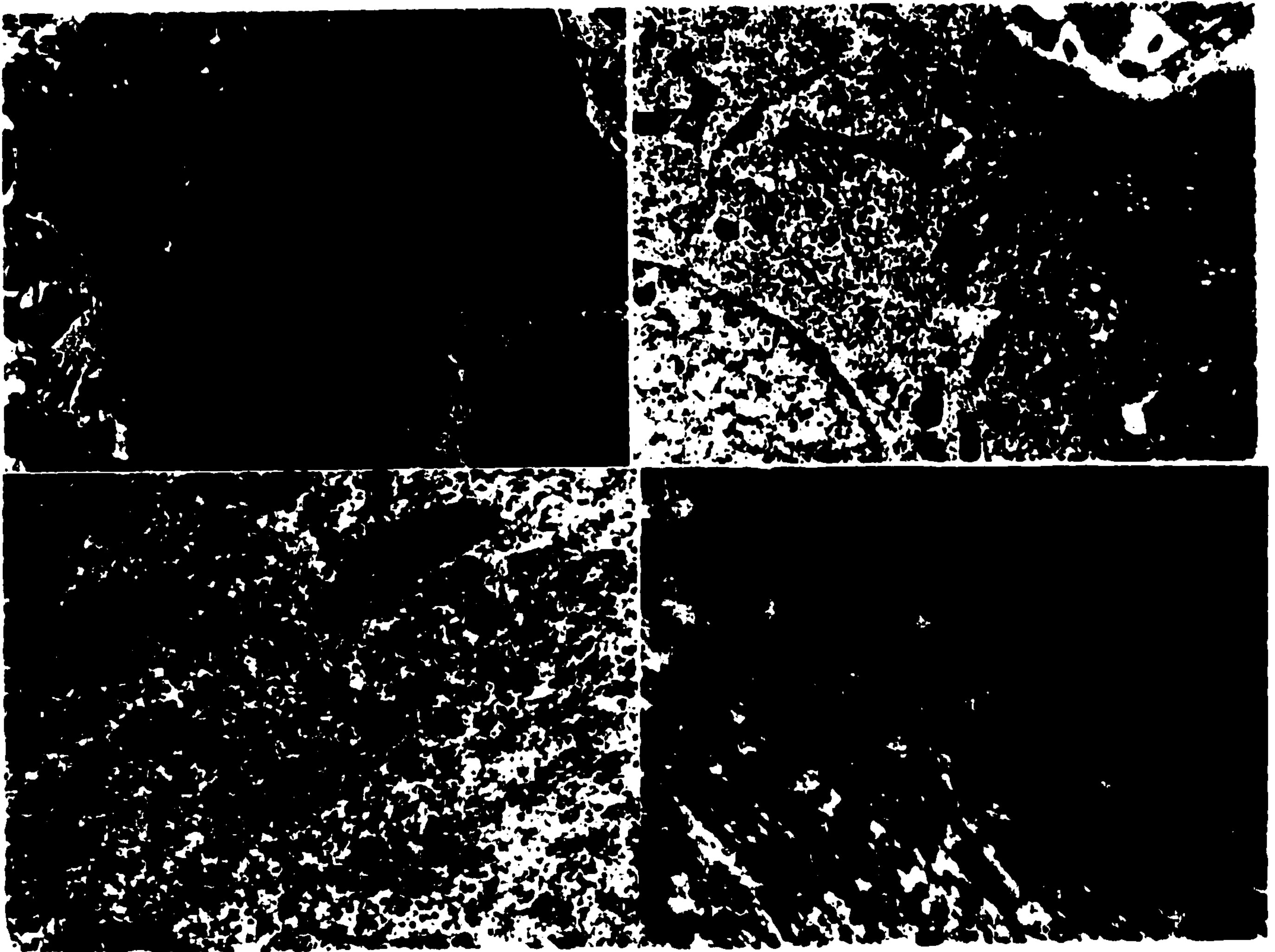

Figure 4. (a) Overview of smooth muscle cell grown in serum containing culture medium. (b) Detail of smooth muscle cell grown in serum-containing culture medium showing abundant mitochondria, and rough endoplasmic reticulum. (c) Detail of rough endoplasmic reticulum showing active protein synthesis. (d) Sparse myofibrils found at the cell periphery.

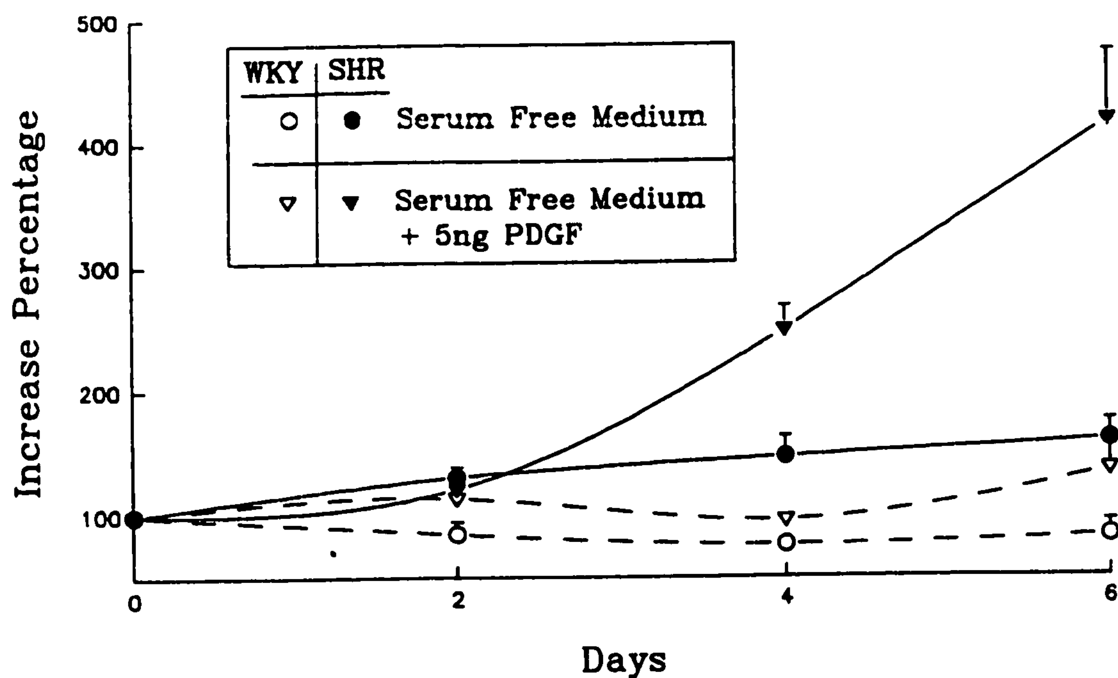

Figure 5.Proliferation of smooth muscle cells derived from SHR and WKY in serum-free medium, and serum-free medium containing 5ng/ml PDGF BB.

Figure 6. (a) Vital staining of artery after mechanical damage illustrating orange-coloured dead cells at the centre of the field.

Figure 6. (b) Vital staining of artery three hours after removal from the body, illustrating some cell damage. (c) Vital staining of artery grown in culture for 1 week showing only live cells.