β-ADRENOCEPTORS, ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND POLYPLOIDY IN THE VASCULAR SMOOTH MUSCLE CELLS FROM DIFFERENT AGE-GROUPS OF SPONTANEOUSLY HYPERTENSIVE AND NORMOTENSIVE WISTAR-KYOTO RATS

By

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TITLE: β-adrenoceptors, adenosine 3',5'-cyclic monophosphate and polyploidy in vascular smooth muscle cells from different age-groups of spontaneously hypertensive and normotensive Wistar-Kyoto rats

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β-adrenoceptors, cAMP and polyploidy in SHR and WKY rats

ABSTRACT

One of the possible contributing factors in the development of hypertension may be an accelerated or premature vascular ageing process, because of some similar structural and functional alterations in the vasculature, including an impaired β-adrenoceptor mediated vascular relaxation and an increase in polyploid smooth muscle cells. The primary objective of this study was to investigate the possible relationship between development of polyploidy and the plasma membrane β-adrenoceptor in cultured smooth muscle cells from the thoracic aortae of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) from 3-4-(prehypertensive), 10-12-(developing hypertensive), and 28-30-weeks (establish hypertensive) of age.

The major findings from this study are: (i) similar to the *in vivo* state, cultured smooth muscle cells from different age-groups of WKY and SHR contain a heterogeneous population of mononucleated and multinucleated cells, as well as diploid and polyploid smooth muscle cells; (ii) the expression of both smooth muscle cell polyploidy and β-adrenoceptor density increases with age in both SHR and WKY, however, this increase

was significantly accelerated in SHR as compared to WKY, suggesting an accelerated or premature ageing process may be involved in SHR as compared to WKY; (iii) both SHR and WKY express functional smooth muscle cell β-adrenoceptors but many of the β-adrenoceptors expressed on cultured SHR smooth muscle cells are not coupled to adenylate cyclase; (iv) elevation of intracellular cAMP levels either by agonist activation of β-adrenoceptors or by direct activation of adenylate cyclase in cultured smooth muscle cells from 3-4-week old WKY and SHR and 10-12-week old WKY resulted in an increase in polyploid cells; (v) a β-adrenoceptor antagonist only partially inhibited the isoproterenol-stimulated increase in polyploid smooth muscle cells in both SHR and WKY; and, (vi) the development of polyploid SMC via the β-adrenoceptor-G_s-protein-adenylate cyclase-cAMP pathway is more efficient in cells from WKY compared to SHR.

From these findings, I conclude that: (i) the vascular β-adrenoceptor mediated signalling pathway plays a role in the development of polyploid smooth muscle cells; and, (ii) additional intracellular pathways, independent of the β-adrenoceptor-cAMP intracellular mediated signalling pathway, are involved in the development of smooth muscle cell polyploidy.

Since it is the resistance arteries (which show no significant increase of polyploid smooth muscle cells with age and/or duration of hypertension) and not the elastic large arteries (aorta) which play a significant role in the development of hypertension, these findings have more relevance to the premature ageing process than to the development of hypertension.

Dedicated to

My Mother, Dad and Sister

For their unending and abundant support, understanding and love

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
βARK	β-adrenoceptor kinase
Ca^{2+}	calcium
cAMP	adenosine 3',5'-cyclic monophosphate
CO	cardiac output
CRE	cAMP response element
CREB	cAMP response element binding protein
DNA	deoxyribonucleic acid
DBP	diastolic blood pressure
GDP	guanosine 5'-diphosphate
G_i	guanosine 5'triphosphate inhibitory protein
G_s	guanosine 5'-triphosphate stimulatory protein
GTP	guanosine 5'-triphosphate
K^+	potassium
mmHg	millimeter of mercury
Na^+	sodium
PKA	protein kinase A

PKC	protein kinase C
PLC	phospholipase C
PR	peripheral resistance
Rp-cAMPS	adenosine 3',5'-cyclic monophosphothioate, Rp-isomer
SBP	systolic blood pressure
SHR	spontaneously hypertensive rats
SMC	smooth muscle cells
Sp-cAMPS	adenosine 3',5'-cyclic monophosphothioate, Sp-isomer
WKY	Wistar-Kyoto rats

CHAPTER I

INTRODUCTION

Chapter 1: Introduction

1. Hypertension

1.1 Human hypertension - overview: essential and secondary

Hypertension is a pathological condition in the human population and is characterized by a persistent elevation of blood pressure. The accepted normal blood pressure is a diastolic blood pressure (DBP) of <90 mm Hg and a systolic blood pressure (SBP) of <140 mm Hg (JNC V, 1993). The use of blood pressure measurements conducted at least twice on two different occasions can be used to categorize hypertension as: (i) mild hypertension: DBP of 90-105 mmHg and/or SBP of 140-180 mmHg; or, (ii) moderate and severe hypertension: DBP of ≥105 mmHg and/or SBP ≥180 mmHg (Zanchetti et al., 1993).

Approximately 90-95% of all people who have hypertension are referred to as having "essential" hypertension, which indicates that the increased blood pressure is of unknown origin. In most essential hypertensive patients, however, there are some common characteristics, such as a strong hereditary tendency, presence of vascular hypertrophy,

defects in ion handling, hyperinsulinemia, and involvement of the renin/angiotensin system (Guyton, 1995).

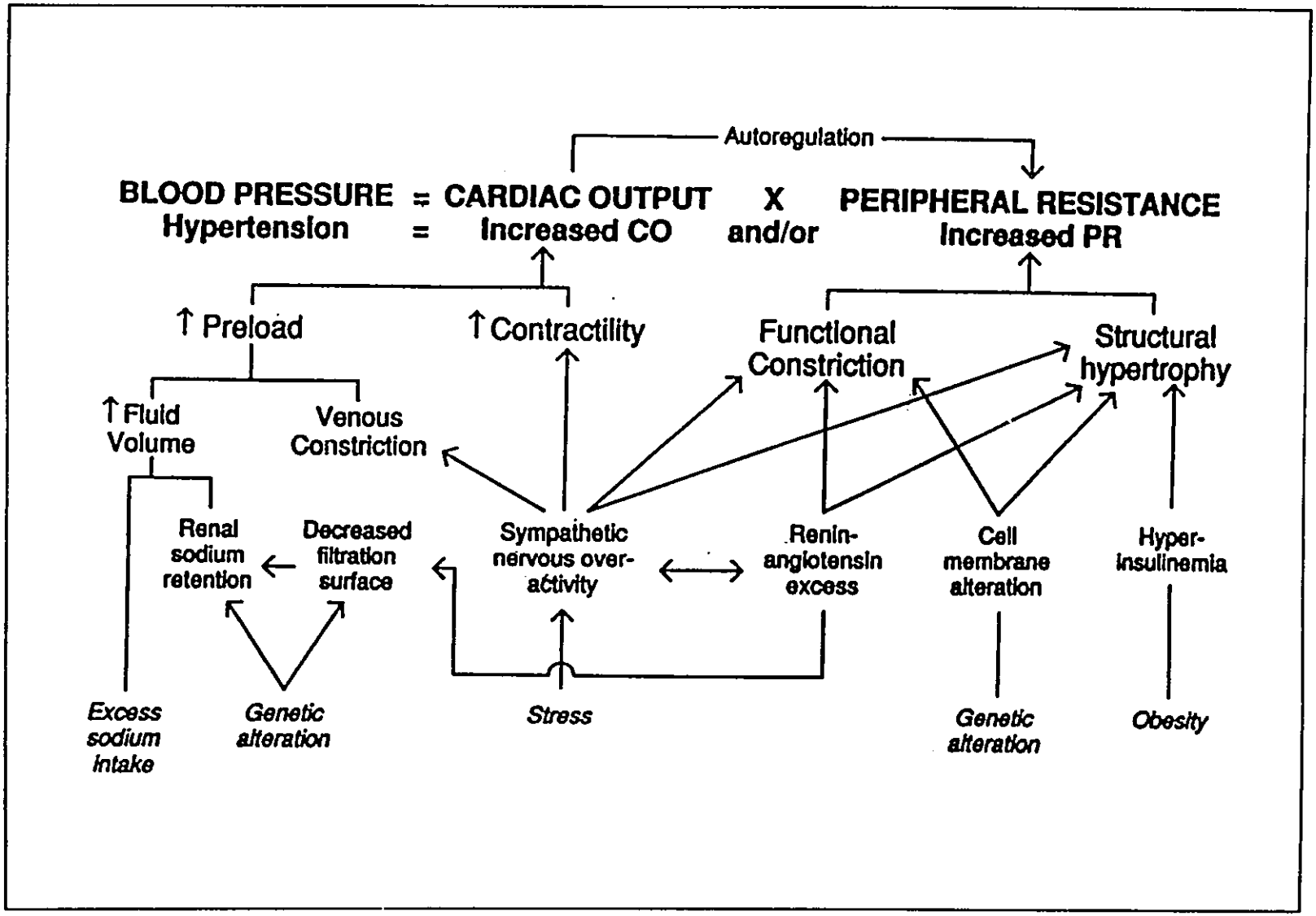
The remainding 5-10% of hypertensive patients have "secondary" hypertension, that is, there is an identifiable underlying disease as the cause of their hypertension. Several diseases that result in secondary hypertension include: (i) chronic renal parenchymal disease (e.g. glomerular disease, diabetic nephropathy, pyelonephritis); (ii) renovascular hypertension (e.g. renal artery stenosis due to atheroma) which may lead to renal ischemia and activation of the renin-angiotensin-aldosterone pathway; (iii) adrenal tumors (mineralocorticoid effect in cortical tumors, or secretion of pressor substances due to tumor of the renal medulla); (iv) oral contraceptives; and, (iv) thyroid disease (Guyton, 1996).

Individuals with hypertension are at an increased risk for cardiovascular diseases, damage to blood vessel linings and organs, strokes, and, kidney failure. The rate of these increased risks corresponds to the degree of blood pressure elevation (Zanchetti et al., 1993). The net effect from the complications of hypertension is a shortened life expectancy (Guyton, 1996).

1.1.1 Human hypertension - Overview of the Pathogenesis of Hypertension

The pumping of the heart (cardiac output (CO)) and the tone of the arteries (peripheral resistance (PR)) provides the pressure required to move blood through the circulatory bed (Kaplan, 1990). The primary determinants of blood pressure (CO and PR) are utimately determined by the complex interactions of a multitude of factors (Frohlich, 1983; Kaplan, 1990; Figure 1).

Figure 1: The primary determinants of blood pressure are determined by the complex interactions of a multitude of factors (Taken from Kaplan, 1990).


Autoregulation
BLOOD PRESSURE = CARDIAC OUTPUT X PERIPHERAL RESISTANCE
Hypertension = Increased CO and/or Increased PR
↑ Preload
↑ Contractility
Functional Constriction
Structural hypertrophy
↑ Fluid Volume
Venous Constriction
Renal sodium retention
Decreased filtration surface
Sympathetic nervous over-activity
Renin-angiotensin excess
Cell membrane alteration
Hyper-insulinemia
Excess sodium intake
Genetic alteration
Stress
Genetic alteration
Obesity

Abnormalities in almost every one of these factors have been attributed to the development of hypertension. Regardless of what starts the process, however, it is clear that the perpetuation of hypertension depends on the development of vascular hypertrophy and/or hyperplasia, which is responsible for the increased tone and enhanced contractility in response to various pressor agents which are believed to play a functional role in the pathogenesis of hypertension (Kaplan, 1990).

1.2 Animal models

1.2.1 Spontaneously hypertensive rats and Wistar-Kyoto rats

There are several genetically hypertensive animal models available for the study of human essential hypertension. Some of these animal models, obtained by a process of selective inbreeding, include the spontaneously hypertensive rat (SHR) (Okamoto et al., 1963), New Zealand genetically hypertensive rat (Phelan et al., 1987), Lyon hypertensive rat (Vincent et al., 1987), Sabra hypertensive rat (Ben-Ishay et al., 1987), Milan hypertensive rat (Bianchi et al., 1987), Fawn-hooded rat (Kuijpers et al., 1987), and the Dahl salt-sensitive rat (Rapp et al., 1985; Iwai et al., 1986; Rapp, 1987; Iwai, 1987). Each of these rat strains have some unique pathophysiological features (i.e. increased sympathetic tone, ionic transport abnormalities etc.) that are linked to the development of hypertension.

The genetically hypertensive strain of SHR rats was selected from the normotensive Wistar-Kyoto strain of rats (WKY) (Okamoto et al., 1963; Yamori et al., 1987). It was observed that a male WKY exhibited high blood pressure, 134 - 175 mmHg, as compared to the normal range of 120 - 140 mmHg for this strain. Mating of this male rat with a female WKY which also exhibited high blood pressure resulted in an inbred strain of rats. Subsequently, most of the SHR colonies are maintained through the process of brother-to-sister mating (Okamoto et al., 1963; Frohlich, 1986; Yamori et al., 1987).

An inherent problem with the SHR model is that this inbred disease strain differs from its control strain in more than just the disease-specific genotype and phenotype. In fact, minisatellite typing (fingerprinting) of genomic DNA revealed multiple polymorphic bands, many more than would be expected to account for the blood pressure phenotype, between WKY and SHR (Samani et al., 1989; Nabika et al., 1991; Johnson et al., 1992; Lindpaintner et al., 1992; St. Lezin et al., 1992). Additionally, SHR strain comparisons may be misleading due to the probability that any two strains will differ in a multitude of traits completely unrelated to mechanisms having to do with blood pressure control (i.e. genetic drift). Finally, the WKY strain was established several years after the establishment of the SHR colony. These inherent problems raises questions with regards to the proper controls for the SHR model. However, in the

absence of a better genetic control, the WKY strain continues to be used as an appropriate model for comparison with SHR.

1.2.2 Characteristics of SHR

The SHR model share many similarities with human essential hypertension, such as: (i) the SHR model is genetically predisposed to hypertension; (ii) there is no specific aetiology; (iii) the control mechanism for blood pressure is multifactorial; (iv) SHR exhibit both salt (sodium, Na^+) sensitivity and altered sodium transport; and, (v) SHR develop similar changes in vasculature, central and peripheral nervous system, and kidneys (Frohlich, 1986).

The development of hypertension in the SHR population can be divided into three stages. The first stage, which is characterized by no measurable differences in blood pressure between SHR and WKY, is termed the prehypertensive stage and lasts up to 3-4 weeks of age after birth. The second stage, which is characterized by a steady increase in blood pressure in SHR as compared to WKY, is termed the developing hypertensive stage and occurs between 7-14 weeks of age. The third stage, referred to as established hypertensive stage, occurs after 14 weeks of age and is characterized by a stable and elevated blood pressure throughout the adult life of the SHR as compared to WKY (Lee, 1986).

1.2.3 Mechanisms of hypertension: Role of vascular structure

The mechanims underlying the development of hypertension in SHR are not clearly known. The hemodynamic hallmark of essential hypertension, however, is an increased peripheral vascular resistance, which seems to be the major factor in the development and maintenance of elevated blood pressure, both in humans and SHR (Lee, 1986; Yamori, 1987). A consistent feature of hypertensive vessels is a thickening of the vessel wall (Bevan et al., 1976; Lee et al., 1983a; Lee et al., 1983b; Lee et al., 1983c; Mulvany et al., 1978; Lee et al., 1985; Lee et al., 1987a). Studies comparing the vascular structure of prehypertensive SHR to age-matched normotensive WKY reveal that in the SHR structural alterations of the blood vessels have already taken place before the elevation of the blood pressure (Lee et al., 1985). This suggests that factors other than transmural pressure are involved in the thickening of the vessel wall. This increase of the smooth muscle cells in the media of the vessels is due to hyperplasia (an increase in cell number) as opposed to hypertrophy (an increase in cell size). This hyperplasia of smooth muscle cells is considered to be a primary vascular change because it occurs before the elevation of blood pressure and also antihypertensive treatment does not prevent it from happening (Lee et al., 1985). In contrast, hypertrophy of smooth muscle cells is considered to be a secondary vascular change because it occurs after the

development of hypertension and antihypertensive treatment prevents these changes from occurring (Lee et al., 1985). The exact mechanisms and/or factors which cause both hyperplastic and hypertrophic growth of smooth muscle cells in hypertension is unclear.

The involvement, however, of the sympathetic nervous system and adrenal medulla have been confirmed since it was shown that bilateral adrenal demedulation of neonatally sympathectomized SHR permanently attenuated the development of hypertension in these rats, as it was able to cause an increase in lumen size of the arteries by preventing the increase in smooth muscle cell number (hyperplasia) (Lee et al., 1991a; Lee et al., 1991b). It has been suggested that the sympathetic nervous system exerts a trophic effect on the smooth muscle cells of resistance and reactive arteries and that the adrenal medulla has an effect on the arteries' lumen size (Lee et al., 1987b; Lee et al., 1987c; Lee et al., 1991a; Lee et al., 1991b). In the development of hypertension, both of these processes are important but their mechanisms of action are unclear.

1.2.4 Cell Culture as a model to study vascular changes in hypertension

In vivo studies investigating the roles of various trophic or inhibitory factors on smooth muscle cell growth is often complicated by possible influences of transmural pressure, and/or interactions between various neurohumoral substances. The use of isolated and cultured smooth muscle cells from hypertensive and normotensive rats as a model to study changes in hypertension offers several advantages, including: (i) a pressure-independent system, where the effects of each factor on smooth muscle cell growth can be tested singly, or in combination; and, (ii) allows for a relatively pure source of smooth muscle cells to be grown in large quantities, thereby allowing the investigation of the pharmacological and physiological properties of receptors from these cells.

The use of the cell culture approach also has some inherent problems. Smooth muscle cells undergo modulation between a contractile phenotype and a synthetic phenotype under culture conditions (Chamley-Campbell et al., 1979; Chamley-Campbell et al., 1981). In the contractile phenotype, smooth muscle cells do not divide when challenged with serum growth factors but respond to mitogens when the smooth muscle cells spontaneously undergo a change to a synthetic phenotype (Chamley-Campbell et al., 1979; Chamley-Campbell et al., 1981). This change in smooth muscle phenotype is reversible and dependent on the cell seeding

density (Chamley-Campbell et al., 1979; Chamley-Campbell et al., 1981).

Smooth muscle cells also exhibit a heterogeneity in culture. Some cells are large while others are smaller and some form monolayers while others grow into multilayers (Lee et al., 1992a). Thus, smooth muscle cells in culture represent a mixed population of cells.

Previous studies using cultured smooth muscle cells have relied on combining cells obtained from individual animals with a given strain. This approach prevents the investigation of individual differences among animals with each strain. Thus, to avoid this problem in the studies conducted in this thesis, cells from each animal were maintained separately, thereby allowing the comparison between cells from individual animals, and between the two strains of rats.

1.3 Ageing, vasculature and hypertension

Blood pressure, particularly systolic blood pressure, tends to increase progressively with age and as a result most people will have the tendency to develop hypertension (Kaplan, 1990). There are many changes in the arterial structural and functional properties as a result of ageing (Lakatta, 1993; Nichols et al., 1987). Functionally, both *in vitro* and *in vivo* studies have indicated that the elastic modulus or stiffness of large arteries increases with age with a reduction in volume elasticity. Arterial stiffness

is also functionally determined by the vascular smooth muscle contractile tone, which in turn is controlled by neurohumoral factors such as catecholamines and angiotensin II (Safar et al., 1987; Simon et al., 1984; Ting et al., 1986). The observation that vasodilators (e.g. nitroprusside) can diminish the increased arterial stiffness in older patients suggests that a component of the increased *in vivo* arterial stiffening with ageing may be due to augmented vascular smooth muscle function (Lakatta, 1993; Carroll et al., 1991). Structurally, this age-associated increase in arterial stiffness is accompanied by: (i) an alteration in the connective tissue composition with an increase in collagen and a decrease in elastin (Lakatta, 1993); and, (ii) an increase in arterial diameter and wall thickness in both humans and rats (Gozna et al., 1974; Lakatta, 1993; Wolinsky et al., 1972; Cliff; 1970; Haudenschild et al., 1981).

Many of the vascular changes seen in association with ageing are also seen associated with hypertension. Some common structural changes include medial and intimal thickening, polyploid cells, and alteration in connective tissue composition (Barret et al., 1983; Haudenschild et al., 1981; Owens et al., 1985). Functionally, however, there may be differences between those associated with ageing and those associated with hypertension. As a result of the structural changes associated with ageing, it has been proposed that there is an increased peripheral vascular resistance with age, however, the peripheral vascular resistance does not

markedly increase with ageing in healthy normotensive individuals (Fleg et al., 1990; Lakatta, 1993).

2. Vascular Smooth Muscle Cells

2.1 The smooth muscle cell: structure and function

The medial layer of adult vessels is composed of highly differentiated cells, which are embryologically derived from mesenchymal cells, called vascular smooth muscle cells (Thyberg et al., 1990; Gabella, 1990). These cells have a number of characteristic features. They are generally spindle shaped, thickest at the nucleus and tapering off at the ends. Cell length varies widely, depending on the species, the tissue and the degree of contraction. Relaxed cells have a length of approximately 40 μm in rat resistance vessels to over 250 μm in rabbit portal mesenteric vein and are roughly 3 to 5 μm in diameter (Mulvany et al., 1985; Berner et al., 1981). The surface area of smooth muscle cells is substantially increased by rows of invaginations, called caveolae, which are approximately 50-80 nm in diameter and 120 nm in length and are usually grouped in rows along the long axis of the cell. The exact function(s) of caveolae is still unclear (Devine, 1971; Gabella, 1979; Gabella, 1981; Devine, 1975). The contractile apparatus of vascular smooth muscle cells consists of thick filaments, thin filaments, intermediate filaments and dense bodies. Thick or myosin

filaments are about 13.5 to 17.5 nm in diameter and are about 2.2 μm in length (Ashton et al., 1975). These filaments, which have a helical arrangement of cross-bridges, tend to lie parallel to each other along the longitudinal axis of the cell. Thin or actin filaments, which are about 5-8 nm in diameter, are arranged around the thick filaments and also lie parallel to the long axis of the cell. Dense bodies are electron dense structures which vary in diameter between 30 to 300 nm and are found throughout the cytoplasm and along the cell membrane (Devine et al., 1971). Dense bodies probably serve as attachment sites for thin filaments (Gabella, 1981; Schollmeyer et al., 1973). Contraction in smooth muscle, like skeletal muscle, is thought to occur via a sliding filament mechanism, that is, through the interactions of thin (actin) and thick (myosin) filaments (Jones, 1981). Intermediate filaments of about 10-11 nm in diameter are usually found surrounding dense bodies and are thought to form a cytoskeleton, providing a means for force transduction between cytoplasmic and membrane dense bodies (Devine et al., 1971; Jones, 1981). Smooth muscle cells with their characteristic features are secured in a network of connective tissue, comprising mainly of collagen and elastin (Mulvany, 1989a; Mulvany et al., 1989b).

In vivo, vascular smooth muscle cells are capable of expressing different phenotypes. Smooth muscle cells may express the contractile phenotype, which is accompanied by 75-80% of the cytoplasm being

occupied by contractile filaments and relatively few Golgi apparatus and free ribosomes (Campbell et al., 1993; Mosse et al., 1985; Manderson et al., 1989). Another phenotypic expression is the synthetic type. This is characterized by the presence of few contractile filaments in the cytoplasm, large amounts of Golgi apparatus, free ribosomes and rough endoplasmic reticulum (Campbell et al., 1993; Campbell et al., 1990). Synthetic vascular smooth muscle cells are usually seen in various cellular processes such as development, repair, and medial thickening (Campbell et al., 1990). Most smooth muscle cells, however, generally express an intermediate morphology between contractile and synthetic phenotypes.

2.2 Vascular smooth muscle cells in culture

Under cell culture conditions, smooth muscle cells undergo phenotypic modulation between contractile phenotype and synthetic phenotype (Chamley-Campbell et al., 1981; Chamley-Campbell et al., 1979). In the contractile phenotype the smooth muscle cells in primary cell culture are not stimulated by growth factors whereas in the synthetic phenotype, smooth muscle cells can be stimulated by mitogens and undergo DNA synthesis, secretion of extracellular proteins and proliferation (Chamley-Campbell et al., 1981; Sjolund et al., 1984; Sjolund et al., 1986). The

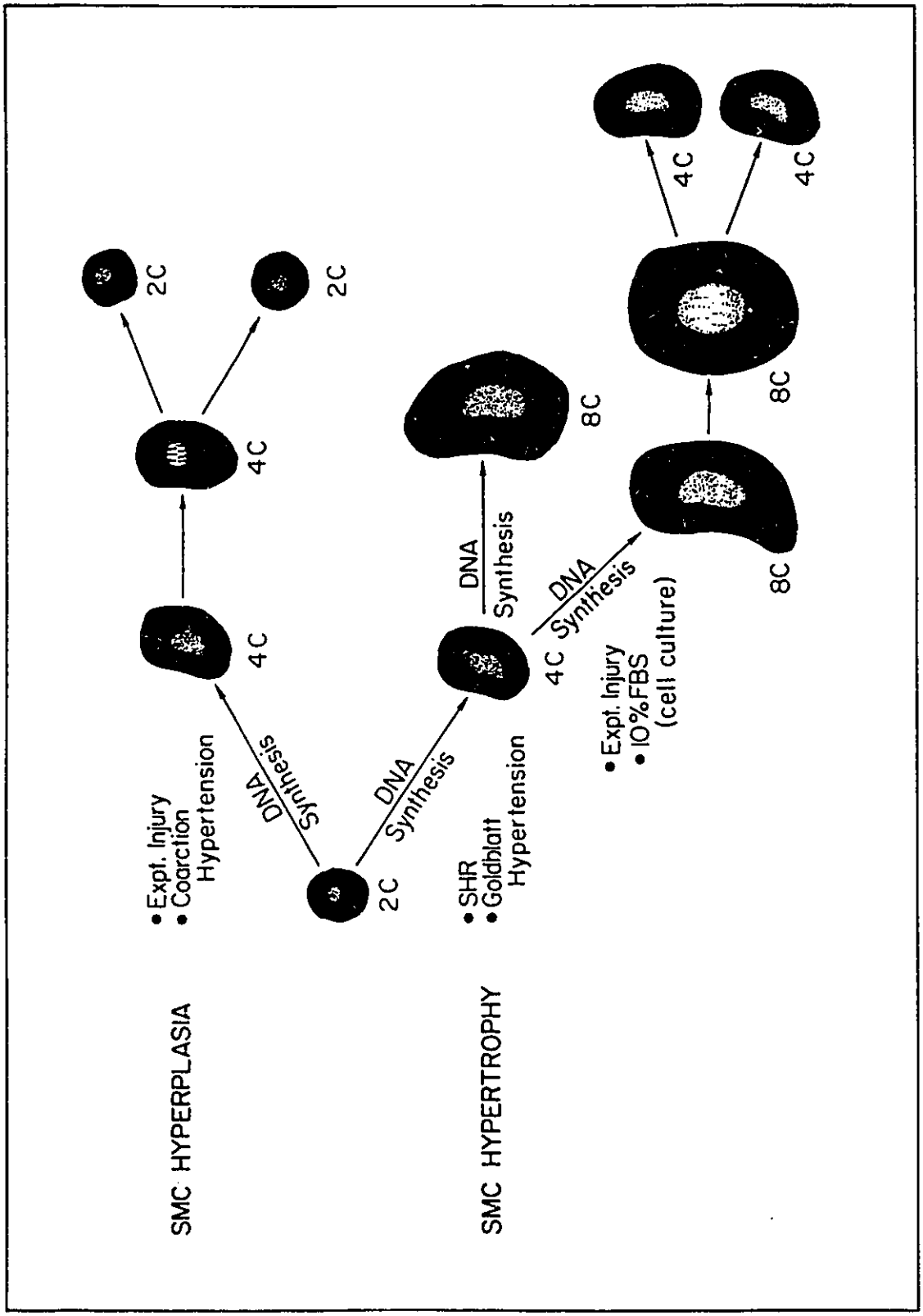
change in smooth muscle phenotype seen in primary culture have been reported to be either reversible or irreversible depending on cell seeding density (Chamley-Campbell et al., 1981; Campbell et al., 1993). Smooth muscle cells that have been subcultured at sparse seeding densities (1×10^3 to 5×10^3 cells/ml) (confluency in 2 weeks) or cells that have migrated from explants in primary culture for several weeks have been reported to be in an irreversible synthetic state; cells seeded at extreme density (1×10^6 cells/ml) (confluency at 1 day) do not undergo a process of phenotypic modulation but remain indefinitely in the contractile state; whereas cells seeded moderately densely (5×10^4 to 1×10^5 cells/ml) (confluency within a week) have been reported to undergo a reversible phenotypic modulation as the cells grow to confluency, involving contractile-synthetic-contractile phenotypic changes (Chamley et al., 1977; Chamley-Campbell et al., 1979). However, the concept of a global, coordinated conversion from one phenotype to another in vascular smooth muscle cells is perhaps a simplistic view of the state of vascular smooth muscle cell differentiation in culture. This is suggested by the fact that cells that have been maintained in culture or subcultured for long periods of time continue to exhibit contractile properties and responses despite the belief that they are in the synthetic state (Murray et al., 1990; Owens et al., 1986; Chamley-Campbell., 1979).

3. Polyploidy

3.1 Polyploid smooth muscle cells: Characteristics

Polyploidy refers to the fact that cells replicate their DNA but do not undergo cell division and hence these cells contain more than the normal diploid (2C, two sets of chromosomes) amount of DNA (that is, tetraploid (4C, four sets of chromosomes) or octaploid (8C, eight sets of chormosomes)) (Owens et al., 1985; Yamori et al., 1987; Figure 2).

Figure 2: The growth of aortic smooth muscle cell *in vivo*. Hypertrophic growth is often accompanied by the development of polyploid cells (Taken from Owens, 1989a).


SMC HYPERPLASIA
•Expt. Injury
•Coarction Hypertension
DNA Synthesis
2C
4C
2C
2C
DNA Synthesis
SMC HYPERTROPHY
•SHR
•Goldblatt Hypertension
4C
DNA Synthesis
8C
•Expt. Injury
•10%FBS
(cell culture)
4C
8C
4C
8C

3.2 Mechanisms of development of polyploid smooth muscle cells

Polyploid smooth muscle cells represent true polyploid cells rather than cells arrested in the G_2 + mitotic/synthetic stages of the cell cycle. When a group of sorted tetraploid cells were cultured, it was found that tetraploid cells do not revert to a diploid state, as would be expected if these cells were G_2 arrested cells, that is, tetraploid smooth muscle cells retained their increased ploidy (DNA) content and cycle as true tetraploid cells when grown (Goldberg et al., 1984). Karyotic analysis confirmed that tetraploid cells contain 4C and 4n DNA content and chromosome number (where C represent the haploid content and n represent the chromosome number) (Goldberg et al., 1984).

The exact mechanism for the formation of polyploid smooth muscle cells is presently unclear, however, a number of mechanisms have been proposed including cell fusion, abnormal mitosis where cytokinesis and/or karyokinesis does not occur and, endoreplication or endoreduplication where DNA synthesis occurs without any evidence of mitosis (Brodsky et al., 1977). Cell fusion, however, seems to be an unlikely mechanism since in SHR, the accumulation of polyploid smooth muscle cells occurs without a change in the number of aortic smooth muscle cell (cell fusion would result in a decrease in cell number) and is associated with an increase in aortic medial DNA content (cell fusion would result in no change in total DNA

content) (Owens et al.,1981; Owens et al., 1983; Owens et al., 1985). Thus, aberrant mitosis and/or endoreplication may be the process whereby polyploid smooth muscle cells develop.

Other factors may also be involved in the development of polyploid smooth muscle cells, such as, catecholamines (Mano et al., 1986; Yamori et al., 1987), angiotensin II (Geisterfer et al., 1988; Black et al., 1988a; Black et al., 1989; Devlin et al., 1995), transforming growth factor β (Owens et al., 1988a) and/or deoxycorticosterone-NaCl treatment (Chobanian et al., 1987). Angiotensin II, for example, has been shown to induce both hypertrophy and polyploidy of vascular smooth muscle *in vitro*, most likely by induction of *c-fos*, a proto-oncogene associated with cellular differentiation and hypertrophy. Further, it has been shown that decreasing the level of angiotensin II by inhibitors of angiotensin converting enzyme reduces vascular smooth muscle polyploidy (Devlin et al., 1995; Black et al., 1989; Geisterfer et al., 1988).

The incidence of polyploidy has also been suggested to be related to high blood pressure since a linear correlation between the blood pressure level and the frequency of polyploid smooth muscle cells in a variety of hypertensive models have been observed (Owens, 1985). Normalization of blood pressure in SHR with antihypertensive treatment prevented further development of polyploidy. Polyploidy, however, may not be a simple response to increased blood pressure since chronic treatment with the β-

adrenoceptor antagonist nadolol did not prevent the development of hypertension in SHR but did decrease the percentage of polyploid smooth muscle cells in the aorta of SHR (Lee et al., 1992b). Additionally, the β-adrenoceptor antagonist propranolol inhibits the development of hypertension-induced polyploidy in aortic smooth muscle cells of deoxycorticosterone-salt hypertensive treated rats (Leitschuh et al., 1987). Thus, there are evidences suggesting that *in vivo* β-adrenoceptors may be involved in the development of smooth muscle cell polyploidy.

3.3 The expression of polyploid smooth muscle cells in hypertension

Vascular smooth muscle cells are capable of two distinct growth processes: hyperplasia (increase in cell number) and hypertrophy (increase in cell size) (Owens, 1989a; Owens, 1989b). In hypertension, hypertrophy commonly occurs in large conduit arteries and is usually accompanied by the development of polyploid (or hyperploidy) smooth muscle cells, with up to 50% of the smooth muscle cells in the vessel wall having more than the normal diploid amount of DNA in the cells (Owens et al., 1985; Yamori et al., 1987). The incidence of polyploid cells increases with both age and duration of hypertension in rats as well as humans (Barrett et al., 1983; Orekhov et al., 1983). In SHR, smooth muscle cell polyploidy was present at 3 months of age and older with approximately 30% of SHR cells being

polyploid by 11 weeks of age as compared to less than 12% polyploid smooth muscle cells in normotensive WKY of similar age (Owens et al., 1982). These polyploid cells are larger (hypertrophied) than normal WKY cells (Owens et al., 1981; Owen et al., 1982).

3.4 The expression of polyploid cells in ageing (non-hypertensive) animals

The development of polyploidy is not only found in hypertensive vascular smooth muscle but is widespread occurring in various non-hypertensive cell types of both human and animal tissues, including uterus, salivary gland, myocytes, urinary bladder, brain, liver, trachea, epidermis and artery (Brodsky et al., 1977; Bodin et al., 1987). The expression of polyploid cells also increases with age (Goldberg et al., 1984; Barrett et al., 1983; Lombardi et al., 1989). For example, in humans the frequency of polyploid cells varied with age from less than 1% at birth to approximately 7% in adult aortic, carotid and iliac vessels (Barrett et al., 1983). It is most likely that the expression of age-related polyploidization is part of the normal growth and development of many tissues (Brodsky et al., 1977).

3.5 Proposed role of polyploid cells

The role or significance of polyploidy smooth muscle cells in hypertension is unclear. In fact, relatively little is known regarding the possible functional alterations associated with smooth muscle cell polyploidy. It has been suggested that the hypertrophy and polyploidy that occurs in the large conduit arteries in SHR many play a role in a decreased compliance of these arteries (Black et al., 1989). The role of polyploidy in the resistance arteries, however, may be an indirect effect since a relatively low incidence of polyploid smooth muscle cells (2-4% compared to approximately 30% in adult SHR aorta) is found in these arteries in both SHR and WKY with no difference between the two strains (Black et al., 1988b; Owens et al., 1988b; Owens et al., 1982).

4. β-adrenoceptors

4.1 The vascular β-adrenoceptor

The initial division of adrenoceptors into α and β was first proposed based on the relative potencies of the adrenergic agonists epinephrine, and norepinephrine in comparison to isoproterenol. The α-adrenoceptors comprised of those receptors which have higher potencies for epinephrine and norepinephrine as compared to isoproterenol, whereas, the β-adrenoceptors comprised of receptors which have a higher potency for

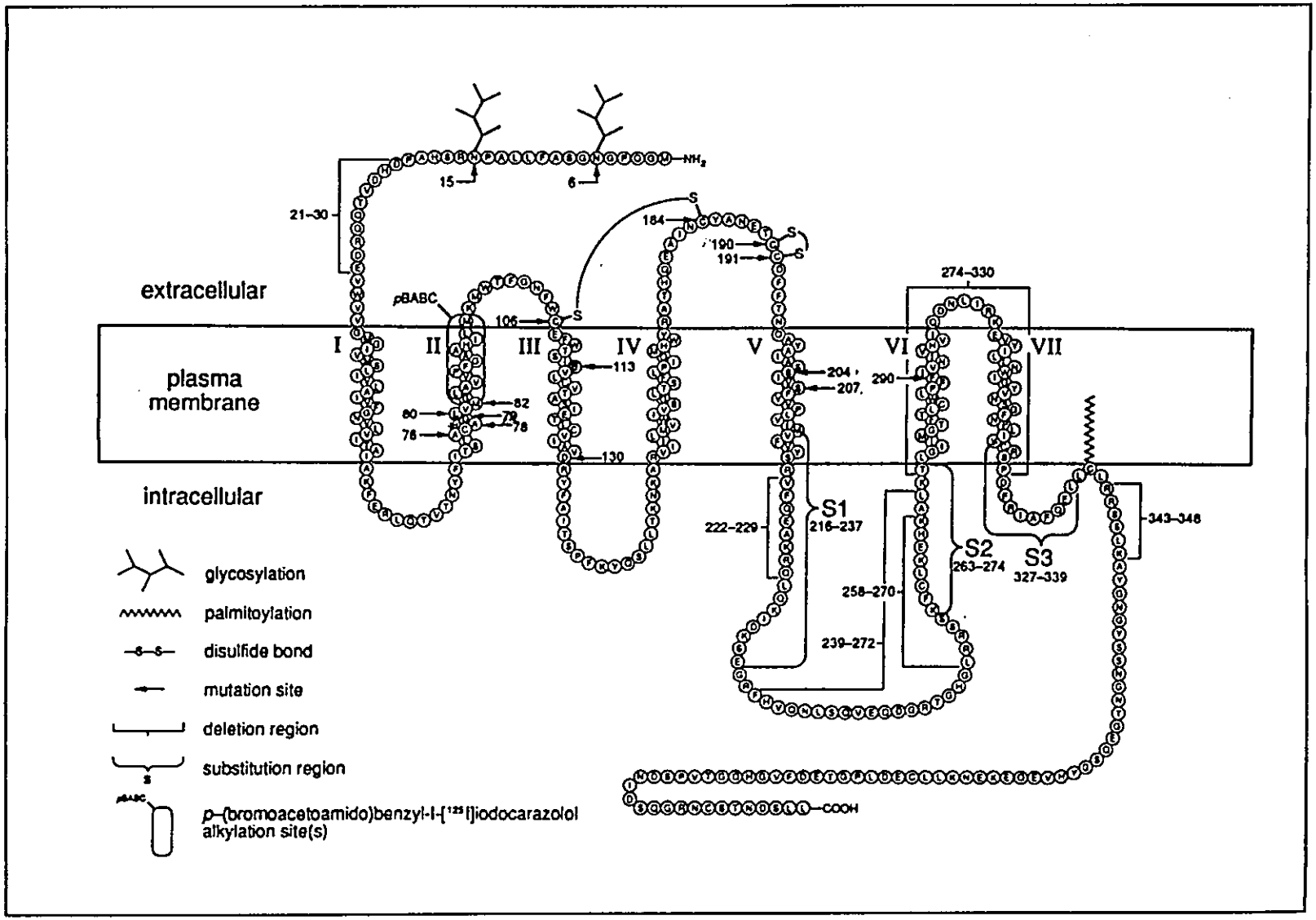
isoproterenol relative to norepinephrine and epinephrine (Ahlquist, 1948). β-adrenoceptors were further divided into β_1- and β_2-adrenoceptors subtypes based on the equal potency of norepinephrine and epinephrine at β-adrenoceptors of the heart or adipose tissue (classified as β_1 subtype), compared to the greater than 100-fold potency of epinephrine over norepinephrine at β-adrenoceptors of smooth muscle cells of the respiratory system or the peripheral blood vessels (classified as β_2 subtype) (Lands et al., 1967). An implicit assumption in this categorization was that a particular tissue or organ contains only one subtype of β-adrenoceptors. This was proved incorrect by the finding of both β_1- and β_2-adrenoceptor subtypes in cat heart (Carlsson et al., 1972). Later studies reported that heterogenous types of β-adrenoceptors may be present not only within the same tissue, but also within a single cellular type (Ebersol et al., 1981). In fact, the presence of both β_1- and β_2-adrenoceptor subtypes within the same tissue or in the same cells have now been documented in various models (Minneman et al., 1979; Brodde et al., 1983; Liang et al., 1986; Brodde et al., 1986; Minneman et al., 1988).

A third β-adrenoceptor subtype, β_3-adrenoceptor, has been shown to be present in white and brown adipose tissue (Tan et al., 1983). These β-adrenoceptor can be stimulated by β-adrenoceptor agonists but differ from both β_1- and β_2-adrenoceptor by their low sensitivity to β-adrenoceptor antagonists.

4.1.1 Structural properties of β-adrenoceptors

The β-adrenoceptor receptor consist of a single polypeptide chain, containing between 412 and 433 amino acid residues, with the presence of seven clusters of hydrophobic amino acids separated by shorter clusters of hydrophilic amino acids (Raymond et al., 1990; O'Dowd et al., 1989; Figure 3). These features confer a structure to the β-adrenoceptor consisting of seven membrane-spanning domains (transmembrane or hydrophobic domains) connected by six shorter hydrophilic loops, three each at the cytoplasmic and extracellular faces of the membrane (O'Dowd et al., 1989; Dohlman et al., 1987; Figure 3). The amino terminal of the receptor molecule extends extracellularly from the first hydrophobic domain, while the carboxyl terminal extends from the intracellular cytoplasmic seventh transmembrane domain (Raymond et al., 1990; Figure 3).

Figure 3: β-adrenergic receptor amino acid sequence and putative membrane topography (Taken from Ostrowski et al., 1992).


extracellular
plasma membrane
intracellular
I
II
III
IV
V
VI
VII
pBABC
15
6
21–30
184
190
191
106
113
82
80
78
76
130
204
207
290
274–330
S1
216–237
222–229
S2
263–274
S3
327–339
258–270
239–272
343–348
glycosylation
palmitoylation
disulfide bond
mutation site
deletion region
substitution region
p–(bromoacetoamido)benzyl-I-[125I]iodocarazolol alkylation site(s)

The use of site-directed mutagenesis to modify the amino acids in β-adreoceptors has provided evidence about the selective involvement of various segments of the β-adrenoceptor molecule in its funciton. The binding sites for catecholamines on the β-adrenoceptor has been proposed to be within the hydrophobic regions of the receptor molecule formed by the transmembrane regions, whereas, the selective coupling of β-adrenoceptors to the activation of adenylate cyclase appears to be determined by sequences present in the third cytoplasmic loop and in the carboxyl terminal end of the molecule extending into the cytoplasm (O'Dowd et al., 1989; Strader et al., 1989).

4.1.2 Functions of vascular β-adrenoceptors

Activation of β-adrenoceptors on blood vessels results in vasodilation. Initially, it was postulated that vascular β-adrenoceptor was of the β_2 subtype. In fact, *in vivo* studies measuring vascular resistance reported a β_2-adrenoceptor-mediated decrease in resistance (Fujimoto et al., 1988). In addition, studies using isolated blood vessels from guinea pig pulmonary artery and human saphenous vein also reported a β_2-adrenoceptor mediated relaxation (Ikezono et al., 1987; O'Donnell et al., 1985). In other blood vessels, however, for example coronary arteries, cerebral arteries and the rabbit facial vein, relaxation is mediated primarily

by β_1-adrenoceptors (Edvinsson et al., 1974; Edvinsson et al., 1976; McPherson et al., 1987). Generally in most blood vessels, the relaxation is mediated primarily by β_2-adrenoceptor with a small β_1-adrenoceptor mediated component (Taira et al., 1977; Cohen et al., 1978; O'Donnell et al., 1985).

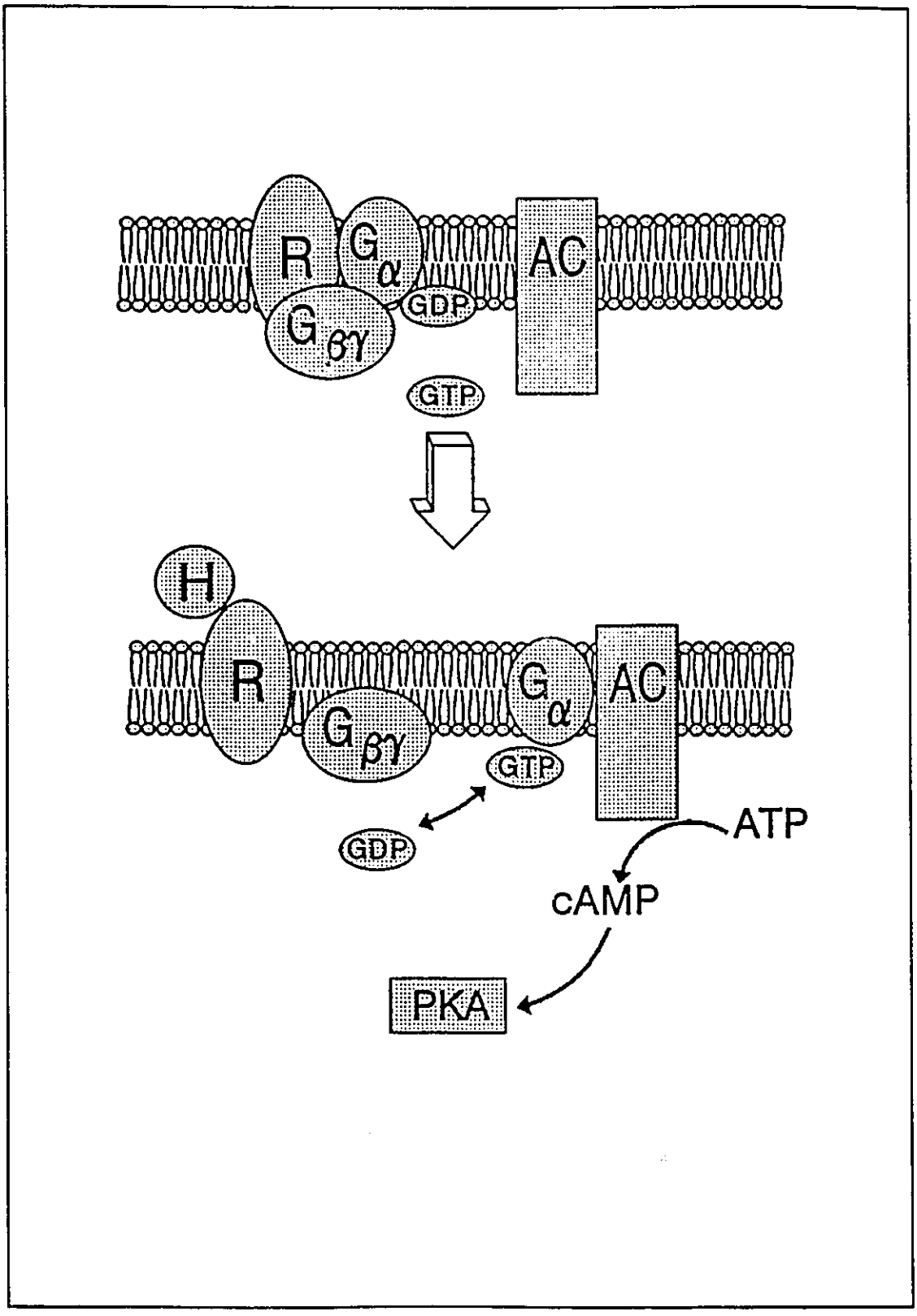
4.1.3 β-adrenoceptor mediated intracellular signal transduction pathway

Adenosine 3',5'-cyclic monophosphate (cAMP) is an intracellular second messenger which mediates the response to β-adrenoceptor activation by agonists. The eventual alteration of intracellular cAMP levels involves the interactions of cell surface receptors, G protein and adenylate cyclase. β-adrenoceptors (β_1 and β_2) are coupled to the activation of adenylate cyclase, whereas the α_2-adrenergic receptor, for example, is coupled to inhibition of adenylate cyclase activity.

Signal transduction between these receptors and adenylate cyclase is mediated by guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins). These G-proteins belong to a larger family of G-proteins that control many other specific effector enzymes including phospholipase C, cyclic guanosine monophosphate, phosphodiesterase, and ion channels (K^+ and Ca^{2+}) (Birnbaumer et al., 1990; Johnson et al., 1989). Two of the G-proteins, G_s and G_i, are involved in the stimulation and inhibition of

adenylate cyclase activity of β- and α_2-adrenoceptors, respectively. G-proteins are heterotrimers consisting of α,β, andγ subunits (Northup, 1985; Weiss et al., 1988; Gilman, 1987). G_s and G_i have similar β and γ subunits with unique α subunits (Weiss et al., 1988; Northup, 1985). Guanosine 5'-triphosphate (GTP) is essential for the activity of these G-proteins, since it facilitates the G-proteins to oscillate between two activity forms, an inactive guanosine 5'-diphosphate (GDP)-bound form and an active GTP-bound form. Occupany of β-adrenoceptor by an agonist activates G-proteins by promoting the deplacement of bound GDP by GTP in the α-subunit of the G-protein. This results in the dissociation of the α-β-γ complex into the β-γ subunit and a complex of α-GTP. The α-GTP regulates the activity of adenylate cyclase by interacting with the catalytic subunit of adenylate cyclase to form an active enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cAMP. In a similar fashion, activation of α_2 (inhibitory) promotes formation of the α-GTP subunit of the G_i-protein, which inhibits adenylate cyclase activity. Additionally, the α-subunit possesses GTPase activity that will hydrolyze the bound GTP. This degradation of GTP allows for the reassociation of the α-β-γ complex and the termination of the control over adenylate cyclase activity (Kebabian, 1992; Weiss et al., 1988; Scarpace et al., 1991; Figure 4).

Figure 4: The β-adrenoceptor (R) mediated intracellular signalling pathway. AC=adenylate cyclase, G=guanosine 5'-triphosphate binding protein, PKA=protein kinase A, ATP=adenosine triphosphate (Taken from Dimensions, 1992).


R
Gα
GDP
Gβγ
AC
GTP
H
R
Gβγ
Gα
AC
GTP
GDP
ATP
cAMP
PKA

The plasma membrane enzyme, adenylate cyclase, of which there are currently eight subtypes, catalyzes the formation of cAMP from ATP (Sibley et al., 1985; Krupinski, 1991; Kebabian, 1992; Iyengar, 1993). Adenylate cyclase activity can be increased independent of activation of β-adrenoceptors by forskolin, a naturally occurring diterpene from *Coleus forskohlii*, which binds directly to the catalytic subunit of adenyate cyclase (Seamon, 1985).

4.1.4 Regulation of β-adrenoceptor functions

The regulation of β-adrenoceptors can be generally categorized into two fundamentally distinct mechanisms: (i) short term alterations in receptor function induced by receptor occupancy by agonists, that is, receptor desensitization; and, (ii) long term regulation of receptor expression at the level of the gene, which involve alterations in the rate of receptor synthesis and/or degradation.

The process of receptor desensitization, which can be demonstrated after prolonged exposure of receptors to agonists, is characterized by a decreased responsiveness of the receptor to agonist stimulation, due to a decrease affinity for the agonist and reduced efficiency of the coupling with adenylate cyclase. Receptor desensitization can be further categorized into

two mechanisms: (i) heterologous desensitization; and, (ii) homologous desensitization (Hollenberg, 1985).

Heterologous desensitization refers to a process whereby the agonist-induced alterations in receptor function is not restricted to the receptors activated by a particular agonist. This method of desensitization seems to be related to a feed-back regulation of the signal-stimulus mechanism by second or third messenger systems common to several receptor types. For β-adrenoceptors, this seems to involve the phosphorylation reactions catalysed by cAMP-dependent protein kinases and/or the specific β-adrenoreceptor kinase (βARK) (Benovic et al., 1986; Lefkowitz et al., 1990). This may involve phosphorylation and functional alterations of the β-adrenoceptor and/or the G_s protein and/or the catalytic subunit of adenylate cyclase. Desensitization occurs as a result of uncoupling of the β-adrenoceptor to the activation of adenylate cyclase.

Homologous desensitization, on the other hand, refers to a process where the attenuation of the response induced by agonist occupancy is specific for the receptor type activated by the given agonist. Homologous desensitization of β-adrenoceptors exhibit three components: (i) rapid, agonist-induced uncoupling of receptors from the activation of adenylate cyclase likely due to receptor phosphorylation; (ii) sequestration of receptors from plasma membrane into intracellular compartments; and, (iii) receptor degradation and/or receptor regeneration/recycling.

The regulation of β-adrenoceptors may also occur by the alteration of β-adrenoceptor density by trophic influences. This regulation involves the selective influence by variations in the physiological levels of natural agonists and hormones on β-adrenoceptors (O'Donnell et al., 1987). For example, it has been reported that decreased levels of norepinephrine result in increased number of β-adrenoceptors (up regulation), while increase levels of norepinephrine result in decreased number of β-adrenoceptors (down regulation). This variation in receptor density was observed for β_1-adrenoceptors, while β_2-adrenoceptor number remained unchanged (Minneman et al., 1979; Ek et al., 1986; Grassby et al., 1986).

4.2 The expression and function of vascular β-adrenoceptors in hypertension

In hypertension, there is a decrease in β-adrenoceptor-mediated vasorelaxation which has been attributed to the development of desensitization of the β-adrenoceptors (Brodde et al., 1992; Ford et al., 1995; Michel et al., 1993; Michel et al., 1994; Bohm et al., 1992; Feldman, 1987). The site of this desensitization, however, is unclear. Some studies reported a reduction in vascular β-adrenoceptor numbers (Michel et al., 1990; Woodcock et al., 1980; Limas et al., 1979). Other studies, however, have detected no alterations in β-adrenoceptors (Asano et al., 1991). It has

also been suggested that desenitization of β-adrenoceptors may be due to altered G protein function, however, direct measurements of vascular G protein levels in hypertension have not supported this view (Asano et al., 1988; Asano et al., 1988; Clark et al., 1993).

4.3 The expression and function of β-adrenoceptors in ageing (non-hypertensive) animals

β-adrenoceptor-mediated vasodilation of blood vessels has been reported in most studies to decrease with age (Ericsson et al., 1975; Fleisch, 1981; Fleisch et al., 1976; Fleisch et al., 1970; Ikezono et al., 1987; O'Donnell et al., 1981; O'Donnell et al., 1984; O'Donnell et al., 1986; Tsujimoto et al., 1986). One explanation for the decreased β-adrenoceptor-mediated action on the vasculature with ageing is an age-associated decrease in β-adrenoceptor density. This possibility has been studied in a variety of tissues including myocardium, vascular smooth muscle and lymphocytes (Narayanan et al., 1982; Tsjimoto et al., 1986; Abrass et al., 1981). In human lymphocytes, rat lymphocytes, heart, lung and mesenteric arteries, the number of β-adrenoceptors have been reported to be unaltered with age (Tsjimoto et al., 1986; Abrass et al., 1981; Abrass et al.,1982; Narayanan et al., 1982). β-adrenoceptor density, however, does not remain

unchanged for all organs. β-adrenoceptor density decreases with age in rat white and brown adipocytes, rat heart and rat brain (Scarpace, 1988; Fan et al., 1985; Friedman et al., 1986). In contrast, it has also been reported that β-adrenoceptor density increases with age in rat heart, liver and lung (Dax et al., 1987; Kusiak et al., 1983; Vanscheeuwijck et al., 1989).

Studies using competition binding between radiolabelled antagonists and unlabelled agonists, which assess the number of high affinity or G-protein coupled receptors, reveal that the number of high affinity or functional receptors decreases with age in most tissues that have been studied, including rat heart, lung and lymphocytes (Feldman et al., 1984; Montamat et al., 1989; Narayanan et al., 1982; Scarpace et al., 1983; Scarpace et al., 1986). It is most likely that this decrease in functional receptors with age may be a contributing factor to the decrease β-adrenoceptor-mediated action with age.

Another explanation for decrease β-adrenoceptor action with ageing may lie with the expression and/or level of G-proteins and/or adenylate cyclase, which ultimately affects the level of cAMP. Some studies have suggested that G_s protein activity is diminished with ageing (Scarpace et al., 1986; Scarpace, 1988; O'Connor et al., 1983; Kusiak et al., 1983). In other studies, G_s protein has been reported to be in excess of the adenylate cyclase catalytic subunit (O'Connor et al., 1983). In older human lymphocytes, G_s protein activity is unchanged compared to younger

individuals, however, the catalytic subunit activity of the adenylate cyclase is lowered (Abrass et al., 1982). Since G_s proteins act as coupling factors to transduce the signal from the receptor to the catalytic subunit of adenylate cyclase, alterations that decrease the number and/or function of either G_s or adenylate cyclase catalytic subunits would result in a decreased production of cAMP, resulting in diminished β-adrenoceptor function with ageing.

4.4 The role of the β-adrenoceptor mediated signalling pathway in DNA synthesis and cell growth

Elevation of intracellular cAMP has been reported to both inhibit and stimulate cell growth (Boynton et al., 1983; Rosengurt, 1986; Kempski et al., 1987; Dumont et al., 1989; Printseva et al., 1992; Jackson et al., 1992). This apparent dual role has been suggest to be due to the fluctuations of intracellular cAMP levels during the different stages of the cell cycle, with a cAMP surge early in the G_1 phase inhibiting cell growth, whereas a cAMP surge late in the S or G_2 phase stimulating cell growth (Boynton et al., 1983). It has been suggested that, with respect to cell growth, the overall role of cAMP is to stimulate events leading to DNA synthesis (Boynton et al., 1983).

This view is supported from studies in cultured rat aortic smooth muscle cells where an increase in adenylate cyclase activity has been reported to precede DNA synthesis (Franks et al., 1984). Further, in cultured aortic smooth muscle cells from SHR and WKY, agonist stimulation of β-adrenoceptors have been found to increase the activities of the enzyme, ornithine decarboxylase, which plays a role in smooth muscle cell hypertrophy and hyperplasia (Kanbe et al., 1983). The effects of cAMP on DNA synthesis and cell growth are thought to be mediated by the activation of protein kinase A (PKA) and by the subsequent phosphorylation of cellular proteins and nuclear transcription factors (Rosengurt, 1986; Dumont et al., 1989; Walsh et al., 1994; Taylor et al., 1992). These findings suggest that activation of smooth muscle cell β-adrenoceptors, with subsequent elevation of intracellular cAMP via stimulation of adenylate cyclase by G_s proteins, may lead to an elevation in DNA synthesis. The molecular mechanisms, however, still needs to be elucidated.

5. Hypertension, smooth muscle cell polyploidy and β-adrenoceptors: Unresolved questions

Despite the research already completed pertaining to polyploid smooth muscle cells and hypertension, several questions still remain unanswered:

1. Does activation of functional β-adrenoceptors, with subsequent elevation of intracellular cAMP levels, mediate the development of polyploidy smooth muscle cells in both hypertensive and non-hypertensive animals?

2. What is/are the molecular mechanism(s) underlying the development of polyploid smooth muscle cells?

3. What are the functional consequences of the presence of polyploid smooth muscle cells in the arteries of hypertensive and non-hypertensive animals?

This thesis focuses on investigating the first question.

CHAPTER II

OBJECTIVES AND HYPOTHESIS

Chapter II: Objectives and Hypothesis

1. Objectives and hypothesis

1.1 General hypothesis:

The activation of SMC β-adrenoceptors leads to the development of polyploid SMC via activation of intracellular messengers.

1.2 Objective 1:

To determine the expression of polyploid cells in cultured aortic smooth muscle cells from 3-4-week (prehypertensive), 10-12-week (developing hypertensive), 28-30-week (establish hypertensive) old SHR and WKY.

Hypothesis:

Smooth muscle cells maintained under standard culture conditions exhibit polyploid cells as observed under *in vivo* conditions.

Results:

Smooth muscle cells from SHR and WKY exhibited a heterogeneous population of cells under culture conditions. Cultured SHR smooth muscle cells exhibited a higher percentage of multinucleated and polyploid cells as compared to WKY at all three age groups. In SHR and WKY, polyploid smooth muscle cell increased with age of the animals.

1.3 Objective 2:

To determine the expression of β-adrenoceptors in cultured smooth muscle cells from 3-4-week (prehypertensive), 10-12-week (developing hypertensive), 28-30-week (establish hypertensive) old SHR and WKY.

Hypothesis:

The expression of β-adrenoceptors is different between SHR and WKY in cultured aortic smooth muscle cells from different age groups.

Results:

Cultured smooth muscle cells from SHR express elevated β-adrenoceptor binding sites (B_{max}) as compared to WKY at all three age groups with no significant difference in receptor affinity (K_d). The increase in β-adrenoceptor number was positively correlated with the increase in polyploid smooth muscle cells and with ageing of SHR and WKY.

1.4 Objective 3:

To determine the functionality of β-adrenoceptors expressed on cultured smooth muscle cells from SHR and WKY from 3-4-week(prehypertensive), 10-12-week (developing hypertensive), 28-30-week (established hypertensive) old SHR and WKY.

Hypothesis:

Stimulation of β-adrenoceptors on cultured smooth muscle cells from SHR and WKY by β-agonists will produce different levels of the intracellular second messenger, adenosine 3',5'-cyclic monophosphate (cAMP).

Results:

Stimulation of cultured smooth muscle cells from SHR and WKY with the β-adrenoceptor agonist, isoproterenol, and the adenylate cyclase activator, forskolin, resulted in similar increases in cAMP levels in both strains of rats in all three age groups. However, treatment with forskolin resulted in significantly higher levels of cAMP production than those obtained with isoproterenol in both SHR and WKY. The addition of the β-adrenoceptor antagonist, dl-propranolol, inhibited the isoproterenol-induced response in both SHR and WKY.

1.5 Objective 4:

To determine the role of β-adrenoceptor activation in mediating the development of polyploid cells in cultured smooth muscle cells from SHR and WKY from 3-4-week (prehypertensive), 10-12-week (developing hypertensive), 28-30-week (established hypertensive) old SHR and WKY.

Hypothesis:

The activation of the β-adrenoceptor mediated intracellular pathway will produce polyploid cells in cultured smooth muscle cells from SHR and WKY.

Results:

Treatment of cultured smooth muscle cells with the β-adrenoceptor agonist, isoproterenol, resulted in an increase in polyploid smooth muscle cells in 3-4-week old SHR and WKY and 10-12-week old WKY when compared to non-stimulated conditions. Addition of the β-adrenergic antagonist, *dl*-propranolol, partially inhibited the isoproterenol-stimulated increase in polyploidy in both SHR and WKY. Treatment of cultured smooth muscle cells with forskolin, an adenylate cyclase activator, or 8-bromo-cAMP, a non-hydrolysable analog of cAMP, resulted in an increase in polyploid smooth muscle cells in both SHR and WKY as compared to basal non-stimulated conditions.

CHAPTER III

MANUSCRIPT # 1

MANUSCRIPT No. 1

INCIDENCE OF MULTINUCLEATED AND POLYPLOID AORTIC SMOOTH MUSCLE CELLS FROM DIFFERENT AGE GROUPS OF SPONTANEOUSLY HYPERTENSIVE RATS

Published in the *Canadian Journal of Physiology and Pharmacology* in 1992

Roop B. Conyers's contribution:

(i) cell culturing

(ii) cell staining

(iii) microdensitiometric scanning

(iv) analysis of data

Incidence of multinucleated and polyploid aortic smooth muscle cells cultured from different age groups of spontaneously hypertensive rats

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LEE, R. M. K. W., CONYERS, R. B., and KWAN, C.-Y. 1992. Incidence of multinucleated and polyploid aortic smooth muscle cells cultured from different age groups of spontaneously hypertensive rats. Can. J. Physiol. Pharmacol. 70: 1496–1501.

Cell size and incidence of multinucleated, polyploid cells in cultured aortic smooth muscle cells from different age groups of spontaneously hypertensive rats (SHR) and normotensive Wistar–Kyoto rats (WKY) were compared. Smooth muscle cells from SHR were generally larger than those from WKY, and the percentage of multinucleated smooth muscle cells was always higher in SHR than WKY in the three age groups of rats studied (3–4, 10–12, and 28–30 weeks). In smooth muscle cells from the 3- to 4-week group, there was a positive correlation between cell diameter and the percentage of multinucleated smooth muscle cells. Microdensitometric measurements also showed that the incidence of polyploid smooth muscle cells was always higher in SHR than WKY in the three age groups. There was a positive correlation between DNA density and nuclear area measurements in all the age groups of SHR and WKY. We conclude that cultured aortic smooth muscle cells from different age groups of SHR and WKY contained heterogeneous populations of cells and that, under our culture conditions, the polyploidy of the smooth muscle cells found *in vivo* was maintained in the SHR and WKY.

Key words: hypertension, cultured smooth muscle cells, aorta, polyploidy.

LEE, R. M. K. W., CONYERS, R. B., et KWAN, C.-Y. 1992. Incidence of multinucleated and polyploid aortic smooth muscle cells cultured from different age groups of spontaneously hypertensive rats. Can. J. Physiol. Pharmacol. 70 : 1496–1501.

On a comparé la taille et le taux de cellules polyploïdes multinucléées dans les cellules musculaires lisses aortiques cultivées de rats spontanément hypertensifs (RSH) et de rats Wystar–Kyoto normotendus (WKY) d'âges différents. Les cellules musculaires lisses des RSH ont été généralement plus grosses que celles des WKY, et le pourcentage de cellules musculaires lisses multinucléées a toujours été plus élevé chez les RSH que chez les WKY des trois groupes d'âge (3–4, 10–12 et 28–30 semaines). Dans les cellules musculaires lisses du groupe âgé de 3–4 semaines, il y eu une corrélation positive entre le diamètre des cellules et le pourcentage de cellules musculaires lisses multinucléées. Des mesures microdensitométriques ont aussi montré que le taux de cellules musculaires lisses polyploïdes était toujours plus élevé chez les RSH que chez les WKY des trois groupes d'âges. Il y a eu une corrélation positive entre l'aire nucléaire et la densité de l'ADN des RSH et des WKY des trois groupes d'âges. Nous concluons que les cellules musculaires lisses aortiques des RSH et des WKY des trois groupes d'âges contenaient des populations hétérogènes de cellules, et que, dans nos conditions de culture, la polyploïdie des cellules musculaires lisses observée dans des conditions *in vivo* a été maintenue chez les RSH et WKY.

Mots clés : hypertension, cellules musculaires lisses cultivées, aorte, polyploïdie.

[Traduit par la rédaction]

Introduction

In hypertension, the causes of medial hypertrophy differ, depending on the types of arteries (Lee and Smeda 1985; Lee 1987). In the aorta of spontaneously hypertensive rats (SHR), medial enlargement is mainly due to hypertrophy and polyploidy of the smooth muscle cells (SMCs) (Owens and Schwartz 1982; Owens *et al.* 1981; Owens 1985, 1987). It was thought that these changes might be a direct consequence of high blood pressure, because they were present only in older SHR and, in addition, antihypertensive treatment prevented these changes from taking place (Lee and Smeda 1985; Lee 1987). However, recent studies suggest that stimulation by catecholamines (Mano *et al.* 1986; Yamori *et al.* 1987), angiotensin II (Geisterfer *et al.* 1988), and by deoxycorticosterone–NaCl treatment (Chobanian *et al.* 1987) also causes polyploidy of the aortic SMCs. Polyploidy is an increase in DNA content within a cell; instead of the normal diploid DNA content (2C, two sets of chromosomes), some cells may contain twice (4C, tetraploid) or four times (8C, octaploid) the normal amount of DNA in each cell. These cells are generally referred to as polyploid (or hyperploid) cells. They are usually larger than normal cells (Owens and Schwartz 1982; Owens *et al.* 1981; Owens 1985, 1987). Under culture conditions, polyploid aortic SMCs from normal rats are capable of undergoing cell division to produce polyploid cells, but with serial subcultivation, there was a gradual loss of the polyploid subpopulation (Goldberg *et al.* 1984*a*). The mechanism or significance of polyploidy is unknown. Polyploidy may occur as a result of either cell fusion or DNA duplication in the cell without subsequent karyokinesis or cytokinesis (Gordon *et al.* 1986). Polyploid SMCs were also found in resistance arteries of SHR and Wistar–Kyoto rats (WKY) (Black *et al.* 1988; Owens *et al.* 1988), but the incidence was relatively low (2–4% (Owens *et al.* 1988) compared with 10–30% in the aorta of adult SHR and WKY (Owens and Schwartz 1982)), and there was no difference between SHR and WKY. In recent studies on the proliferative characteristics of cultured aortic SMCs from different age groups of SHR and WKY (R. M. K. W. Lee, N. Blaes, M. R. Buchanan, and P. Ernst, unpublished), we have noticed the presence of multinucleated SMCs. The primary objectives of this study were to investigate (*i*) whether there is a difference between SHR and WKY in the incidence of multinucleated, polyploid SMCs and cell size in culture and (*ii*) whether SMCs derived from different age groups of SHR and WKY differ in cell size and in number of nuclei.

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Materials and methods

Secondary cultures of SMCs derived from the thoracic aortae of SHR and WKY in the prehypertensive (3–4 weeks), developing hypertensive (10–12 weeks), and established hypertensive (28–30 weeks) phases of hypertension were used. These rats were obtained from colonies maintained at the animal facility of McMaster University. They originated from the Charles River (St. Constant, Que.) strains more than 10 years ago. Cells derived from each rat were propagated separately so that we could compare the difference between cells from different animals. In each set of experiments, SMCs from one SHR and one WKY were cultured simultaneously under identical conditions. For each rat, cells in passage 6 or 7 were cultured in two Falcon flasks (25 cm^2) containing Dulbecco's modified Eagle medium (DMEM), with 10% fetal calf serum (FCS) and 1% antibiotic–antimycotic solution. The cells were seeded at a density of 2×10^4 cells/mL. Plating efficiency for both SHR and WKY was ≥95%. When confluency was reached, the growth cycle of the cells was synchronized by replacing the culture medium with DMEM containing only 0.5% FCS, and the cells were grown in this medium for 48 h. Cells in one of the two flasks were harvested by trypsinization and used for Coulter counter measurements of cell size. When cultured cells were trypsinized, the cells became rounded, so that their volume could be measured easily with a Coulter counter. The Coulter counter was calibrated using polystyrene microspheres 20–27 μm in diameter. The cell diameter was calculated from the cell volume determined by the Coulter counter, using the formula

$$d = 2 \times \sqrt[3]{\left(V \times \frac{3}{4\pi}\right)}$$

where d is the diameter of the cell (μm) and V is the volume (μm^3) from Coulter counter measurements. The cells in the second flask were fixed with 10% neutral Formalin and subsequently stained with haematoxylin. Under high power phase contrast, the number of SMCs containing one or more nuclei in each field was counted. This same process was repeated until the total number of cells counted exceeded 500. The percentage of cells containing one nucleus and those containing more than one nucleus was calculated. Care was taken not to count two closely situated cells as binucleated cells.

In separate experiments, SMCs in passage 7 from the three age groups of rats were grown until confluent, and their growth was synchronized as before. The cells were fixed with 10% neutral Formalin for 1 h at room temperature and rinsed in distilled water. Nuclear DNA was demonstrated by the Feulgen reaction, using the following schedule: hydrolysis in 5 M HCl for 1 h at room temperature (Dixon and Stead 1977); two rinses in distilled water; staining in Schiff's reagent prepared by the method of de Tomasi for 1 h (Pearse 1968); three rinses in freshly prepared bisulphite solution for 3 min each; one rinse in distilled water; dehydration in ethanol; and mounting in mineral oil (Pearse 1968). A Vickers M-85 scanning microdensitometer (Vickers Instruments, England) was used to measure the intensity of the Feulgen staining. Integrated scans of individual intact nuclei were carried out at 545 nm without background subtraction. Areas of the nuclei were also measured simultaneously. From each preparation, 200 nuclei were measured at random. Chicken erythrocytes stained the same way were used as standards, since each erythrocyte is known to contain 2.5 pg of DNA (Black *et al.* 1988).

Student's unpaired *t*-tests, one-way analysis of variance, and linear regression analyses were carried out to compare SHR with WKY and also to compare various age groups of SHR and WKY. Values of p less than 0.05 were considered significant.

Results

Physical characteristics of the rats from which the aortic SMCs were obtained for culture are given in Table 1. At 3–4 weeks, blood pressure (BP) and heart rate of SHR and WKY were similar, but the body weight of WKY was higher than that of SHR. In the two older groups of animals, the body weight of SHR and WKY became similar, while BP was significantly higher in SHR than WKY.

TABLE 1. Physical characteristics of SHR and WKY used in cell cultures (mean ± SEM)

	Age group (weeks)		
	3–4	10–12	28–30
Blood pressure (mmHg, tail)			
SHR	109±4	185±10	193±4
WKY	99±6	125±3	122±2
p	ns	0.001	<0.0001
Heart rate (min^{-1})			
SHR	400±6	360±12	360±9
WKY	380±20	353±9	352±9
p	ns	ns	ns
Body weight (g)			
SHR	34±3	248±2	391±16
WKY	58±4	270±11	289±16
p	0.01	ns	ns

In the cultures derived from the two strains, many SMC types could be recognized structurally. At confluency, most of the mononucleated cells were of uniform size (Fig. 1*a*). Some large cells were found interspersed among the smaller mononucleated cells (Fig. 1*b*). Some of these large cells were mononucleated (Fig. 1*b*), whereas others were multinucleated (Figs. 1*c*, 1*d*). Postconfluency, these large cells maintained their monolayer characteristics and did not form clusters of multilayered cells (Fig. 1*c*). At this stage, the nuclei of these large cells were generally larger than nuclei of the smaller cells, and hence on the basis of size alone, two distinct cell types could easily be recognized (Figs. 1*b*, 1*c*).

Comparison of cell diameters between SHR and WKY for each age group, based on Coulter counter measurements, showed that in the prehypertensive (3–4 weeks old) and young adult (10–12 weeks) groups, SMCs from SHR were significantly larger than those from WKY (14.1 ± 0.6 μm for SHR and 12.1 ± 0.3 μm for WKY, in the prehypertensive group, $p < 0.001$; 12.7 ± 0.4 μm for SHR versus 11.0 ± 0.2 μm for WKY in the young adult group, $p < 0.01$). However, in the older adult group (28–30 weeks), the average diameter of the SMCs was similar for SHR and WKY (13.6 ± 0.3 μm for SHR and 13.0 ± 0.9 μm for WKY, ns). Analysis of variance (with Bonferroni correction for multiple comparisons) showed that within each strain, there was no difference in cell diameter among different age groups.

The percentage of SMCs that were multinucleated was always higher in the SHR than WKY in the three age groups (Table 2). Analysis of variance showed that within each strain, there were differences among the various age groups of SHR and WKY ($p < 0.0001$ for both SHR and WKY). In both strains of rats, the differences among different age groups within each strain were due to a higher percentage of multinucleated cells in the 10- to 12- and 28- to 30-week-old groups as compared with the 3- to 4-week-old group ($p \leq 0.001$). There was no difference between the 10- to 12- and 28- to 30-week-old groups within each strain. Correlation of the percentage of multinucleated SMCs with cell diameter was positive only in the 3- to 4-week-old group ($r = 0.724$, $p < 0.001$).

Microdensitometric measurements of the SMCs from the

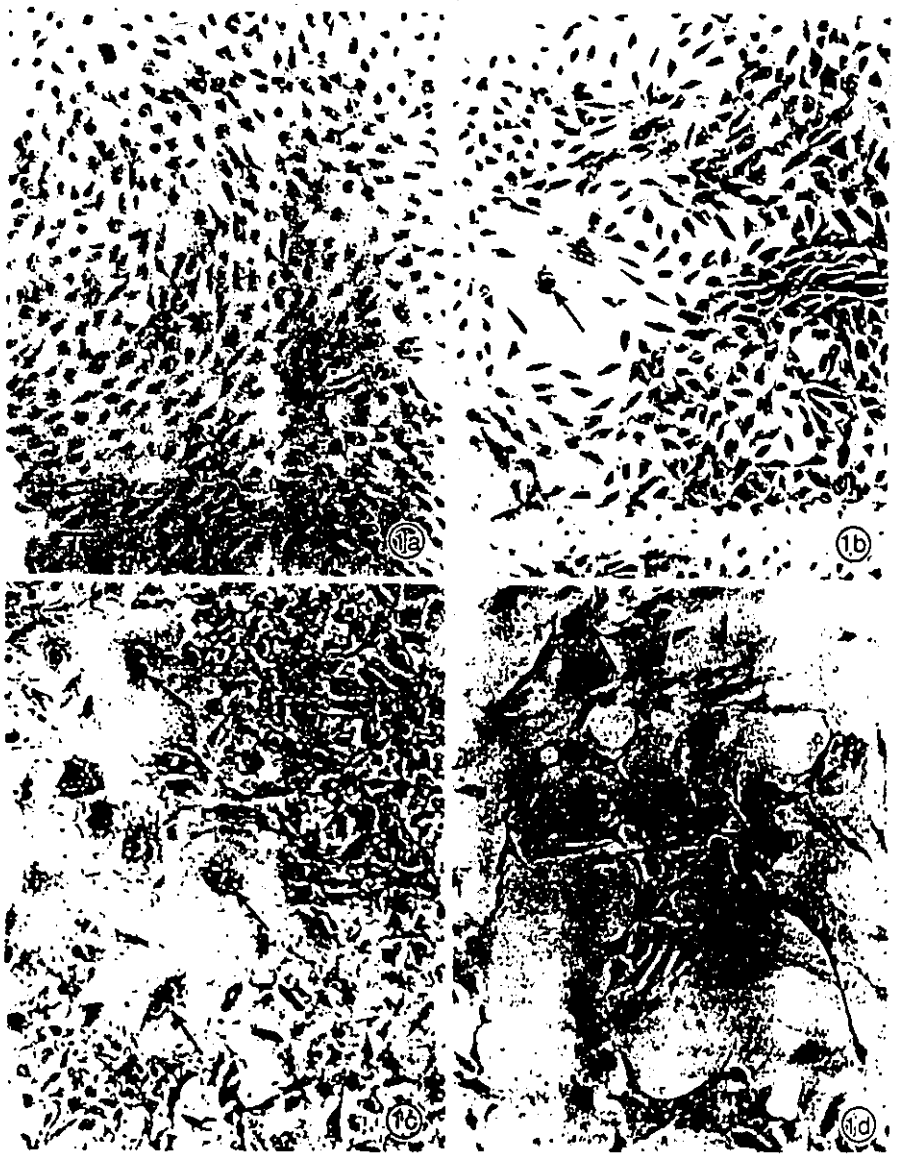

FIG. 1. (*a*) SMCs from 28-week-old WKY at confluency. Note that most of the cells are mononucleated. Bar represents 100 μm for all the micrographs. (*b*) As in Fig. 1*a*, showing some large cells (arrow). (*c*) SMCs from 12-week-old SHR, showing large, binucleated smooth muscle cells (arrows). (*d*) SMCs from 4-week-old SHR, showing binucleated SMCs

three age groups showed that in the WKY, most of the cells were at the 2–20 intensity units range, whereas in the SHR, there were many cells with high intensity readings. Under our measurement conditions and using the chicken erythrocytes as standards, 1 intensity unit was equivalent to 0.46 pg of DNA. We therefore chose cells with 20 intensity units or less (i.e., ≤9.20 pg DNA) as diploid cells (2C); cells with 21–40 intensity units (9.66–18.40 pg DNA) as tetraploid (4C); and those with >41 units (>18.86 pg DNA) as octaploid (8C). These values are within the range previously reported for SMCs freshly isolated from the aortae of SHR and WKY (Owens *et al.* 1981; Owens 1985). On the basis of these criteria, it was found that there was always a significantly higher population of polyploid SMCs in SHR than WKY in the three age groups of rats (Table 2). Analysis of variance showed that within each strain, there was a significant difference among the three age groups in percentage of 2C and 4C cells ($p \leq 0.001$ for both SHR and WKY), but not in 8C cells ($p \geq 0.07$). Among the SHR age groups, there was a trend for an age-dependent decrease in percentage of 2C cells, with a corresponding increase in 4C cells. In contrast, among the WKY age groups, there was no significant difference in percentage of 2C and 4C

TABLE 2. Percentage of cells with multiple nuclei and percentage of polyploidy in SMCs from different age groups of SHR and WKY

Age group (weeks)	Rat group	Cells with multiple nuclei (%)	2C SMCs (%)	4C SMCs (%)	8C SMCs (%)
3–4	SHR	2.8±0.5	64.8±4.9	23.4±2.5	11.8±2.5
	WKY	0.6±0.1	92.3±2.3	6.8±2.1	0.90±0.30
	p	<0.0005	<0.001	<0.001	<0.004
10–12	SHR	7.8±0.7	48.5±9.6	47.6±10.6	3.9±3.2
	WKY	5.2±0.1	99.5±0.5	0.5±0.5	0
	p	<0.03	<0.002	<0.004	
28–30	SHR	7.7±0.6	21.2±1.7	74.1±1.2	4.7±0.7
	WKY	4.8±0.9	35.0±1.2	64.7±1.1	0.3±0.3
	p	<0.05	<0.003	<0.005	<0.005

NOTE: Values are means ± SEM. For the 3- to 4-week age group, $n = 6$ for SHR and WKY; for the 10- to 12-week age group, $n = 4$ for SHR and WKY; for the 28- to 30-week age group, $n = 4$ for SHR and $n = 3$ for WKY.

cells between the 3–4- and 10–12-week-old groups. However, a significantly lower percentage of 2C and a higher percentage of 4C cells were found in the 28- to 30-week-old group compared with the two younger groups (Table 2). DNA content was positively correlated with nuclear area measurements in all the age groups of SHR and WKY ($r \geq 0.90$, $p < 0.001$).

Discussion

The major findings in this study are (*i*) cultured aortic SMCs from different age groups of SHR and WKY contained heterogeneous populations of cells; (*ii*) in the three age groups studied, there was always a higher percentage of multinucleated cells in SHR than WKY, and in the 3- to 4-week-old group, there was a positive correlation between cell size and percentage of multinucleated SMCs; and (*iii*) the incidence of polyploid cells, as determined by microdensitometry, was higher in SHR than WKY in the three age groups studied.

Previous studies using cultured aortic SMCs from SHR and stroke-prone SHR have shown that SMCs from hypertensive animals tended to grow faster than those from WKY (Kanbe *et al.* 1983; Yamori *et al.* 1981, 1984; Clegg *et al.* 1986; Hadrava *et al.* 1989; Scott-Burden *et al.* 1989). This suggested that, apart from BP and neurohormonal factors, there is a genetic difference between SMCs from hypertensives and normotensives affecting their ability to proliferate. It has been recognized that aortic SMCs contain heterogenous population of cells with different morphological and growth characteristics (see below). It is therefore desirable to maintain such heterogeneity in cell culture studies, so that the growth characteristics of SMCs in culture conditions and also in the presence of various growth-promoting agents can be studied to understand the genetic basis of various disease states such as hypertension. However, in recent studies on growth characteristics of SMCs from prehypertensive and developing hypertensive SHR and age-matched WKY (R. M. K. W. Lee, N. Blaes, M. R. Buchanan, and P. Ernst, unpublished), we found that, depending on the age group as well as the source of the cells, cells from WKY generally tended to proliferate faster than those from the SHR. This difference may be partially due to the difference in plating efficiency of the cells from SHR as compared with WKY. In cultured SMCs from the 3- to 4-week-old group, the rate of attachment of the cells at different times after seeding was always faster in WKY than SHR, which corresponded to a faster rate of cell proliferation in WKY than SHR; however, in the 10- to 12-week-old group, the difference in cell proliferation between SHR and WKY was variable, depending on the experimental pairs, but cells that attached to the substrate faster always proliferated faster (R. M. K. W. Lee, N. Blaes, M. R. Buchanan, and P. Ernst, unpublished). Another factor is the heterogeneity of the SMCs from the aorta, as discussed below.

Many recent studies have recognized that aorta of normal rats contained mixed populations of SMCs, which are different from each other morphologically as well as functionally (Owens and Schwartz 1982; Owens *et al.* 1981; Owens 1985, 1987; Goldberg *et al.* 1984*a*; Gordon *et al.* 1986; Haudenschild and Grunwald 1985; Rosen *et al.* 1985, 1986*b*; Bodin *et al.* 1987). A significant proportion of aortic SMCs from normal and hypertensive animals are polyploid (Owens and Schwartz 1982; Owens *et al.* 1981; Owens 1985, 1987; Goldberg *et al.* 1984*a*; Gordon *et al.* 1986; Rosen *et al.* 1985, 1986*a*, 1986*b*). The percentage of polyploid SMCs in intact aortae from SHR as compared with WKY varied depending on age, being lower in younger than older animals. The percentages varied among different studies. Rosen *et al.* (1986*a*) found 3.5% polyploid cells in SHR and 4.5% in WKY in neonatal rats compared with 22.5% in SHR and 13.5% in WKY in 3- to 12-month-old animals; Owens and his colleagues found <1% polyploid cells in both SHR and WKY in the 12-week-old group (Owens 1985) compared with 15–21% in SHR and 8% in WKY in the 20-week-old group (Owens 1987; Owens *et al.* 1981). In aortae from normal rats, there are heterogeneous populations of SMCs with respect to their proliferative ability (Haudenschild and Grunwald 1985), which might be related to the ploidy of the cells. Polyploid cells are known to proliferate much more slowly than normal diploid cells (Rosen *et al.* 1985, 1986*b*). In addition, SMCs from different segments of the thoracic aortae in SHR and WKY differed in cell size, ploidy, and proliferation rate (Bodin *et al.* 1987). It is evident that, depending on the composition of the cells obtained from each rat, the growth characteristics of the cell culture can be quite different. Cultures containing a higher proportion of polyploid cells, as in the case of our prehypertensive SHR, would proliferate at a slower rate than those containing more diploid cells (R. M. K. W. Lee, N. Blaes, M. R. Buchanan, and P. Ernst, unpublished).

In this study, we used the number of nuclei in a cell as another measure of the ploidy of the cells. We recognized that

by this method we might be underestimating the incidence of polyploidy, because mononuclear polyploid cells are not included. Our results indeed showed that the percentage of polyploid cells determined by microdensitometry is higher than multinucleated cells. However, the two results are complementary to each other, because with the microdensitometric method, polyploid cells in the form of multinucleated cells are missed, because the nuclei are measured individually, while counting the number of nuclei does not take into account mononucleated polyploid cells. A question that arises is whether multinucleated cells are present *in vivo*. Owens *et al.* (1981) found that in SMCs isolated from the aortae of 7-month-old rats, the percentage of binucleated SMCs was higher in SHR (2.49 ± 0.27) than WKY (0.44 ± 0.13), which is similar to our results from cultured SMCs in the 3- to 4-week-old group. We also found that multinucleated cells tended to be larger and cells with larger nuclei tended to have a larger cell attachment area, facts which are consistent with the results of Rosen *et al.* (1985). However, significant differences in cell diameter between SHR and WKY as determined using a Coulter counter were found only in the 3- to 4- and 10- to 12-week-old groups. A significant correlation of the percentage of multinucleated SMCs with cell diameter was found only in the 3- to 4-week-old group. This may be related to the limitation of our Coulter counter for cell size estimation.

The functional significance of polyploidy and the formation of multinucleated cells in the aortic SMC is unknown. It is also not known how these two processes are related. Nevertheless, the occurrence of polyploid SMCs may be related in part to BP. Regression analyses of SHR treated with various types of antihypertensives showed a high degree of correlation between BP and the frequency of polyploid SMCs and medial smooth muscle content in the SHR (Owens 1987). The order of effectiveness of drugs in preventing the development of polyploidism in SHR was the same as their potency in lowering BP. However, further analyses indicated that captopril had an effect over and above that predicted by its antihypertensive efficacy, suggesting that other factors, such as angiotensin II, may be important in modulating aortic medial hypertrophy in SHR (Owens 1987). Our results suggest that the development of polyploid SMCs in the aorta may be part of the ageing process, because of the presence of a higher percentage of multinucleated and polyploid SMC in older rats (≥ 10 weeks) as compared with 3- to 4-week-old rats. This is consistent with the findings in *normal* humans and animals that, during the ageing process, there is a tendency for the frequency of 4C SMCs to increase (Lombardi *et al.* 1989; Barrett *et al.* 1983; Goldberg *et al.* 1984*b*). In SHR, results suggest that this process occurs sooner than in WKY (premature ageing?). An increase in polyploid SMC had already occurred at 10–12 weeks in the SHR, whereas in WKY, a significant age-dependent reduction in 2C SMCs occurred only in the 28- to 30-week-old group. In certain branch levels of the mesenteric arteries, an age-dependent (45 vs. 78–80 months) increase in the frequency of polyploid SMCs was found in SHR and WKY (Lombardi *et al.* 1989). However, there was no difference between SHR and WKY in the percentage of polyploid cells at any mesenteric branch level at either age (Lombardi *et al.* 1989). These results indicate that, depending on the vessel types, the rate of age-dependent increase in polyploid SMCs may differ between SHR and WKY.

In conclusion, we have demonstrated that, in cultured aortic SMCs from three age groups, the incidence of polyploid cells was consistently higher in SHR than WKY. This suggests that the *in vivo* level of polyploidy of SMCs from SHR is maintained in culture. The higher incidence of polyploid SMCs from SHR may explain the slower growth rate and reduced plating efficiency of the SMCs from SHR compared with WKY.

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CHAPTER IV

MANUSCRIPT # 2

MANUSCRIPT No. 2

ALTERATIONS IN β-ADRENOCEPTORS AND POLYPLOIDY IN CULTURED AORTIC SMOOTH MUSCLE CELLS FROM DIFFERENT AGE GROUPS OF SPONSTANEOULSY HYPERTENSIVE RATS AND WISTAR-KYOTO RATS

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Roop B. Conyers's contribution:

(i) cell culturing

(ii) cell staining

(iii) microdensitiometric scanning

(iv) receptor binding

(v) anaylsis of data

(vi) preparation of first draft of manuscript

Alterations in beta-adrenoceptors and polyploidy in cultured aortic smooth muscle cells from different age groups of spontaneously hypertensive rats and Wistar–Kyoto rats

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Objective: The relationship between the number of β-adrenoceptors and polyploidy in cultured aortic smooth muscle cells derived from different age groups of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats were examined.

Design: The number of β-adrenoceptors, the percentage of multinucleated cells and the incidence of polyploidy from cultured smooth muscle cells derived from SHR and WKY rats aged 3–4, 10–12 and 28–30 weeks were measured. The effect of passaging of the cells on the expression of β-adrenoceptors and polyploidy on cultured smooth muscle cells from both SHR and WKY rats was also investigated.

Methods: Receptor binding experiments were carried out using [^{125}I]-monoiodocyanopindolol with osmotically lysed cultured aortic smooth muscle cells to investigate the properties of vascular β-adrenoceptors in SHR and WKY rats. The proportion of polyploid smooth muscle cells was determined by frequency distribution analyses of Feulgen DNA microdensitometric measurements.

Results: The incidence of polyploid smooth muscle cells was consistently higher in cells cultured from SHR than in those from WKY rats in all three age groups, with a positive correlation between polyploidy and age in SHR. Furthermore, in all three age groups the number of β-adrenoceptor binding sites was also higher in cultured smooth muscle cells from SHR than in those from WKY rats. There was no significant difference in the receptor affinity. The increase in β-adrenoceptor number was associated with an increase in polyploidy, and both of these changes were positively correlated both with the age of the rats from which these cells were derived and with the number of passages.

Conclusions: Under cell culture conditions the expression of β-adrenoceptor density increases with the number of passages in both SHR and WKY rats. Smooth muscle cells derived from older SHR and WKY rats have a greater propensity to develop polyploidy. This trend is significantly accelerated in cultured smooth muscle cells derived from SHR compared with those from WKY rats, suggesting a premature ageing process. These findings suggest that, in cultured smooth muscle cells from SHR and WKY rats, β-adrenoceptors may influence the expression of polyploidy.

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Keywords: Beta-adrenoceptor, polyploidy, cultured vascular smooth muscle, aorta, hypertension.

Introduction

Hypertension is a pathological condition that is characterized by persistent elevation in blood pressure, which is due to an increase in the total peripheral resistance, especially in the resistance vessels [1–3]. A common feature of hypertensive vessels is medial thickening, which can be due to either hyperplasia or hypertrophy of the

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smooth muscle cells [3–5]. It is believed that such structural changes may contribute to the increased reactivity of blood vessels to constrictor stimuli, which leads to an increase in blood pressure [1,2].

Medial thickening in the aorta of spontaneously hypertensive rats (SHR) is primarily the result of hypertrophy of the smooth muscle cells [6,7]. Furthermore, hypertrophy of smooth muscle cells is often accompanied by the development of polyploidy (or hyperploidy), with up to 50% of the smooth muscle cells in the vessel wall having more than the normal diploid amount of DNA in the cells (i.e. tetraploid or octaploid) [7,8].

Polyploid smooth muscle cells have the ability to undergo cell division to produce polyploid smooth muscle cells [9]. It has been suggested that this might occur as a result of a failure of cytokinesis or karyokinesis [10]. However, the exact mechanism underlying the development of polyploidy in hypertension is not known. High blood pressure is thought to be one of the causes of polyploidy, because these cells are present only in older SHR, and antihypertensive treatment prevents these changes [3,11]. However, recent reports have suggested that catecholamines [8,12], angiotensin II [13] and deoxycorticosterone–salt treatment [14] may all play a role in the development of polyploidy.

We have recently described [15] the presence of polyploidy in cultured aortic smooth muscle cells from SHR and normotensive Wistar–Kyoto (WKY) rats. The finding that chronic treatment with β-antagonists inhibits or attenuates the development of polyploidy in the aorta of deoxycorticosterone–salt-included hypertensive rats [16], and in SHR and WKY rats [17], suggests that polyploidy may be expressed via a β-adrenoceptor-mediated signal transduction mechanism.

The primary objectives of the present study were to characterize vascular β-adrenoceptors in cultured aortic smooth muscle cells derived from SHR and WKY rats, and to examine the relationship between the number of β-adrenoceptors and polyploidy in these cells. Furthermore, under culture conditions smooth muscle cells are known to undergo phenotypic modulation, which is characterized by changes including a loss of contractile ability, a decrease in contractile filaments and an increase in the amount of synthetic organelles [18]. Therefore, we studied the effects of repeated passaging on the phenotypic expression of polyploidy and β-adrenoceptor density in smooth muscle cells derived from different age groups of SHR and WKY rats.

Materials and methods

All chemicals, unless specified, were obtained from Sigma Chemical Co. (St Louis, Missouri, USA).

Source of smooth muscle cells

The SHR and WKY rats were obtained from colonies maintained at the animal facility of McMaster University. They were originally obtained from Charles River strains more than 10 years ago. The blood pressure of these rats was measured by the pneumatic tail-cuff method. Smooth muscle cells were obtained from the thoracic aortae of SHR and WKY rats, at the prehypertensive stage (age 3–4 weeks), developing hypertensive stage (age 10–12 weeks) and established hypertensive stage (age 28–30 weeks), using the explant method described below.

Briefly, the rats were killed by cervical dislocation. A segment of thoracic aorta (approximately 2 cm) was removed and placed in Hanks' buffer containing antibiotics (1000 u/ml penicillin and 1000 μg/ml streptomycin) and antimycotic agents (25 μg/ml fungizone). Blood was removed by flushing with Hanks' buffer. After removal of the fat and surrounding connective tissue by dissection, the aorta was cut open longitudinally. Endothelial cells were removed by rubbing with the sides of a fine forceps. Under a dissecting microscope, small strips of medial layer were removed from the adventitia using a fine watchmaker's forceps. These strips were transferred to Petri culture dishes (60×15 mm) and cut into smaller pieces using a razor blade. Two or three drops of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Burlington, Ontario, Canada) containing 20% fetal bovine serum (Gibco BRL) and 1% antibiotic–antimycotic solution (Gibco BRL) were placed on these cut pieces, and a small coverslip was placed on these drops to facilitate the attachment of the tissue to the culture disc. After 24 h incubation in an atmosphere of 95% air and 5% carbon dioxide at 37°C, 5 ml of the DMEM containing 10% fetal bovine serum and 1% antibiotic–antimycotic solution was added to each culture dish. The culture medium was changed every 3–5 days. It usually took 2–4 weeks for the cells to migrate out from the tissues. Once confluency was reached (designated the primary culture), the cells were harvested by trypsinization using 0.25% trypsin (Gibco BRL) at 37°C. As soon as most of the cells were detached from the disc, 10 ml culture medium was added to the dish to deactivate the trypsin. These cells were passaged using the conventional method.

Passages of smooth muscle cells from these primary cultures were stored in liquid nitrogen. Cells derived from each rat were propagated separately, in order to compare differences between cells from different rats. In culture these cells exhibit the characteristic hill-and-valley growth pattern of smooth muscle cells [18]. Positive staining with monoclonal anti-α-smooth muscle actin (>85%) and the presence of a basal lamina and contractile filaments under transmission electron microscopy analysis confirmed that the cells in culture were smooth muscle cells. Positive staining for von Willebrand factor, which is characteristic of endothelial cells, was not detected in these cultured cells.

For each experiment cells from WKY rats and SHR were cultured under identical conditions in DMEM, which contained 10% fetal bovine serum and 1%

antibiotic–antimycotic solution, at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Depending on the experiment, cells were either synchronized with serum-poor medium (DMEM containing 0.5% fetal bovine serum) for 48 h or harvested at confluency by trypsinization (0.25% trypsin).

Flow cytometry

Preliminary experiments were carried out to determine the proportion of cells in different phases of the cell cycle as a result of synchronization with serum-poor medium. Smooth muscle cells were either synchronized with serum-poor medium (DMEM containing 0.5% fetal bovine serum) or serum-free medium (defined as the medium) for 48 and 72 h or harvested at confluency by trypsinization (0.25% trypsin). Cells were resuspended in orthophosphate buffer saline containing 320 μmol/l calcium and 60 μmol/l magnesium, fixed in 70% ethanol for 30 min at 4°C, centrifuged, washed in the buffer and resuspended in 2 ml buffer containing 20 μg/ml propidium iodide (a dye specific for nucleic acids) and 10 μg/ml ribonuclease A (to prevent binding of propidium iodide to RNA) for 30 min at 37°C. These cells were mechanically dispersed by passing through a needle (inside diameter 0.25 mm, outside diameter 0.46 mm), and analysed using FACScan flow cytometry (Becton Dickinson and Co., Mississauga, Ontario, Canada) with an argon laser emitting 15 mW at wavelength 488 nm. For each sample the data on 25 000 events (cells) were acquired. The percentage of cells in each phase of the cell cycle was calculated using the sum of broaden rectangle model [19].

Multinucleated cell measurements

Smooth muscle cells in passages 2, 5 and 7 from WKY rats and SHR in all three age groups were synchronized with 0.5% fetal bovine serum in DMEM for 48 h. The cells were fixed with 10% neutral formalin and then stained with haematoxylin. The number of smooth muscle cells containing one or more nuclei was counted under a high-power phase-contrast microscope. Extreme care was taken not to count two closely situated cells as one. For each experiment at least 500 cells were counted. The percentage of cells containing one nucleus and of those containing two or more nuclei were calculated.

Microdensitometric measurements

Synchronized WKY rat and SHR smooth muscle cells from passages 2, 5 and 7 were fixed with 10% neutral formalin for 1 h at room temperature (approximately 21°C) and rinsed in distilled water. Nuclear DNA was stained by the Feulgen reaction (hydrolysis in 5 mol/l hydrochloric acid for 1 h at room temperature [20], two rinses in distilled water, stained in Schiff's reagent prepared by the method of de Tomasi for 1 h [21], three rinses in freshly prepared bisulphite solution for 3 min each, rinsed in distilled water, dehydrated in ethanol and mounted in mineral oil [21]). A scanning microdensitometer (model M-85; Vickers Instruments, UK) was used to measure the intensity of the Feulgen staining. Intergrated scans of individual intact nuclei were measured at wavelength 545 nm. The instrument provides simultaneous measurement of nuclear area, so that the areas of the nuclei were also recorded. More than 200 nuclei were measured at random for each preparation. Chicken erythrocytes, known to have a DNA value of 2.5 pg each, were used as standards [22].

Binding studies

Cultured smooth muscle cells from passages 6 and 7 of the three age groups of SHR and WKY rats were utilized for binding studies. At confluency, cells were washed with Hanks' balanced salt solution (pH 7.4) and then harvested using 0.25% trypsin. The cells were divided into aliquots for the purpose of cell counting, size determination, propagation and freezing for the radioligand binding assay on the following day. For binding studies the cells were sedimented by centrifugation. The supernatant containing culture medium and trypsin was aspirated, and distilled water was then added to lyse the cells. We have previously shown [23] that such osmotic lysing of the cells eliminated the apparently high non-specific binding resulting from the trapping of the hydrophobic ligand inside the cells. For the binding assay the concentration of cells was maintained at $(4–7)\times10^6$ cells/ml (>150 μg/100 μl protein). Binding assays were performed in either triplicate or duplicate using [^{125}I]-(–)iodocyanopindolol (DuPont NEN Research Products, Boston, Massachusetts, USA) at various concentrations in TRIS buffer (pH 7.4) at 37°C. Specific binding was defined as the difference between the total binding and the binding in the presence of excess cold D,L-propranolol. The binding reaction was stopped by the addition of 2.5 ml ice-cold buffer. Smooth muscle cell-bound radioligand was separated by a millipore filtration method using Whatman GF/F filters (Baxter Diagnostics Corporation, Canlab Division, Mississauga, Ontario, Canada), and was washed repeatedly (5×2.5 ml) with ice-cold buffer. Saturation experiments were conducted using eight concentrations of [^{125}I]-(–)iodocyanopindolol in the concentration range 0–150 pmol/l for 90 min at 37°C in triplicate. Analysis of data was carried out using the computerized program EBDA (Elsevier–Biosoft, Cambridge, UK). The maximal binding and dissociation constant were expressed as binding sites per cell and pmol/l, respectively.

Polyploidy and β-adrenoceptors

In order to evaluate the degree to which polyploidy contributes to the increase in the number of β-adrenoceptor binding sites per cell, correlation of maximal binding values (y axis) with degree of polyploidy (x axis) at various passages (3, 5 and 7) for SHR and WKY rats aged 10–12 weeks was determined. Based on the linear correlation, it was possible to determine the theoretical maximal binding value (y intercept) at 0% polyploidy. Using this method, we estimated the number of binding sites per cell for a diploid cell, and calculated the number of binding sites per cell for polyploid cells, because the binding experiments yielded a maximal binding value of total binding sites in each population of cells.

Statistics

Values are expressed as means±SEM. Differences between multiple mean values were assessed using analysis of variance with Bonferroni correction, and $P<0.05$ was considered statistically significant.

Results

Physical characteristics

At age 3–4 weeks systolic blood pressure and heart rate were similar for the SHR and WKY rats, but the body weight of the WKY rats was higher than that of the SHR (Table 1). At age 10–12 weeks systolic blood pressure was significantly higher in the SHR than in the WKY rats, whereas heart rate and body weight were not significantly different between the two strains (Table 1). Similarly, at age 28–30 weeks systolic blood pressure was significantly higher in the SHR than in the WKY rats, whereas heart rate and body weight were not significantly different between the strains (Table 1).

Table 1. Physical characteristics of the spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats at different ages.

	SBP (mmHg)	Heart rate (beats/min)	Body weight (g)
Age 3–4 weeks			
SHR	109±4	400±6	34±3
WKY rats	99±6	380±20	58±4
Age 10–12 weeks			
SHR	185±10***	360±12	248±2
WKY rats	125±3	353±9	270±11
Age 28–30 weeks			
SHR	193±4****	360±19	341±16
WKY rats	122±2	352±9	289±16

Values are expressed as means±SEM (n=4 for all groups except WKY rats aged 28–30 weeks, for which n=3). ***$P<0.001$, ****$P<0.0001$, versus WKY rats. SBP, systolic blood pressure.

Flow cytometry

Synchronization of the smooth muscle cells with serum-poor medium for 48 h reduced the percentage of cells in the synthetic phase to <20% both for SHR and for WKY rats in all three age groups. Complete reduction of cells in the synthetic phase of the cell cycle was not obtained even after 72 h in serum-poor medium. This finding is consistent with other studies [24,25], which have reported that it was possible to obtain completely quiescent smooth muscle cells neither for SHR nor for WKY rats after synchronization for 3 days with defined serum-free medium.

Measurements of multinucleated smooth muscle cells

There was a significantly higher population of multinucleated cells in SHR than in WKY rats in all three passages and age groups ($P<0.05$) except for passage 2 in the groups aged 3–4 and 28–30 weeks (Fig. 1). Analysis of variance for each passage showed that, within each strain, there was a significant difference in the number of nuclei between passages (passage 5 $P<0.003$, passage 7 $P<0.001$, both for SHR and for WKY rats) among the various age groups except in passage 2 both for SHR and for WKY rats. Analysis of variance for each age group showed that, both for SHR and for WKY rats at age 10–12 weeks and for only SHR at age 28–30 weeks, there was a significant difference (age 10–12 weeks $P<0.01$, age 28–30 weeks $P<0.02$) in the number of nuclei between passage 7 and the lower two passages. A trend towards a passage-dependent increase in multinucleated cells was also observed both for SHR and for WKY rats of the group aged 10–12 weeks, but for only SHR of the group aged 28–30 weeks.

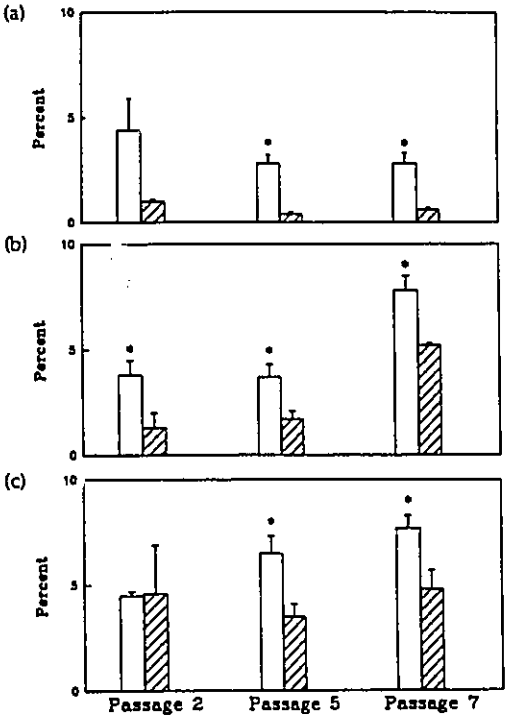

Fig. 1. The percentage of cultured thoracic aortic smooth muscle cells with multiple nuclei from spontaneously hypertensive rats (SHR, □) and Wistar-Kyoto (WKY, ▨) rats aged (a) 3–4 weeks, (b) 10–12 weeks and (c) 28–30 weeks. Values are expressed as means±SEM (n=4 both for SHR and for WKY rats, except for passages 5 and 7 of groups aged 3–4 weeks, for which n=6 each, and for passages 5 and 7 of WKY rats aged 28–30 weeks, for which n=3). *$P<0.05$, versus WKY rats.

Multinucleated smooth muscle cells both from SHR and from WKY rats usually occupied a larger surface area than the diploid cells.

Microdensitometric measurements

The frequency distribution of microdensitometric measurements of smooth muscle cells from the three age groups showed that most of the cells from WKY rats were within the 2–20 intensity units (IU) range, whereas

cells from SHR were within a higher intensity range. Using chicken erythrocytes as the standard, it was determined that 1 IU was equivalent to 0.46 pg DNA. Based on these results, we have defined cells with ≤20 IU (≤9.20 pg DNA) as diploid cells, cells within the range 21–40 IU as tetraploid cells (9.66–18.40 pg DNA) and cells with ≥41 IU (>18.86 pg DNA) as octaploid cells. These values are within the range previously reported in smooth muscle cells freshly isolated from the aortae of SHR and WKY rats [6,7].

Among the three age groups there was a significantly higher population of polyploid smooth muscle cells in SHR than in WKY rats in all of the passages except for passage 2 in the group aged 28–30 weeks (Fig. 2). Analysis of variance showed that, within each strain, for all passages (except passage 2 in the group aged 28–30 weeks), there was a significant difference among the three age groups in the percentage of tetraploid cells ($P \leq 0.03$ both for SHR and for WKY rats), but not in the octaploid group.

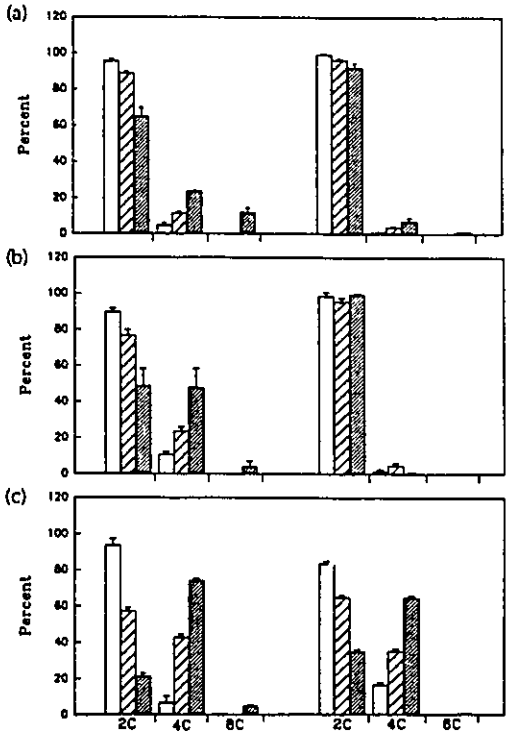

Fig. 2. Age- and passage-dependent changes in the percentage of polyploidy in cultured smooth muscle cells from (left) spontaneously hypertensive rats (SHR) and (right) Wistar–Kyoto rats aged (a) 3–4 weeks, (b) 10–12 weeks and (c) 28–30 weeks. Values are expressed as means ± SEM (n = 4 for both strains, except for passages 5 and 7 of the group aged 3–4 weeks, for which n = 6, and for passages 5 and 7 for the groups aged 28–30 weeks, for which n = 3). □, Passage 2; ▨, passage 5; ▩, passage 6; 2C, diploid cells; 4C, tetraploid cells; 8C, octaploid cells.

Furthermore, there was a trend for an age- and passage-dependent increase in tetraploid smooth muscle cells in SHR (Fig. 2). In contrast, among the WKY rat groups there was no significant difference in the percentages of diploid and tetraploid cells between the groups aged 3–4 and 10–12 weeks in all three passages. However, a significantly higher percentage of tetraploid cells was found in the three passages of the WKY rats aged 28–30 weeks than in those of WKY rats aged 3–4 and 10–12 weeks. A comparison of the nuclear area and DNA content of smooth muscle cells always revealed a positive correlation ($r \geq 0.9$, $P \leq 0.001$) in all of the age groups and passages of SHR and WKY rats studied.

Binding studies

Preliminary [^{125}I]-(−)iodocyanopindolol binding experiments were conducted to optimize the conditions of the binding assay. It was determined that binding of [^{125}I]-(−)iodocyanopindolol was clearly detected when the binding assay mixture contained at least 3.0×10^6 cells/ml (150 μg/100 μl protein). As a result, all further assays were carried out under these conditions.

[^{125}I]-(−)iodocyanopindolol saturation binding experiments were completed both for SHR and for WKY rats at passages 6 and 7 in all three age groups. Linear transformation of Scatchard data indicated a straight line both for SHR and for WKY rats (Fig. 3), suggesting a single class of binding sites for β-adrenoceptor both for SHR and for WKY rats. Furthermore, there was a consistent and significantly higher maximal binding value for SHR than for WKY rats in all three age groups (Table 2), suggesting a higher density (an increased number of binding sites per cell) of β-adrenoceptors in the smooth muscle cells of SHR than in those of WKY rats. There was no significant difference in the dissociation constant (receptor affinity for the radioligand) between SHR and WKY rats within any age group studied. However, within each strain there was a significant difference in dissociation constant between the group aged 3–4 weeks and the two older groups (Table 2). Hill coefficient analyses consistently yielded a Hill coefficient of >0.95 both for SHR and for WKY rats in all three age groups, indicating a single non-interacting binding site. There was no evidence of cooperativity or the presence of multiple binding sites.

A correlation of the maximal binding values with polyploidy for each age group (at passage 7) showed a positive relationship both for SHR ($r = 0.96$, $P = 0.179$) and for WKY rats ($r = 0.99$, $P = 0.033$; Fig. 4). When the maximal binding values from separate passages of the group aged 10–12 weeks were compared with the degree of polyploidy, we found a positive correlation for SHR smooth muscle cells ($r > 0.98$, $P = 0.0049$); that is, the number of binding sites increased with passaging of smooth muscle cells in the culture for SHR.

Polyploidy and β-adrenoceptors

Correlation of maximal binding values (y axis) with degree of polyploidy (x axis) at various passages (2 and 3,

Table 2. [125I]-(–)-iodocyanopindolol saturation binding data in cultured vascular smooth muscle cells from spontaneously hypertensive rats and Wistar–Kyoto (WKY) rats at different ages.

	Spontaneously hypertensive rats			Wistar–Kyoto rats		
Age	B_{max} (mmHg)	K_d (pmol/l)	n_h	B_{max} (mmHg)	K_d (pmol/l)	n_h
3–4 weeks	705±8	38±2	0.969	465±29	24±5	0.982
10–12 weeks	1280±120	13±2	0.993	347±35	12±3	0.980
28–30 weeks	1633±273	18±4	0.985	1065±12	8±1	0.945

Values are expressed as means±SEM (n=4 for all groups except WKY rats aged 28–30 weeks, for which n=3). B_{max}, maximal binding; K_d, dissociation constant; n_h, Hill coefficient.

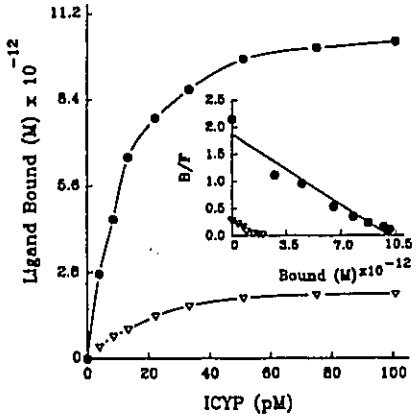

Fig. 3. [125I]-(–)-iodocyanopindolol (ICYP) saturation binding data in cultured (passages 6 and 7) thoracic aortic smooth muscle cells from spontaneously hypertensive rats (●) and Wistar–Kyoto (▽) rats aged 10–12 weeks. The inset shows a linear transformation of the saturation binding data. n=4 for both strains. B/F, bound:free ligand ratio.

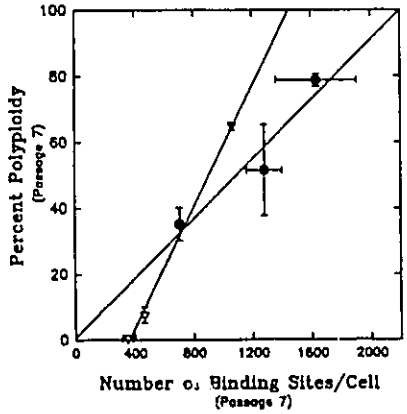

Fig. 4. Correlation of maximal binding (passage 7) and percentage of polyploid smooth muscle cells (passage 7) from spontaneously hypertensive rats (●; r=0.96) and Wistar–Kyoto rats (▽; r=0.99) aged 3–4, 10–12 and 28–30 weeks. For maximal binding measurements, n=4 for both strains for all three age groups. For polyploidy measurements of both strains n=6 for the group aged 3–4 weeks, n=4 for the group aged 10–12 weeks and n=3 for the group aged 28–30 weeks. Values are expressed as means±SEM.

5, and 6 and 7) for the smooth muscle cells of SHR and WKY rats aged 10–12 weeks yielded a linear equation, $y=mx+b$, where m is the slope of the line and b is the intercept at $x=0$ (i.e. the maximal binding value when the percentage polyploidy is zero; Fig. 5). Based on this linear correlation equation, the calculated maximal binding value (y intercept) at zero polyploidy (i.e. diploid smooth muscle cells only) was 911 sites/cell for SHR and 337 sites/cell for WKY rat smooth muscle cells (Fig. 5). For WKY rats this value was close to the measured value of 347 sites/cell (Table 2) for a population of smooth muscle cells from rats aged 10–12 weeks with 0.5±0.5% polyploidy (essentially 0% polyploidy). Using this approach, it was determined that polyploid smooth muscle cells in the SHR aged 10–12 weeks at passage 7 had 1627 sites/cell; that is, those polyploid smooth muscle cells exhibited a twofold increase in β-adrenoceptor numbers compared with diploid cells in the SHR. For WKY rats aged 10–12 weeks (at passage 7) there were essentially no polyploid smooth muscle cells (0.5±0.5%), which reflected diploid smooth muscle cells with approximately 337–347 sites/cell.

Discussion

The major findings of the present study, under cell culture conditions, were the following. Aortic smooth muscle cells from SHR contained significantly elevated numbers of polyploid cells and β-adrenoceptor sites compared with those from WKY rats, and there was a positive correlation between polyploidy and an increase in β-adrenoceptor density. Both in SHR and in WKY rats, polyploidy and β-adrenoceptor numbers increased with the age of the rats from which the cells were derived, and with the passage number of smooth muscle cell cultures. Both of the age- and passage-dependent effects were accelerated in cultured SHR smooth muscle cells compared with those from WKY rats. The following discussion focuses on the significance of these findings and their relevance to the pathophysiology of hypertension.

In the present study we used the measurement of multinucleated smooth muscle cells and microdensitometric measurements to estimate the number of polyploid cells.

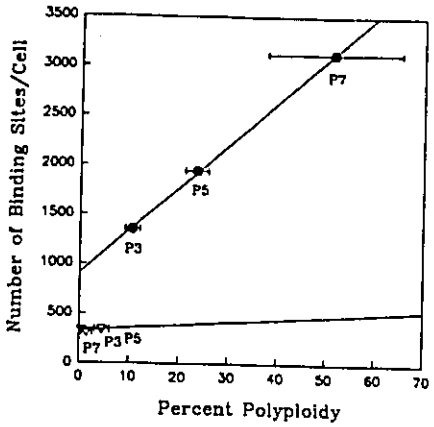

Fig. 5. Correlation of maximal binding and the percentage of polyploid smooth muscle cells at the third (P3), fifth (P5) and seventh (P7) passages for spontaneously hypertensive rats (●) and Wistar–Kyoto rats (▽) aged 10–12 weeks. Values are expressed as means ± SEM. For spontaneously hypertensive rats: r = 0.99, y intercept = 911; for Wistar–Kyoto rats: r = 0.45, y intercept = 327.

Our finding that, at higher passage number, the percentage of multinucleated smooth muscle cells was significantly higher in SHR than in WKY rats for all three age groups is consistent with *in vivo* findings in which it was reported that the percentage of binucleated smooth muscle cells was higher in SHR than in WKY rats [6]. We also observed that, in the smooth muscle cells from WKY rats aged 28–30 weeks, the number of multinucleated cells at passage 2 had increased to the same level as in the SHR, which suggests the effect of ageing on these cells.

The evaluation of polyploidy by frequency distribution of Feulgen DNA densitometric measurements again revealed a significantly higher proportion of polyploid smooth muscle cells in SHR than in WKY rats, and a positive correlation between polyploidy and age for SHR smooth muscle cells for all three age groups. These findings are similar to those reported under *in vivo* conditions, in which the percentage of polyploid smooth muscle cells in intact aorta from SHR compared with WKY rats varied with age [7,26]. We also found that there was a positive correlation of polyploidy with passaging of smooth muscle cells in culture. With increasing number of passages, smooth muscle cells from the three age groups of SHR showed a passage-related increase in polyploidy, whereas in the WKY rats such an increase was found only in smooth muscle cells derived from rats aged 28–30 weeks. These findings are interesting, because it has been reported [27] that polyploid smooth muscle cells proliferate at a much lower rate than normal diploid cells. The present observation that cultured smooth muscle cells derived from rats of different ages display different characteristics with passaging is relevant and useful, because it might reflect different growth characteristics of smooth muscle cells from rats of different ages *in vivo*. In normal humans [28] and rats [9,29] there is an age-dependent increase in the frequency of tetraploid cells. The presence of a passage-related increase in the number of polyploid smooth muscle cells in cells derived from younger SHR than from WKY rats, and the higher percentage of multinucleated smooth muscle cells in the higher passages of smooth muscle cells in the SHR than in the WKY rats in the three age groups, are indicative of a premature ageing process in the SHR compared with the WKY rats.

The exact mechanism and functional significance of polyploidy is not known. Owens [7] reported that smooth muscle cell polyploidy is a possible response to increased blood pressure. Owens observed a linear correlation between the blood pressure level and the frequency of polyploidy in a variety of hypertensive models. Normalization of blood pressure in SHR treated with antihypertensive drugs was also effective in preventing further development of smooth muscle cell polyploidy. Other studies [16,17] have indicated that polyploidy is not simply a response to increased blood pressure. Lee *et al.* [17] reported that chronic treatment with nadolol (a non-selective β-antagonist) did not prevent the development of hypertension in SHR but did decrease the percentage of polyploid smooth muscle cells in the aorta both of SHR and of WKY rats. Leitschuh *et al.* [16] found that propranolol inhibited the development of hypertension-induced polyploidy in aortic smooth muscle cells of deoxycorticosterone–salt hypertensive treated rats. Both of those reports suggest that *in vivo* β-adrenoceptors may be implicated in the development of smooth muscle cell polyploidy. However, the manner in which these changes relate to hypertension is not clear.

Our finding of a single class of binding sites for β-adrenoceptors both in SHR and in WKY rats is consistent with our previous results [23] in cultured smooth muscle cells and in purified membrane preparations from aorta both of SHR and of WKY rats using [^{125}I]-(–)-iodocyanopindolol as the ligand. In the present study we found higher β-adrenoceptor numbers of SHR than in WKY rats for all three age groups. Results from previous studies on β-adrenoceptors in the smooth muscles of hypertensives were inconsistent. There were reports of decreased β-adrenoceptor numbers in SHR arteries [30] and in cultured arterial smooth muscle cells [31]. In contrast, no differences in β-adrenoceptor numbers were found by Asano *et al.* [32] in femoral artery tissue preparations. In our previous study using purified membrane preparations from aortic muscle strips of adult SHR and WKY rats [23] we found no difference between β-adrenoceptor density in SHR and that in WKY rats. The reasons for these contradictory findings are still not clear. It is possible that the age of the rats might be one of the contributing factors, as we found in the present study. It is also possible that, under cell culture conditions, the differences between smooth muscle cells from SHR and WKY rats became exaggerated.

In the present study we found that the increase in β-adrenoceptor numbers was associated with an increase

in polyploidy of smooth muscle cells, and that these changes were positively correlated with the age of the rats from which the cultures were derived, and the passage of smooth muscle cell cultures. The increased expression of β-adrenoceptors in polyploid cells may be related to cell size, because we have observed that multinucleated smooth muscle cells tended to occupy a larger surface on the culture dish, and our calculations show that, in the SHR, more binding sites were present in polyploid cells than in diploid cells. Furthermore, our calculations also show that differences in β-adrenoceptor binding sites are also strain-related, because, among the diploid cells, smooth muscle cells from SHR had approximately threefold more receptors per cell than WKY rats. It is not known whether diploid smooth muscle cells from SHR are larger than those from WKY rats. The increase in β-adrenoceptor expression appeared to be accelerated in the SHR, because the changes that were seen in the WKY rats aged 28–30 weeks (i.e. increased receptor density compared with the other age groups) had already occurred in the SHR rats aged 10–12 weeks. Thus, as in the case of polyploidy discussed above, the accelerated increase in β-adrenoceptor numbers of the cultured smooth muscle cells from SHR might indicate a premature ageing process in these rats.

We speculate that an increased density of β-adrenoceptors might lead to polyploidization of the cells through their role in promoting DNA synthesis. Smooth muscle cell β-adrenoceptors are known to be coupled to adenylate cyclase via G_s proteins, so that stimulation of these receptors can lead to an elevation of intracellular cyclic AMP (cAMP) levels [33,34]. Because an increase in adenylate cyclase activity (cAMP production) has been found to precede DNA synthesis in rat aortic smooth muscle cells in culture [35], it is possible that an elevation in cAMP level leads to an increase in DNA synthesis. Another possibility is that β-adrenoceptor-mediated effects are independent of cAMP production. In turkey erythrocytes β-adrenoceptor stimulation was found to activate phospholipase C in a manner that was independent of cAMP production [36]. It has been suggested [36] that the phospholipase C activation might be due to direct coupling of β-adrenoceptors to a G protein (G_{PLC}). The activation of phospholipase C will eventually lead to activation of protein kinase C, which has been reported to play a role in DNA synthesis [36]. Regardless of the route by which an increase in DNA synthesis caused by β-adrenoceptor stimulation might occur, polyploidy of the smooth muscle cells may develop in cells in which DNA synthesis has taken place without subsequent karyokinesis or cytokinesis [10].

In conclusion, we have found that cultured aortic smooth muscle cells derived from SHR of different ages expressed characteristics different from those of WKY rats. Smooth muscle cells from SHR exhibited increased polyploidy and elevated β-adrenoceptor numbers compared with those from WKY rats. In both strains of rats, polyploidy and β-adrenoceptor expression increased with the age of the rats from which the smooth muscle cells were derived, and this effect was significantly accelerated in smooth muscle cells from SHR compared with those from WKY rats. Cell polyploidy was correlated positively with β-adrenoceptor density in smooth muscle cells from both strains of rats, suggesting that the β-adrenoceptor-mediated signal transduction might be implicated in the development of smooth muscle cell polyploidy in culture.

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CHAPTER V

MANUSCRIPT # 3

MANUSCRIPT No. 3

FUNCTIONAL β-ADRENOCEPTORS IN CULTURED AORTIC SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE AND WISTAR-KYOTO RATS

Submitted to the *American Journal of Physiology*

Roop B. Conyers's contribution:

(i) cell culturing

(ii) cAMP measurements via radioimmunoassay

(iii) analysis of data

(iv) preparation of first draft of manuscript

Functional ß-Adrenoceptors in Cultured Aortic Smooth Muscle Cells from Spontaneously Hypertensive and Wistar-Kyoto Rats

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ABSTRACT

Functional ß-adrenoceptors in cultured aortic smooth muscle cells from spontaneously hypertensive and Wistar-Kyoto Rats.
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The objective of this study was to investigate the relationship between ß-adrenoceptor density and functional responses in cultured aortic smooth muscle cells (SMC) from 3-4-, 10-12-, and 28-30-week old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Cultured SMC were stimulated with the non-selective ß-adrenoceptor agonist, isoproterenol, or the adenylate cyclase activator, forskolin. Changes in intracellular cAMP levels were measured using a radioimmunoassay. Isoproterenol and forskolin stimulation resulted in increased cAMP levels in both strains of rats in all three age-groups. However, treatment with forskolin resulted in significantly higher cAMP levels than those obtained with isoproterenol in both SHR and WKY. Further, the addition of the ß-adrenoceptor antagonist, dl-propranolol, inhibited the isoproterenol response in both SHR and WKY. Thus, SMC from SHR and WKY express functional ß-adrenoceptors, as identified by agonist-stimulated increases in cAMP levels, and their selective inhibition by a ß-adrenoceptor antagonist. However, the increases in the numbers of low-affinity ß-adrenoceptors (as measured by antagonist binding) identified in all three age groups of SHR SMC in our previous studies (Lee et al. 1992a; Conyers et al. 1995) are not paralleled by similar increases in the functional, high affinity ß-adrenoceptors (as measured by agonist stimulated receptor activation of adenylate cyclase) in these cells, indicating that many of the low-affinity ß-adrenoceptors expressed on SMC of SHR are not coupled to G_s-adenylyl cyclase, and these may be desensitised.

Key words: **ß-adrenoceptors, cAMP, polyploidy, smooth muscle cells, hypertension**

INTRODUCTION

A common feature of vascular changes in hypertension is a thickening of the blood vessel wall (Lee and Smeda, 1985; Lee, 1987). This structural change may lead to increased blood pressure by contributing to the increased reactivity of blood vessels to constrictor stimuli (Folkow, 1979; Mulvany et al. 1978). In the aorta of spontaneously hypertensive rats (SHR), vessel wall thickening is largely due to hypertrophied smooth muscle cells (SMC) which are accompanied by polyploid DNA content and enhanced synthetic function (Owens et al. 1981; Owens, 1985). The term polyploid or hyperploid cells refers to cells with greater DNA content than normal cells, which have a diploid DNA content. In chronically hypertensive animals, up to 50% of the SMC in the vessel wall may have an increase in DNA content within a cell (Owens, 1985; Yamori et al. 1987). The incidence of polyploidy may be related to high blood pressure since polyploid SMC are mainly present in older SHR, and antihypertensive treatment prevents these changes (Lee and Smeda, 1985; Lee, 1987; Owens, 1987). In addition, catecholamines (Mano et al. 1986; Yamori et al. 1987), angiotensin II (Geisterfer et al. 1988), transforming growth factor (Owens et al. 1988) and/or deoxycorticosterone/NaCl treatment (Chobanian et al. 1987) may play a role in the development of polyploidy.

In previous reports, we have shown that development of polyploid SMC in SHR and Wistar Kyoto rats (WKY) may be part of the ageing process,

with accelerated ageing occurring in SHR. We found that SMC from 10-12 week-old SHR already exhibited an increased percentage of polyploid SMC compared to WKY, and this was seen only in older (28-30 week-old) WKY rats (Lee et al. 1992a; Conyers et al. 1995). We have also identified and characterized ß-adrenoceptors in cultured SMC from both SHR and WKY at 3-4, 10-12, and 28-30 weeks of age (Kwan and Lee, 1990; Conyers et al. 1995). We found that although receptor affinity did not differ between SHR and WKY among the various age-groups, there was an increase in ß-adrenoceptor binding sites in SHR compared to WKY in all three age-groups (Conyers et al. 1995). Further, we have shown that the increase in polyploid SMC in SHR and WKY was positively correlated with an increase in ß-adrenoceptor numbers (Conyers et al. 1995), suggesting that polyploidy may be mediated by the expression of functional ß-adrenoceptors. This is supported by previous studies from our laboratory (Lee et al. 1992b). Chronic treatment of both SHR and WKY rats from gestation to 28 weeks with nadolol (a non-selective ß-adrenoceptor antagonist), decreased significantly the percentage of polyploid SMC but did not prevent the development of hypertension (Lee et al. 1992b). In addition, propranolol (a non-selective ß-adrenoceptor antagonist) has been shown to inhibit the development of polyploidy in aortic SMC of deoxycorticosterone-NaCl-induced hypertensive rats (Leitschuh and Chobanian, 1987). This further supports the notion that the expression of functional ß-adrenoceptors is

required for the development of smooth muscle polyploidy. The exact mechanism, however, of how ß-adrenoceptors alter SMC polyploidy is unclear.

It is well established that binding of agonists (catecholamines) to ß-adrenoceptors results in activation of adenylate cyclase via a stimulatory guanyl-nucleotide binding protein (G_s) (Benovic et al. 1988; Dohlman et al. 1987). This activation leads to the formation of the intracellular second messenger adenosine 3',5'-cyclic monophosphate (cAMP). It has been suggested that cAMP plays a role in DNA synthesis by regulating the phosphorylation of nuclear proteins, which in turn bind to cAMP response elements within the promoters of cAMP-inducible genes and thus mediate their induction (Karin and Smeal, 1993; Spaulding, 1993).

The objective of the present study was to determine whether the ß-adrenoceptors expressed in cultured aortic SMC from SHR and WKY rats were functional. We measured isoproterenol stimulated cAMP levels in cultured SMC obtained from 3-4, 10-12, and 28-30 weeks old animals from both strains of rats. The ß-adrenoceptor density was correlated with functional responses of SMC from the three age groups of SHR and WKY, to determine if a relationship exists between these two parameters.

MATERIALS AND METHODS

All chemicals, unless specified, were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Using the explant method as described previously (Lee et al. 1992a; Conyers et al. 1995), cultured SMC was obtained from the thoracic aortae of SHR and WKY at the pre-hypertensive stage (3-4 weeks), developing hypertensive stage (10-12 weeks) and established hypertensive stage (28-30 weeks) of hypertension development. These rats were obtained from colonies maintained at the animal facility of McMaster University. They were originally obtained from Charles River strains. Passages of SMC from these primary cultures were stored frozen in liquid nitrogen. Cells derived from each rat were propagated separately, in order to compare differences between cells from different animals. For each experiment, cells from WKY and SHR were cultured under identical conditions with Dulbecco's Modified Eagle Medium (D-MEM) (GIBCO BRL, Burlington, Ontario, Canada), with 10% fetal bovine serum (FBS) (GIBCO BRL, Burlington, Ontario, Canada) and 1% antibiotic-antimycotic solution (GIBCO BRL, Burlington, Ontario, Canada) at 37°C in an atmosphere of 95% air and 5% CO_2. Depending upon the experiment, cells were either synchronized with serum poor medium (0.5% FBS) for 48 hours or harvested at confluency by trypsinization (0.25% trypsin) (GIBCO BRL, Burlington, Ontario, Canada).

Measurement of cAMP levels were conducted in cultured SMC from 3-4-, 10-12-, and 28-30-week old SHR and WKY. SMC in passages 6-7 were grown in culture medium and synchronized with 0.5% FBS for 48 hours. Prior to measurement of cAMP levels, the medium was aspirated and the cells were washed twice with Hank's buffered salt solution (HBSS) (pH=7.41) and subsequently equilibrated with 0.45ml HBSS for 15 minutes at 37°C. All drug solutions were prepared to give a final volume of 0.5ml. The cAMP-specific phosphodiesterase inhibitor, Ro 20-1724 (RBI Chemicals, Natick, MA, USA), was added at the time of each drug addition and to a final concentration of 300 μM. Initial experiments were conducted in cultured SMC from 10-12-week old SHR and WKY to determine the isoproterenol-stimulated cAMP levels, using different concentrations of isoproterenol (1×10^{-4} to 1×10^{-8} M) and also the time course of the isoproterenol-induced cAMP increases.

The effects of the following drugs were tested: 10 μM forskolin, 1 μM isoproterenol, 10 μM isoproterenol, 1 μM isoproterenol and 10 μM dl-propranolol, 10 μM isoproterenol and 100 μM dl-propranolol. All samples were incubated for 2 minutes at 37°C and the reaction was stopped with 0.5 ml ice-cold 10% (w/v) trichloroacetic acid. The 1ml incubation volume was transferred to microcentrifuge tubes and concentrated in a speed vac concentrator. The remaining precipitate was resuspended in 0.05M acetate

buffer and the level of cAMP was determined using a scintillation proximity radioimmunoassay kit (Amersham, Oakville, Ontario, Canada).

The *n* value reflects the number of separately maintained cultured cells from different animals used for each experiments. All data points within each experiment were measured in triplicates. All values are expressed as means ± standard error of the mean (SEM). Student's unpaired t-tests and one-way analysis of variance with Bonferroni correction were utilized to determine differences between SHR and WKY. A p value of ≤ 0.05 was considered significant.

RESULTS

Measurement of cAMP levels in the presence of the phosphodiesterase inhibitor (RO 20-1724) showed a significantly higher basal level of cAMP in SHR compared to WKY in the 10-12-week old age group, but not in the other two age groups (Table 1). Experiments using SMC from 10-12-week old SHR and WKY showed that isoproterenol caused a concentration-dependent increase in cAMP levels (Figure 1) and maximal increases in cAMP were obtained by 1 minute in WKY, and by 2.5 minute in SHR SMC with 10 μM isoproterenol (Figure 2). Based on these results, we have chosen to use two minutes of stimulation with 1 and 10 μM isoproterenol. These concentrations exceed the ED_{50} for isoproterenol stimulated increases of cAMP in human cultured tracheal SMC (Hall et al. 1992), rat aorta

(Schoeffter and Stoclet, 1982), and rat mesenteric arteries (Tsujimoto et al. 1986).

In the 3-4-week old group, the addition of 1 μM isoproterenol resulted in an approximately 2.0 fold increase in cAMP levels in SMC from SHR and WKY, whereas 10 μM isoproproterenol gave an approximately 2.7 fold increase in SMC from WKY and 2.5 fold increase in SMC of SHR (Table 1). The addition of dl-propranolol to SMC from SHR and WKY inhibited the isoproterenol-induced increases in cAMP levels to basal levels in both strains of rats. Treatment of SMC from 3-4 week-old rats with 10 μM forskolin gave an increase in cAMP levels of approximately 5.0 fold in WKY and 3.6 fold in SHR. There was no difference between SHR and WKY in relation to the responses to isoproterenol and forskolin stimulation.

In the 10-12-week old group, basal cAMP levels were significantly elevated in SMC of SHR compared to those of WKY ($p<0.05$, Table 1). Stimulation by 1 μM isoproterenol resulted in an approximately 2.0 fold increase in cAMP levels in WKY and 1.5 fold increase in SHR, whereas 10 μM isoproterenol gave approximately 2.5 fold increase in cAMP levels in WKY and 1.6 fold increase in SHR. In this age group the addition of dl-propranolol inhibited the isoproterenol-induced increase in cAMP to basal levels in SMC from both SHR and WKY. Treatment of SMC with 10 μM forskolin resulted in an increase in cAMP levels by approximately 4.6 fold

in WKY and 3.3 fold in SHR. There was no difference between SHR and WKY in relation to the responses to isoproterenol and forskolin stimulation.

In the 28-30-week old group, stimulation by 1 μM isoproterenol gave an approximately 2.0 fold increase in cAMP levels in SMC from both, SHR and WKY; whereas the addition of 10 M isoproterenol resulted in an approximately 2.4 fold increase in WKY and a 2.0 fold increase in SHR (Table 1). The addition of dl-propranolol inhibited the isoproterenol-induced increases in cAMP to basal levels in SMC from both SHR and WKY. Treatment of SMC with 10 μM forskolin resulted in an increase in cAMP levels by approximately 6.0 fold in WKY and 3.6 fold in SHR. There was no difference between SHR and WKY in relation to the responses to isoproterenol and forskolin stimulation.

Within each age group, there was no significant difference in the percentage of isoproterenol stimulated increase in cAMP levels with respect to the total levels of cAMP in SMC from both SHR and WKY (Table 2).

Binding experiments conducted in previous studies revealed a consistent and significantly higher number of binding sites (B_{max}) for SHR as compared to WKY at all three age groups (Conyers et al. 1995) (Table 2).

DISCUSSIONS

The major findings in this study are: (i) SMC from both SHR and WKY express functional ß-adrenoceptors, as identified by both ß-agonist-stimulated increases in cAMP levels, and the selective inhibition of these by the ß-antagonist, dl-propranolol; (ii) the isoproterenol stimulated increases in cAMP levels are similar in SHR and WKY SMC at 3-4, 10-12 and 28-30 weeks of age, indicating that the expression of the high-affinity, functional ß-adrenoceptors is comparable in SMC of the three age groups in both SHR and WKY; (iii) forskolin stimulated cAMP levels are significantly higher than those obtained with isoproterenol in SMC from all three age groups in both strains of rats, indicating that direct stimulation of adenylate cyclase (i.e. independent of ß-adrenoceptors) leads to much higher levels of cAMP than those obtained with ß-agonist activation; (iv) the increases in the numbers of low-affinity ß-adrenoceptors (as measured by antagonist binding) identified in all three age groups of SHR SMC in our previous studies (Lee et al. 1992a; Conyers et al. 1995) are not paralleled by similar increases in the funtional, high affinity ß-adrenoceptors (as measured by agonist induced coupling of the receptor to activation of adenylatel cyclase) in these cells, indicating that many of the low-affinity ß-adrenoceptors expressed on SMC of SHR are not coupled to the stimulatory guanylate-nucleotide-binding protein (G_s), and these may be desensitised.

The following discussion will focus on the significance of the expression of functional ß-adrenoceptors in SMC from SHR and WKY rats, a rationale for the lack of correlation between the expression of low-, and high- affinity ß-adrenoceptors in SMC from both strains of rats, and the possible mechanisms underlying ß-adrenoceptor-mediated development of SHR SMC polyploidy, and their relevance to hypertension.

In cultured aortic SMC, we have previously reported a significantly elevated density of ß-adrenoceptor in SHR compared to WKY at 3-4, 10-12, and 28-30 weeks of age (Conyers et al. 1995). Further, within the SHR group only, there was a trend for an age-dependent increase in ß-adrenoceptor density. In contrast, in the WKY SMC a significant increase in ß-adrenoceptors was found only in the 28-30-week old group, but not in the 3-4- and 10-12- week old groups. The expression of ß-adrenoceptor in SMC from both SHR and WKY therefore increased with age, and this effect was significantly greater in SMC from SHR compared to those from WKY. We have identified a single, low affinity binding site, with no difference in ligand affinity in SMC obtained in either strain of rats, and in any of the age groups. Further, we found no evidence of cooperativity or of multiple binding sites in any of the SMC studied.

In all the age groups studied, stimulation of SMC ß-adrenoceptors with isoproterenol resulted in increased levels of cAMP, and this elevation in cAMP was blocked by the ß-antagonist dl-propranolol. It is well

established that in many tissues including SMC, binding of agonists to ß-adrenoceptors results in the activation of adenylate cyclase via the G_s, and this in turn leads to elevated intracellular cAMP levels (Benovic et al. 1988; Dohlman et al. 1987). Our findings in cultured SMC from both strains of rats at the three ages confirm the functional coupling of ß-adrenoceptor sites via G_s proteins to adenylate cyclase.

Basal cAMP levels measured in the presence of the phosphodiesterase inhibitor RO 20-1724 in SMC from 10-12-week old SHR were significantly higher compared to those of WKY. This may simply reflect the fact that SHR SMC have a greater number of receptors coupled to stimulation of adenylate cyclase (such as prostaglandin E_2 and A_2 adenosine receptors), and these receptors may be activated by various agonists present in the culture media. Alternately, since intracellular cAMP levels are also regulated by receptors coupled to the inhibition of adenylate cyclase (i.e. α_2-adrenoceptors and A_1 adenosine receptors) via inhibitory guanyl-nucleotide-binding proteins (G_i), factors present in the cell culture media may act via these receptors and decrease cAMP levels in WKY SMC. Since the final intracellular cAMP levels will depend on the net activities of the stimulatory and the inhibitory pathways, an increase in the adenylate cyclase activation, and or a decrease in adenylate cyclase inhibition in SHR SMC may account for the above observations. Elevated cAMP accumulation has been reported in SHR mesenteric arterioles *in vitro* at different stages of

hypertension (4,6,12 and 18 weeks) compared to those of WKY (Dusseau and Hutchins, 1982). Increased basal adenylate cyclase activities have also been found in SHR mesenteric artery plasma membrane preparations compared to those of WKY (Clark et al. 1993). However, these authors found no differences in $G_{i\alpha}$ protein levels between the two strains of rats. In contrast, Srivastava et al. (Thibault and Anand-Srivastava, 1992) found decreased basal adenylate cyclase activities in rat aorta preparations from SHR compared to WKY, and elevated levels of $G_{i\alpha}$ protein levels in these tissues. It is also possible that an elevated basal level of cAMP we have found in this study may be due to the altered activities of phosphodiesterase enzymes which hydrolyse cAMP. Thus, a decreased cAMP-specific phosphodiesterase activity in SHR SMC would result in elevated intracellular cAMP levels under basal, unstimulated conditions compared to those in WKY. Another intriguing possibility is that SHR SMC may have an altered, constitutively active ß-adrenoceptors, so that under basal (agonist-independent) conditions adenylate cyclase may be activated by this mutant ß-adrenoceptors, without any agonist binding (Lefkowitz et al. 1993). An increase in basal levels of cAMP in SHR SMC compared to WKY may ultimately lead to increased cAMP-dependent phosphorylation of several intracellular target proteins, and possibly to altered cell growth. Our results suggest that alterations in any or all of the above processes may contribute to the observed elevated basal cAMP levels we detect in SHR

SMC. The exact mechanisms involved, however, are unknown at this point. It is also unclear to us why an elevated basal level of cAMP was present only in the SMC from SHR as compared with WKY in the 10-12-week old group, and not in other age groups.

The trend of an age-related increase in ß-adrenoceptor numbers observed in cultured SMC from SHR and WKY was not reflected in a parallel age-related increase in the functional expression of ß-adrenoceptors in the three age-groups of the two strains of rats. There may be several explanations to account for these results in the aging SMC: the efficiency of coupling of ß-adrenoceptors-G_s-adenylate cyclase may decline, due to desensitization of the ß-adrenoceptors; the inhibitory adenylate cyclase signalling pathway (i.e. via α_2-adrenoceptors and A_1 adenosine receptors coupled to G_i proteins) may be enhanced; and/or the activities of the cAMP-specific phophodiesterase enzymes may increase. Our findings that forskolin treatment of SMC from SHR and WKY resulted in a much greater stimulation of adenylate cyclase activities than those induced by ß-adrenoceptor agonist, supports the hypothesis that there may be an inefficient coupling of ß-adrenoceptors-G_s-adenylate cyclase in all these cells. Since forskolin activates the catalytic subunit of the adenylate cyclase directly, which bypasses the ß-adrenoceptor-G_s-adenylate cyclase complex, these findings confirm that the level of adenylate cyclase expression is not the limiting factor in this pathway.

Previous studies have reported that ß-adrenoceptor-stimulated cAMP levels were decreased in vascular SMC from SHR compared to WKY in aortic tissue extracts (Bhalla et al. 1978), in aortic tissue membranes (Bhalla and Sharma, 1982), in intact aorta (Sands et al. 1976), in femoral artery strips (Asano et al. 1988) and in cultured SMC from the thoracic aorta (Sands et al. 1976). In contrast, Nabika et al. (Nabika et al. 1985) found very large increases in isoproterenol stimulated cAMP levels in cultured SMC from thoracic aorta, but no significant differences were detected between SHR and WKY in stimulated cAMP levels. Recently, Grammas et al. (Grammas et al. 1994) reported a significant increase in ß-adrenoceptor numbers in cultured SMC from SHR compared to WKY, the magnitude of isoproterenol-stimulated cAMP levels, however, were similar in SMC from the two strains of rats. An elevated number of ß-adrenoceptors was found in renal membrane preparations from SHR, but these were not accompanied by enhanced cAMP formation (Michel et al. 1994). These findings are in agreement with our results, and support the idea that there may be an alteration in the ß-adrenoceptor signalling pathway in cultured SMC. The possible components which are altered may include the G proteins or other sites, distal to the ß-adrenoceptor-G_s-adenylate cyclase complex, such as the cAMP-specific phosphodiesterases, and/or the various intracellular kinases (e.g. protein kinase A).

A decrease in ß-adrenoceptor-mediated function has been well documented in hypertension (Brodde and Michel, 1992; Ford et al. 1995) and in ageing (Arribas et al. 1994). Several authors have attributed the loss of function to the development of desensitization of the ß-adrenoceptors, and their signalling pathways (Michel et al. 1993; Michel et al. 1994; Bohm et al. 1992). The fact that fetal bovine serum used in our tissue culture media contained detectable levels of catecholamines (unpublished results), indicates that ß-agonist-induced desensitization of the ß-adrenoceptors may be an important contributor to the observed results.

The role of ß-adrenoceptors and cAMP in SMC growth and polyploidy is unclear. In cultured aortic SMC in SHR and WKY, ß-adrenoceptor activation has been reported to lead to increases in ornithine decarboxylase (an essential enzyme of cell regulation and differentiation) activity for polyamine biosynthesis (Kanbe et al. 1983; Wagner et al. 1994). Further, an increased adenylate cyclase activity has also been found to precede DNA synthesis in rat aortic SMC in culture (Franks et al. 1984). Recently, elevation of cAMP levels in cultured rat aortic SMC were also found to lead to an accumulation of the mRNA of the early genes *c-fos* and *c-jun* (Hultgardh-Nilsson et al. 1994). Taken together, these findings suggests that elevations in intracellular cAMP levels may increase DNA synthesis. Recently, we reported that cultured SMC from 10-12-week old SHR exhibited a significantly higher proportion of polyploidy SMC compared to

those of WKY (Conyers et al. 1995). Since in the present study we also detected elevated basal cAMP levels in these cells, it is plausible that the higher levels of cAMP in SMC of SHR may contribute to the increased DNA synthesis. Regardless of the mechanisms which mediate increases in SMC DNA synthesis, polyploidy of SMC develops in cells where DNA synthesis has taken place without subsequent karyokinesis or cytokinesis (Gordon et al. 1986). The exact mechanisms underlying ß-adrenoceptor-mediation of SMC growth, and the relevance of these processes to the hypertension-related SMC hypertrophy and polyploidy are at present still unknown.

Alternatively, it is possible that the ß-adrenoceptor mediated effects on SMC polyploidy are independent of cAMP production. Venance et al. (1993) have found that the addition of forskolin or isoproterenol to cultured aortic fibroblasts increased cAMP levels in SHR and WKY. However, they noted that the epidermal growth factor-stimulated DNA synthesis in these cells was enhanced by isoproterenol but inhibited by forskolin, suggesting that isoproterenol binding to ß-adrenoceptors may also activate a second transduction pathway that is cAMP independent (Venance et al. 1993). In turkey erythrocytes, ß-adrenoceptor stimulation was found to activate phospholipase C, and this was independent of cAMP production (Vaziri and Downes, 1992). It has been suggested that the phospholipase C activation may be due to direct coupling of ß-adrenoceptors to a G protein (G_{PLC}) (Vaziri and Downes, 1992). The activation of phospholipase C eventually

leads to activation of protein kinase C, which has been reported to play a role in DNA synthesis (Hoshina et al. 1990).

In conclusion, we have found that cultured SMC from SHR and WKY express functional ß-adrenoceptors as identified by agonist-stimulated increases in cAMP levels. However, the age-related increases in these low-affinity ß-adrenoceptor sites are not reflected in a parallel increase of the high affinity ß-adrenoceptor sites. We suggest that in cultured SMC from SHR the ß-adrenoceptor mediated signalling pathway is altered and may be desensitised. This may be due to alterations in the expressions of, or in the activities of any of the components of this pathway (e.g. G-proteins, cAMP-specific phosphodiesterases, various protein kinases), or in the various receptor-mediated signalling pathways which ultimately regulate intracellular cAMP levels.

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Legend to Figures

Figure 1. Isoproterenol-induced changes in cAMP levels after a two minute stimulation of cultured aortic smooth muscle cells from 10-12-week-old age SHR and WKY. n = 4 for both SHR and WKY.

Figure 2. Time course of 10 μM isoproterenol-induced changes in cAMP levels of cultured aortic smooth muscle cells from 10-12-week-old age SHR and WKY. n = 4 for both SHR and WKY.

Figure 1. Isoproterenol-induced changes in cAMP levels after a two minute stimulation of cultured aortic smooth muscle cells from 10-12-week-old age SHR and WKY. n = 4 for both SHR and WKY.

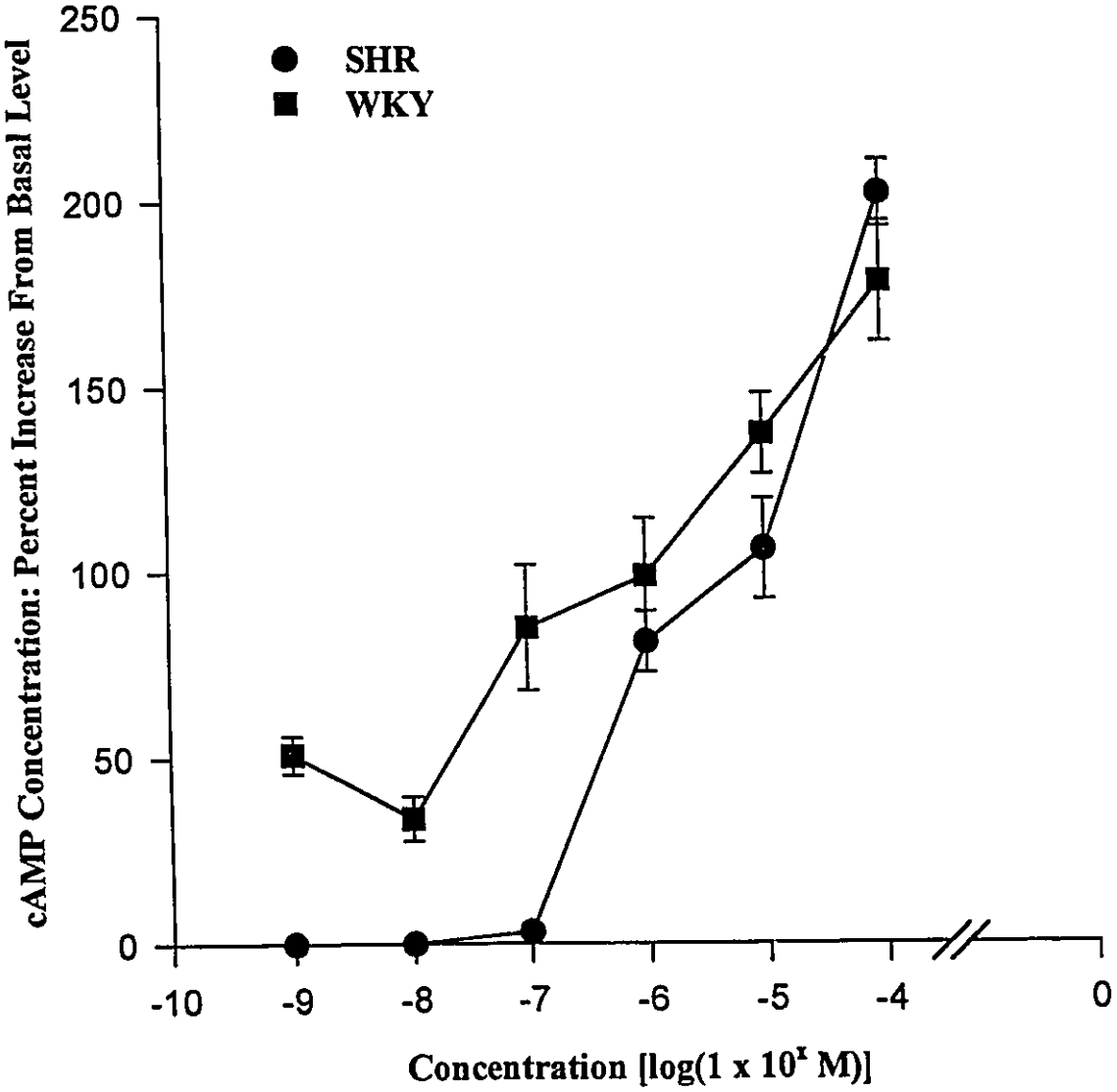
250
200
150
100
50
0
SHR
WKY
cAMP Concentration: Percent Increase From Basal Level
-10
-9
-8
-7
-6
-5
-4
0
Concentration [log(1 x 10^x M)]

Figure 2. Time course of 10 μM isoproterenol-induced changes in cAMP levels of cultured aortic smooth muscle cells from 10-12-week-old age SHR and WKY. n = 4 for both SHR and WKY.

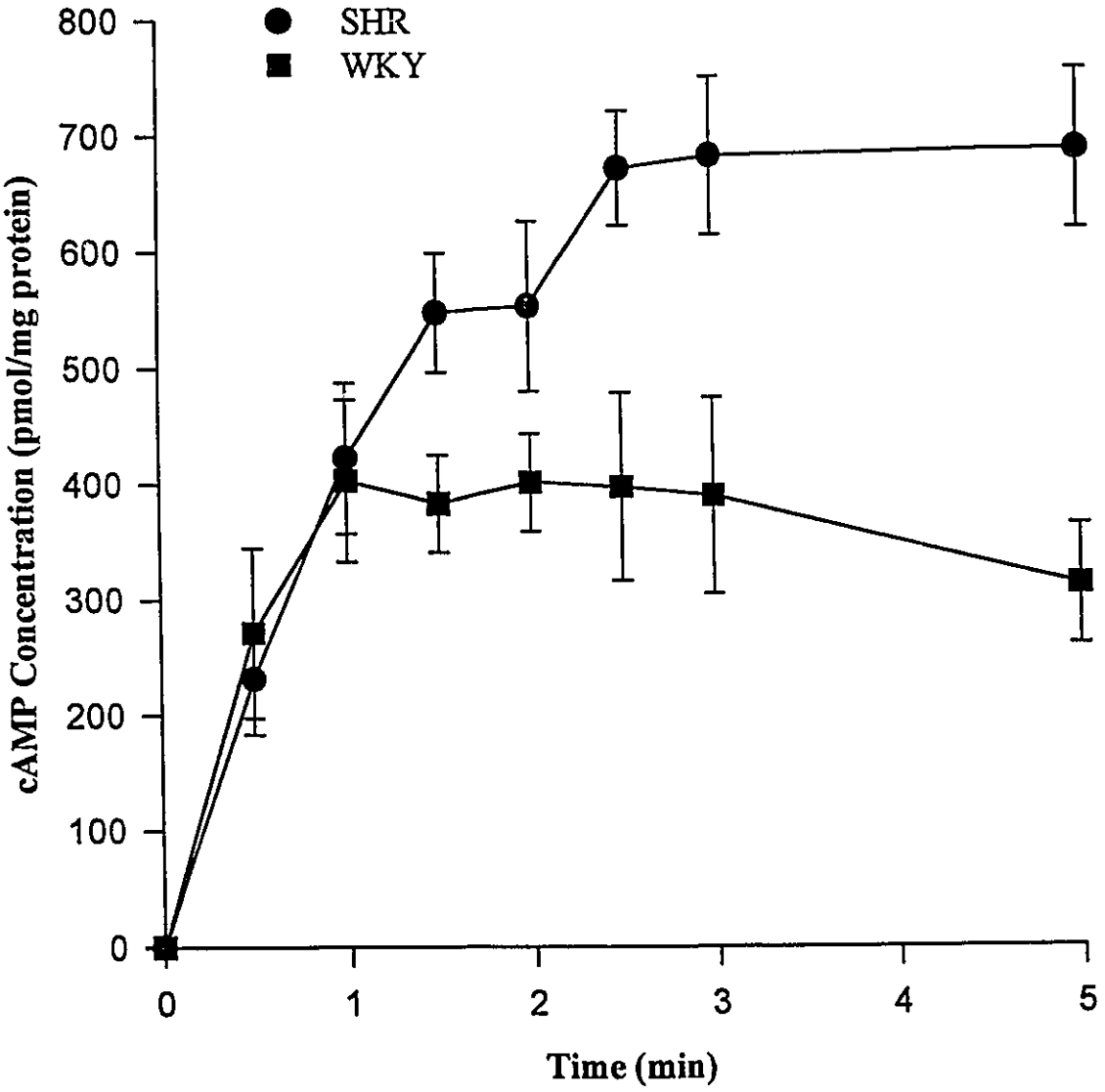
SHR
WKY
800
700
600
500
400
300
200
100
0
cAMP Concentration (pmol/mg protein)
0
1
2
3
4
5
Time (min)

Table 1. cAMP levels in Cultured Aortic SMC from WKY and SHR (mean ± S.E.M)

3-4 Week-Old Age-Group

	WKY (n=4) (pM/μg protein)	Fold increase	SHR (n=4) (pM/μg protein)	Fold increase	*p*
Basal	276 ± 46		299 ± 44		NS
1 μM Isoproterenol	557 ± 84	2.0	619 ± 127	2.1	NS
10 μM Isoproterenol	750 ± 152	2.7	762 ± 156	2.5	NS
1 μM Isoproterenol + 10 μM dl-propranolol	316 ± 57	Basal	252 ± 36	Basal	NS
10 μM Isoproterenol + 100 μM dl-propranolol	276 ± 69	Basal	274 ± 65	Basal	NS
10 μM Forskolin	1381 ± 356	5.0	1067 ± 130	3.6	NS

10-12 Week-Old Age-Group

	WKY (n=4) (pM/μg protein)	Fold increase	SHR (n=4) (pM/μg protein)	Fold increase	*p*
Basal	212 ± 55		446 ± 11		< 0.05
1 μM Isoproterenol	425 ± 43	2.0	652 ± 37	1.5	NS
10 μM Isoproterenol	527 ± 48	2.5	724 ± 101	1.6	NS
1 μM Isoproterenol + 10 μM dl-propranolol	303 ± 57	Basal	322 ± 30	Basal	NS
10 μM Isoproterenol + 100 μM dl-propranolol	300 ± 80	Basal	375 ± 52	Basal	NS
10 μM Forskolin	986 ± 164	4.6	1459 ± 288	3.3	NS

28-30 Week-Old Age-Group

	WKY (n=3) (pM/μg protein)	Fold increase	SHR (n=3) (pM/μg protein)	Fold increase	*p*
Basal	242 ± 37		258 ± 31		NS
1 μM Isoproterenol	493 ± 101	2.0	484 ± 110	1.9	NS
10 μM Isoproterenol	579 ± 159	2.4	529 ± 46	2.1	NS
1 μM Isoproterenol + 10 μM dl-propranolol	229 ± 37	Basal	274 ± 60	Basal	NS
10 μM Isoproterenol + 100 μM dl-propranolol	314 ± 91	Basal	231 ± 34	Basal	NS
10 μM Forskolin	1027 ± 107	4.2	936 ± 39	3.6	NS

Table 2. ß-adrenoceptor binding sites and cAMP levels in Cultured Aortic SMC from WKY and SHR (mean ± S.E.M)

3-4 Week-Old Age-Group

	WKY (n=4)	SHR (n=4)	*p*
*Bmax (binding sites/cell)	465 ± 29	705 ± 8	< 0.05
cAMP Levels % increase of total			
1 μM Isoproterenol	20 ± 8	30 ± 13	NS
10 μM Isoproterenol	34 ± 14	43 ± 16	NS

10-12 Week-Old Age-Group

	WKY (n=4)	SHR (n=4)	*p*
*Bmax (binding sites/cell)	347 ± 35	1280 ± 120	< 0.05
cAMP Levels % increase of total			
1 μM Isoproterenol	22 ± 8	14 ± 3	NS
10 μM Isoproterenol	32 ± 8	19 ± 8	NS

28-30 Week-Old Age-Group

	WKY (n=3)	SHR (n=3)	*p*
*Bmax (binding sites/cell)	1065 ± 12	1633 ± 273	< 0.05
cAMP Levels % increase of total			
1 μM Isoproterenol	25 ± 11	25 ± 12	NS
10 μM Isoproterenol	33 ± 16	29 ± 6	NS

* Taken from (Conyers et al. 1995)

CHAPTER VI

MANUSCRIPT # 4

MANUSCRIPT No. 4

THE INDUCTION OF POLYPLOID CELL DEVELOPMENT BY β-ADRENOCEPTOR DEPENDENT AND INDEPENDENT PATHWAYS IN CULTURED VASCULAR SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE AND NORMOTENSIVE RATS

To be submitted to *American Journal of Physiology*

Roop B. Conyers's contribution:

(i) cell culturing

(ii) cell staining

(iii) microdensitometric scanning

(iv) data analysis

(v) preparation of first draft of manuscript

The induction of polyploid cells by cAMP dependent and independent pathways in cultured vascular smooth muscle cells from spontaneously hypertensive and normotensive rats

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Key words: β-adrenoceptors, cAMP, polyploidy, vascular smooth muscle cells, hypertension

ABSTRACT

The induction of polyploid cells by cAMP dependent and independent pathways in cultured vascular smooth muscle cells from spontaneously hypertensive and normotensive rats

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The objective of this study was to investigate the possible role of ß-adrenoceptor activation in the development of polyploidy in cultured aortic smooth muscle cells (SMC) from 3-4-, 10-12-, and 28-30-week-old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Cultured SMC were stimulated with the non-selective ß-adrenoceptor agonist, isoproterenol, or the adenylate cyclase activator, forskolin, or the membrane permeable analog of adenosine 3',5'-cyclic monophosphate (cAMP), 8-bromo-cAMP. Changes in the development of polyploid SMC were measured by scanning microdensitometry. When compared to basal non-stimulated conditions, isoproterenol, forskolin and 8-bromo-cAMP resulted in a significant increase in polyploid SMC in both WKY and SHR at 3-4-weeks of age, and in WKY only at 10-12-weeks of age. Further, the addition of the ß-adrenoceptor antagonist, *dl*-propranolol, marginally reduced the isoproterenol-stimulated increase in polyploidy, suggesting that alternative intracellular pathway independent of the ß-adrenoceptor signalling pathway may be involved in the development of SMC polyploidy. These results support a role for the intracellular cAMP signalling pathway in mediating the development of polyploidy in cultured SMC from both SHR and WKY.

INTRODUCTION

In hypertension, there is an increase smooth muscle cell (SMC) mass in the media of large arteries in hypertensive animals as compared to normotensives[1,2]. In the aorta of spontaneously hypertensive rats (SHR), vessel wall thickening is largely due to hypertrophied smooth muscle cells (SMC) which are accompanied with polyploid DNA content[3-5]. Polyploidy refers to an accumulation of DNA within SMC as a result of DNA synthesis without subsequent karyokinesis and/or cytokinesis[6]. The mechanism of polyploidy development is unclear but may be related to high blood pressure[1,2,7], catecholamines[3,8], angiotensin II[9], transforming growth factor[10] and/or deoxycorticosterone/NaCl treatment[11].

Previous studies from our lab have shown that the development of polyploid SMC in SHR and Wistar Kyoto rats (WKY) may be part of the ageing process, with accelerated ageing occurring in SHR. We found that SMC from 10-12 week-old SHR already exhibited an increased percentage of polyploid SMC compared to WKY, and this was seen only in older (28-30 week-old) WKY rats[12,13]. We have also identified and characterized ß-adrenoceptors in cultured SMCs from both SHR and WKY at 3-4, 10-12, and 28-30 weeks of age[13,14]. We found that there are no significant differences in receptor affinity between SHR and WKY among the various age-groups, but, there is an increase in ß-adrenoceptor binding sites in SHR as compared to WKY at all three age-groups[13]. Further, we have shown

that the increase in polyploid SMC in SHR and WKY is positively correlated with an increase in ß-adrenoceptors[13], suggesting that polyploidy may be mediated by the functional expression of ß-adrenoceptors. This hypothesis is supported by other studies from our laboratory[15]. We have shown that chronic treatment of both SHR and WKY rats from gestation to 28 weeks with nadolol (a non-selective ß-antagonist), significantly decreased the percentage of polyploid SMC but did not prevent the development of hypertension[15]. In addition, propranolol (ß-adrenoceptor antagonist) has been shown to inhibit the development of polyploidy in aortic SMC of deoxycorticosterone-NaCl-induced hypertensive rats[16]. Taken together, these findings support the hypothesis that polyploidy may be mediated via the action of ß-adrenoceptors[16].

The primary objective of this study was to investigate the possible role of ß-adrenoceptor activation in the development of polyploidy in cultured aortic SMC from both SHR and WKY rats.

MATERIALS AND METHODS

All chemicals, unless specified, were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Source and culture conditions of smooth muscle cells

The source of SMC and the culture conditions utilized in this study have been previously described[12,13]. Briefly, using the explant method, cultured SMC was obtained from the thoracic aortae of spontaneously hypertensive rats (SHR) and Wistar-Kyoto Rats (WKY) at the pre-hypertensive (3-4 weeks) and developing hypertensive (10-12 weeks) stages of hypertension development. Passages of SMC from these primary cultures were stored in liquid nitrogen. Cells derived from each rat were propagated separately, in order to compare differences between cells from different animals. For each experiment, cells from WKY and SHR were cultured under identical conditions with Dulbecco's Modified Eagle Medium (D-MEM) (GIBCO BRL, Burlington, Ontario, Canada) containing 10% fetal bovine serum (FBS) (GIBCO BRL, Burlington, Ontario, Canada) and 1% antibiotic-antimycotic solution (GIBCO BRL, Burlington, Ontario, Canada) at 37°C in an atmosphere of 95% air and 5% CO_2.

Polyploidy and microdensitometric measurements

Cultured SMC in passages 6-7 from 3-4- and 10-12-week old SHR and WKY were grown in culture medium and synchronized with 0.5% FBS for 48 hours. These cultured cells were then incubated for 72 hours in 2% FBS culture medium with various drug additions. The effects of the following drugs were tested: 10 μM isoproterenol, 10 μM isoproterenol and

100 μM dl-propranolol, 10 μM forskolin, and 100 μM 8-bromo-cAMP. Culture medium containing various drugs was changed every 24 hours. Following the 72 hours drug incubation period these cultured SMC were synchronized with 0.5% FBS for 48 hours. This step was done in order to arrest the cultured SMC in the G_o/G_1 stage of the cell cycle. These cells were then evaluated for polyploidy via scanning microdensitometry, a method that has been previously described[12,13].

Statistics

The values are expressed as means ± standard error of mean (SEM). Student's unpaired t-tests and one-way analysis of variance were utilized to determine differences between SHR and WKY. A p value of <0.05 was considered significant.

RESULTS

Microdensitometric measurements

Microdensitometric measurements of the cultured SMC revealed a significantly ($p<0.01$) higher proportion of polyploid SMC in SHR as compared to WKY at both 3-4- and 10-12-weeks of age (Table 1, Table 4).

Cultured 3-4-week old SMC incubated with the ß-adrenergic agonist isoproterenol resulted in a significant increase in polyploid SMC in both WKY ($p<0.0005$) and SHR ($p<0.005$) when compared to basal non-stimulated conditions (Table 1 and Table 2). Further, treatment with isoproterenol resulted in a 23.5 ± 2.5 % and 16.6 ± 5.4 % increase in polyploidy in WKY and SHR respectively when expressed as an increase from basal levels (Table 3). The ß-adrenergic antagonist *dl*-propranolol marginally reduced the isoproterenol-stimulated increase in polyploidy in both WKY and SHR (Table 2). When calculated as a percentage change in relation to the basal non-stimulated levels of polyploid SMC, treatment with *dl*-propranolol resulted in only <12% reduction of isoproterenol-stimulated increase in total polyploid (4C+8C) SMC in both SHR and WKY (Table 3). Both forskolin, an adenylate cyclase activator, and 8-bromo-cAMP, a non-hydolysable analog of adenosine 3',5'-cyclic monophosphate, caused a significant increase in polyploid SMC in both WKY ($p<0.02$) and SHR

($p<0.05$) (Table 1 and Table 2) as compared to basal non-stimulated conditions. Forskolin resulted in a 17.6 ± 3.6 % and 14.0 ± 4.4 % increase in polyploid SMC in WKY and SHR respectively, whereas 8-bromo-cAMP caused a 22.6 ± 4.2 % and 9.5 ± 4.6 % increase in WKY and SHR respectively (Table 3).

Cultured 10-12-week old SMC incubated with isoproterenol resulted in an increase in polyploid SMC in WKY ($p<0.005$) only when compared to basal non-stimulated conditions (Table 1 and Table 2). Further, isoproterenol resulted in a 14.4 ± 2.2 % increase in polyploid smooth muscle cells in WKY when expressed as an increase from basal levels (Table 6). Additionally, *dl*-propranolol marginally reduced the isoproterenol-stimulated increase in polyploidy in WKY (Table 4, Table 6). However, when calculated as a percentage change from basal non-stimulated conditions, *dl*-propranolol significantly reduced (from 14.4 ±2.2% to 3.3 ± 2.5%; $p<0.03$) the isoproterenol-stimulated increase in total polyploidy (4C+8C) SMC (Table 3). Further, both forskolin and 8-bromo-cAMP caused a significant increase in polyploid SMC in WKY only ($p<0.03$, Table 5) (Table 4, Table 6) as compared to basal non-stimulated conditions. In fact, forskolin resulted in a 13.5 ± 2.6 % increase, whereas 8-bromo-cAMP caused a 19.0 ± 4.2 % increase polyploidy (Table 4). In 10-12-week old SHR, however, isoproterenol, forskolin or 8-bromo-cAMP did not cause any significant increase in polyploid smooth muscle cells (Table 4).

Cultured 28-30-week old SMC incubated with isoproterenol, forskolin or 8-bromo-cAMP did not significantly increase polyploid SMC in either SHR or WKY (Table 7 and Table 8). However, when calculated as a percentage change from basal non-stimulated conditions, isoproterenol, forskolin and 8-bromo-cAMP resulted in a 10.5±1.4, 20.4±2.1 and 13.9±1.4 percentage increase in total polyploid SMC in WKY, respectively. Further, *dl*-propranolol was ineffective in decreasing the isoproterenol-stimulated increase in total polyploidy SMC in WKY. In SHR such changes in total polyploidy SMC as compared to basal conditions was not observed.

DISCUSSIONS

The major findings in this study are: (i) ß-adrenergic activation via agonist stimulation resulted in a significant increase in polyploid cells in cultured SMC from 3-4-week old WKY and SHR and 10-12-week old WKY; (ii) elevation of intracellular cAMP levels via isoproterenol stimulation, forskolin stimulation and/or 8-bromo-cAMP results in an increase of polyploidy in cultured SMC at 3-4-weeks from WKY and SHR and at 10-12-weeks from WKY only; (iii) the development of polyploid SMC via the ß-adrenergic receptor-G_s-protein-adenylate cyclase-cAMP pathway is more efficient in WKY as compared to SHR; and, (iv) more than one intracellular

pathway may be involved in the development of SMC polyploidy, because dl-propranolol only caused a partial inhibition of isoproterenol-stimulated increase in polyploid SMC in both WKY and SHR. This discussion will focus on the possible roles of ß-adrenoceptor signal transduction pathway in mediating the development of polyploidy.

Previous studies from our lab have shown that the increase in polyploid SMC in SHR and WKY in culture is positively correlated with the increased expression of ß-adrenoceptors[13], suggesting that the functional expression of ß-adrenoceptors may mediate the development of polyploidy.

In this study, the potential for a causative role of functional ß-adrenoceptors in the development of polyploidy was confirmed since an elevation in intracellular cAMP levels, either by isoproterenol, forskolin or 8-bromo-cAMP, resulted in increases in polyploid SMC in both SHR and WKY.

Interestingly, the addition of dl-propranolol did not completely block the isoproterenol stimuated increases in polyploidy. This may reflect activation of alternative pathways independent of ß-adrenoceptor signalling pathway.

Previous studies from our lab have shown that there is a significant elevation of ß-adrenoceptor density in SHR as compared to WKY[12,13]. However, from this study it was also found that isoproterenol, forskolin and 8-bromo-cAMP all caused a greater increase of polyploid SMC (4C+8C) in

WKY as compared to SHR. This may suggest that the coupling efficiency of ß-adrenoceptor-G_s-adenylate cyclase-cAMP dependent protein kinases (PKA) is greater in WKY as compared to SHR, despite an increase in ß-adrenoceptor density in SHR as compared to WKY. This is consistent with the findings that in the hypertensives, there is a functional uncoupling of the receptor from the adenylate cyclase complex[17]. Additionally, this may also suggest that an alternative mechanism might be acting in causing polyploidy in SMC from WKY as compared to SHR.

In 10-12-week and 28-30-week old SHR, the use of isoproterenol, forskolin and 8-bromo-cAMP did not affect the development of smooth muscle cell polyploidy, unlike the WKY SMC. It is possible that the lack of effect on the 10-12-week and 28-30-week old SHR may be due to a ceiling effect, that is, this population of smooth muscle cells have attained the maximum level of polyploid cells that can be stimulated.

The role of ß-adrenoceptors and cAMP in the development SMC polyploidy is unclear. In SMC, binding of agonist to ß-adrenoceptors results in activation of adenylate cyclase via a stimulatory guanine-nucleotide dependent regulatory protein (G_s)[18,19]. This activation leads to the formation of intracellular cAMP. As we have shown in this study, an increase level of intracellular cAMP either by agonist stimulation of ß-adrenoceptor, or by direct elevation via a membrane permeable non-hydrolyzable cAMP analog, or by increase adenylate cyclase activity results

in increase DNA synthesis in cultured SMC. This finding is consistent with other studies where cAMP has been established as a promoter of cell growth[13,20-24]. The underlying mechanisms of cAMP mediated cell growth and hyperploidy may involve the activation of cAMP-dependent protein kinases (PKA) and the regulation of a family of nuclear proteins (CREB)[25,26]. Elevation of intracellular cAMP results in binding of cAMP to the regulatory subunit of PKA. This in turn results in dissociation of the PKA catalytic subunit which then translocates to the nucleus where it phosphorylates nuclear proteins (known as cAMP recognition/response element binding (CREB) proteins). These phosphorylated proteins (CREB) in turn binds to cAMP response element (CRE) within the promoters of cAMP-inducible genes and thereby mediates their induction[25,26]. There are a number of genes that can be induced in this way and hence elevated cAMP may result in increased transcription[25,26]. Alternatively, it is possible that the ß-adrenoceptor mediated effects are independent of cAMP production. For example, in turkey erythrocytes, ß-adrenoceptor stimulation was found to activate phospholipase C (PLC) in a manner that was independent of cAMP production[27]. It has been suggested that the PLC activation may be due to direct coupling of ß-adrenoceptors to a G protein (G_{PLC})[27]. The activation of PLC eventually leads to activation of protein kinase C (PKC), which has been reported to play a role in DNA synthesis[28]. Another possible mechanism may involve the intracellular

messenger calcium (Ca^{2+}). Recent studies suggest that agonist-binding to β-adrenoceptors leads to an elevation in intracellular Ca^{2+} and this may be mediated by the α_s-subunit of G_s proteins (Brown et al., 1989; Xiong et al., 1995; Sperelakis et al., 1994), or the β,γ-subunits of G_s proteins (Clapham et al., 1993; Muller et al., 1995) or by cAMP (Cooper et al., 1995) or by PKA (Brown et al., 1989). Further, it has been proposed that smooth muscle cell β-adrenoceptors may directly increase intracellular Ca^{2+} by activation of L-type Ca^{2+} channels (Sperelakis et al., 1994; Takuwa et al., 1988; Yamaguchi et al, 1995; Brown et al., 1989). Regardless of the cellular mechanism, this increase in intracellular Ca^{2+} in turn leads to the activation of PKC which plays a role in DNA synthesis (Hoshina et al., 1990). Regardless of the route through which an increase in DNA synthesis in response to ß-adrenoceptor stimulation may occur, polyploidy of the SMC may develop in cells where DNA synthesis has taken place without subsequent karyokinesis or cytokinesis[6].

In conclusion, results from this study support a role for the cAMP intracellular signalling pathway in mediating the development of polyploidy in cultured SMC from both SHR and WKY.

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Table 1. The effects of increased intracellular cAMP levels on the percentage of polyploidy in cultured SMC from 3-4 Week old SHR and WKY (n=4). *p* represents the statistical comparison between SHR and WKY at the given condition.

Condition	Rat Group	DNA Content 2C SMC	4C SMC	8C SMC
Basal	WKY	90.2 ± 2.0	9.8 ± 2.0	0
[A]	SHR	72.0 ± 3.0	25.7 ± 3.1	2.3 ± 0.3
	p	<0.001	<0.01	
10 μM Isoproterenol	WKY	66.7 ± 2.9	32.9 ± 2.7	0.4 ± 0.2
[B]	SHR	55.4 ± 5.7	41.2 ± 4.6	3.4 ± 2.8
	p	ns	ns	ns
100 μM dl-propranolol	WKY	78.8 ± 5.2	20.2 ± 5.4	1.0 ± 1.0
& 10 μM Isoproterenol	SHR	63.7 ± 5.3	35.6 ± 5.9	0.7 ± 0.7
[C]	*p*	ns	ns	ns
10 μM Forskolin	WKY	72.6 ± 4.9	26.3 ± 5.1	1.1 ± 0.4
[D]	SHR	58.0 ± 3.1	41.7 ± 3.2	0.3 ± 0.3
	p	ns	ns	ns
100 μM 8-bromo-cAMP	WKY	67.6 ± 6.2	29.6 ± 4.8	2.8 ± 1.6
[E]	SHR	62.5 ± 2.3	34.1 ± 3.9	3.4 ±2.1
	p	ns	ns	ns

Table 2. Statistical analysis of polyploid smooth muscle cell measurements from Table 1.

		p Value		
p value	**Rat Group**	**2C SMC**	**4C SMC**	**8C SMC**
A vs B	WKY	< 0.0005	< 0.0005	ns
	SHR	< 0.05	< 0.05	ns
A vs C	WKY	ns	ns	ns
	SHR	ns	ns	ns
A vs D	WKY	< 0.02	< 0.03	ns
	SHR	< 0.02	< 0.02	ns
A vs E	WKY	< 0.02	< 0.01	ns
	SHR	< 0.05	ns	ns
B vs C	WKY	ns	ns	ns
	SHR	ns	ns	ns

Table 3. Increase in polyploid SMC from 3-4-week old SHR and WKY. Results are expressed as a percentage increase by the various conditions as compared to basal unstimulated conditions.

Condition	Rat Group	DNA Content 4C + 8C SMC
10 μM Isoproterenol	WKY	23.5 $\pm$ 2.5
	SHR	16.6 $\pm$ 5.4
	p	ns
100 μM dl-propranolol	WKY	11.4 $\pm$ 4.2
& 10 μM Isoproterenol	SHR	8.3 $\pm$ 4.4
	p	ns
10 μM Forskolin	WKY	17.6 $\pm$ 3.6
	SHR	14.0 $\pm$ 4.4
	p	ns
100 μM 8-bromo-cAMP	WKY	22.6 $\pm$ 4.2
	SHR	9.5 $\pm$ 4.6
	p	ns

Table 4. The effects of increased intracellular cAMP levels on the percentage of polyploidy in cultured SMC from 10-12 Week old SHR and WKY (n=4). *p* represents the statistical comparison between SHR and WKY at the given condition.

Condition	Rat Group	DNA Content 2C SMC	4C SMC	8C SMC
Basal	WKY	91.7 ± 1.7	8.3 ± 1.7	0
[A]	SHR	57.6 ± 4.8	39.9 ± 5.9	2.5 ± 1.7
	p	<0.0005	<0.002	
10 μM Isoproterenol	WKY	77.3 ± 2.8	22.7 ± 2.8	0
[B]	SHR	56.0 ± 3.9	38.2 ± 3.4	5.8 ± 2.1
	p	<0.004	<0.001	
100 μM dl-propranolol	WKY	88.4 ± 3.4	11.6 ± 3.4	0
& 10 μM Isoproterenol	SHR	66.9 ± 4.1	32.6 ± 4.1	0.5 ± 0.5
[C]	*p*	<0.007	<0.008	
10 μM Forskolin	WKY	78.2 ± 3.5	21.5 ± 3.2	0.3 ± 0.3
[D]	SHR	60.9 ± 4.8	36.3 ± 3.1	2.8 ± 2.0
	p	<0.03	<0.02	ns
100 μM 8-bromo-cAMP	WKY	72.7 ± 6.7	27.3 ± 6.7	0
[E]	SHR	54.8 ± 1.5	43.2 ± 2.1	2.0 ±0.7
	p	<0.04	ns	

Table 5. Statistical analysis of polyploid smooth muscle cell measurements from Table 4.

		p Value		
p value	**Rat Group**	**2C SMC**	**4C SMC**	**8C SMC**
A vs B	WKY	< 0.005	< 0.005	ns
	SHR	ns	ns	ns
A vs C	WKY	ns	ns	ns
	SHR	ns	ns	ns
A vs D	WKY	< 0.01	< 0.01	ns
	SHR	ns	ns	ns
A vs E	WKY	< 0.03	< 0.03	ns
	SHR	ns	ns	ns
B vs C	WKY	ns	ns	ns
	SHR	ns	ns	ns

Table 6. Increase in polyploid SMC from 10-12-week old SHR and WKY. Results are expressed as a percentage increase by the various conditions as compared to basal unstimulated conditions.

Condition	Rat Group	DNA Content 4C + 8C SMC
10 μM Isoproterenol	WKY	14.4 $\pm$ 2.2
	SHR	1.6 $\pm$ 1.6
	p	$<$0.003
100 μM dl-propranolol	WKY	3.3 $\pm$ 2.5
& 10 μM Isoproterenol	SHR	no increase
	p	
10 μM Forskolin	WKY	13.5 $\pm$ 2.6
	SHR	no increase
	p	
100 μM 8-bromo-cAMP	WKY	19.0 $\pm$ 4.2
	SHR	2.8 $\pm$ 2.8
	p	$<$0.02

Table 7. The effects of increased intracellular cAMP levels on the percentage of polyploidy in cultured SMC from 28-30 Week old SHR and WKY (n=3, except where noted). *p* represents the statistical comparison between SHR and WKY at the given condition.

Condition	Rat Group	DNA Content 2C SMC	4C SMC	8C SMC
Basal	WKY	40.2 ± 3.3	51.3 ± 3.4	8.5 ± 1.5
[A]	SHR	27.2 ± 3.6	64.1 ± 3.7	8.7 ± 1.1
	p	ns	ns	ns
10 μM Isoproterenol	WKY	33.9 ± 2.1	62.8 ± 2.2	3.3 ± 1.1
[B]	SHR (n=2)	25.7 ± 4.4	68.1 ± 1.1	6.2 ± 3.4
	p	ns	ns	ns
100 μM dl-propranolol	WKY	31.9 ± 2.1	62.0 ± 2.1	6.1 ± 1.7
& 10 μM Isoproterenol	SHR (n=2)	33.5 ± 2.7	62.8 ± 3.3	3.7 ± 1.2
[C]	*p*	ns	ns	ns
10 μM Forskolin	WKY	28.0 ± 1.8	65.3 ± 2.6	6.7 ± 1.3
[D]	SHR (n=2)	29.7 ± 2.8	62.9 ± 2.1	7.4 ± 2.7
	p	ns	ns	ns
100 μM 8-bromo-cAMP	WKY	35.6 ± 4.8	54.2 ± 6.5	10.2 ± 1.7
[E]	SHR	26.8 ± 1.8	68.2 ± 4.8	5.0 ± 3.0
	p	ns	ns	ns

Table 8. Statistical analysis of polyploid smooth muscle cell measurements from Table 7.

		p Value		
p value	**Rat Group**	**2C SMC**	**4C SMC**	**8C SMC**
A vs B	WKY	ns	ns	ns
	SHR	ns	ns	ns
A vs C	WKY	ns	ns	ns
	SHR	ns	ns	<0.04
A vs D	WKY	ns	ns	ns
	SHR	ns	ns	ns
A vs E	WKY	ns	ns	ns
	SHR	ns	ns	ns
B vs C	WKY	ns	ns	ns
	SHR	ns	ns	ns

Table 9. Increase in polyploid SMC from 28-30-week old SHR and WKY. Results are expressed as a percentage increase by the various conditions as compared to basal unstimulated conditions.

Condition	Rat Group	DNA Content 4C + 8C SMC
10 μM Isoproterenol	WKY	10.5 $\pm$ 1.4
	SHR	2.1 $\pm$ 0.4
	p	$<$0.005
100 μM dl-propranolol	WKY	13.9 $\pm$ 1.4
& 10 μM Isoproterenol	SHR	no increase
	p	
10 μM Forskolin	WKY	20.4 $\pm$ 2.1
	SHR	no increase
	p	
100 μM 8-bromo-cAMP	WKY	7.7 $\pm$ 1.7
	SHR	no increase
	p	

CHAPTER VII

DISCUSSION

Chapter VII: Discussion

1. Hypertension

1.1 Premature Ageing as a possible mechanism for the development of hypertension

The processes of ageing and hypertension, as previously discussed (Introduction 1.3), share similarities with respect to structural and functional vascular changes. In human arteries, arterial stiffness, which is believed to be due to changes within the vascular media that manifest as an increase in pulse wave velocity, increases with age and is considered to be a major factor in the age-related increase in systolic pressure (Avolio, 1992; Cooper et al., 1994). In fact, in populations where the decrease in arterial compliance with age is blunted, the associated increase in blood pressure with age is also blunted (Avolio, 1985). With this in mind, it is therefore possible that hypertension is associated with an accelerated or premature ageing process.

One of the structural changes associated with vascular ageing is an increase in polyploid smooth muscle cells (Barrett et al., 1983). In human arterial tissue (aortic, carotid and iliac vessels), the frequency of polyploid smooth muscle cells increased from less than 1% at birth to a mean of 7%

in adults (Barrett et al., 1983). In fact, the development of polyploid cells appears to be an important phenomenon common to the ageing process, as evidenced by the increase in polyploid cells with age in a number of tissues (Medvedev, 1986; Brodsky et al., 1977; Dice, 1993). In hypertensive animals, such as the SHR model, increases in polyploid smooth muscle cells in the aorta have been reported to occur relatively early in the animal's life (Owens et al., 1982; Lombardi et al., 1989; Black et al., 1989). Based on these observations, I propose that a premature ageing process may be a possible mechanism for the development of hypertension. I therefore evaluated the expression of polyploidy in cultured smooth muscle cells from different age-groups of hypertensive (SHR) and non-hypertensive (WKY) animals (Chapter 2, Paper 1).

1.2 The expression of polyploid smooth muscle cells in ageing hypertensive animals

I found that when grown and maintained under culture conditions, smooth muscle cells from 3-4-weeks (prehypertensive), 10-12-weeks (developing hypertensive), and 28-30-weeks (established hypertensive) old SHR and WKY all exhibit a heterogenous populations of cells as defined by the expression of both mononucleated and multinucleated cells, as well as diploid and polyploid smooth muscle cells. (Lee et al., 1992a; Conyers et

al., 1995). It was found that there was a higher percentage of both multinucleated and polyploid smooth muscle cells in SHR as compared with WKY at three age-groups (Lee et al., 1992a; Conyers et al., 1995). These findings are consistent with *in vivo* findings from SHR and WKY, where the percentage of polyploid smooth muscle cells in intact aortae from SHR as compared to WKY increased with the age of the animal (Owens et al., 1981; Owens, 1985; Rosen et al., 1986a; Rosen et al., 1986b). For example, one study reported that the percentage of polyploid smooth muscle cells in SHR versus WKY was 3.5% and 4.5% respectively in neonatal rats as compared to 22.5% and 13.5% respectively in 12-month-old animals (Rosen et al., 1986a). The parallel findings of our *in vitro* results with the *in vivo* results may allow extension of findings from *in vitro* studies of polyploidy to the *in vivo* polyploidy state.

I found that polyploidy in cultured smooth muscle cells varied with ageing (Lee et al., 1992a; Conyers et al., 1995). In fact, in WKY, there was a relatively low percentage of polyploid smooth muscle cells at 3-4- and 10-12-weeks of age, however, by 28-30-weeks of age, there was a significant elevation in polyploid cells. In contrast, smooth muscle cells from SHR had already exhibited a significant elevation in polyploid smooth muscle cells by 10-12-weeks of age (Conyers et al., 1995). Thus, the increase in polyploidy seen in the older (28-30-weeks of age) WKY smooth muscle cells, was detected at an earlier stage in the younger (10-12-weeks of age) SHR.

Another important observation was the finding that there was a positive correlation of polyploid cell expression with passaging of smooth muscle cells in culture (Conyers et al., 1995). With increasing passaging in culture (P2, P5, P6-7), SHR smooth muscle cells from all three age-groups showed a passage-related increase in polypolidy cell expression, whereas, in WKY such a passage-related increase was found only in smooth muscle cells from 28-30-weeks (Conyers et al., 1995). If we view passaging of cells in culture as a model of *in vivo* ageing, then, the observed passage-related increases in polyploidy in cultured smooth muscle cells can be considered to be an indication of the ageing of smooth muscle cells.

Our findings of: (i) significant elevation of polyploid smooth muscle cells in SHR by 10-12-weeks as compared to similar increases occurring only in older (28-30-weeks) WKY; (ii) the presence of a passage-related increase in the percentage of polyploid smooth muscle cells in all three age-groups of SHR (3-4-, 10-12-, 28-30-weeks of age) as compared to similar changes occurring only in older (28-30-weeks) WKY; and, (iii) the higher percentage of multinucleated smooth muscle cells in cultured SHR smooth muscle cells as compared to WKY at all three age-groups, supports the notion that the development of polyploid smooth muscle cells in SHR may be an indicator that a premature or accelerated ageing process may be occurring in SHR as compared to the WKY rats.

The exact mechanism(s) for polyploid smooth muscle cell development is unknown. However, as discussed previously (Introduction 3.2), polyploidy may develop as a result of an abnormal cell division process. For example, the occurence of mitosis with the failure of cytokinesis and/or karyokinesis and/or endoreplication (Brodsky et al., 1977). This aspect (cell division processes) of polyploid smooth muscle cell development, however, was not investigated in this study.

One possible molecular mechanism may involve the activation of membrane bound receptors, that is, polyploidy development may be a membrane receptor-mediated response. In fact, several studies have suggested that vascular β-adrenoceptors may be involved in the development of smooth muscle polyploidy (Lee et al., 1992b; Leitschuh et al., 1987). With this in mind, I evaluated the expression of β-adrenoceptors in cultured smooth muscle cells from different age-groups of hypertensive (SHR) and non-hypertensive (WKY) animals.

1.3 The expression of smooth muscle cell β-adrenoceptors in ageing hypertensive animals

I found that cultured smooth muscle cells from both SHR and WKY at 3-4-, 10-12-, and 28-30-weeks of age all express a single, low affinity β-adrenoceptor binding site in culture (Conyers et al., 1995). More

significantly, I found that cultured SHR smooth muscle cells express more β-adrenoceptor binding sites as compared to WKY in all three age-groups (Conyers et al., 1995). Previous studies on vascular smooth muscle cell β-adrenoceptors have reported conflicting findings, with some studies showing a decrease in β-adrenoceptor number in SHR as compared to WKY (Jazayeri et al., 1989; Limas et al., 1979), while others have reported no differences in β-adrenoceptor numbers (Kwan et al., 1990; Asano et al., 1991).

In the SHR strain, I found that in the cultured smooth muscle cells there was an increase in the expression of β-adrenoceptors from 3-4-weeks to 28-30-weeks, that is, a trend for an age-related increase in β-adrenoceptors (Conyers et al., 1995). Such a trend was not seen in the WKY strain. There was, however, a significant increase in WKY smooth muscle cell β-adrenoceptor number in the 28-30-week old group as compared with the 3-4- or 10-12-week old groups (Conyers et al., 1995). In addition to age, I found that the passage of smooth muscle cell cultures was also a determining factor in the expression of β-adrenoceptors. In 10-12-week old SHR and WKY, the expression of β-adrenoceptor numbers increased with increasing passaging, more so in SHR than WKY (Conyers et al., 1995).

The changes in β-adrenoceptors density seen in SHR and WKY at the three age-groups suggests that the increase in β-adrenoceptor expression

is accelerated in SHR as compared to WKY, since the increased receptor density measured in the older (28-30-weeks) WKY rats had already been observed in the younger (10-12-weeks) SHR rats.

These findings further support the hypothesis that a premature ageing process might be a possible mechanism underlying the development of hypertension.

Additionally, the *parallel* pattern of change seen in β-adrenoceptor density and polyploidy measurements in both SHR and WKY supports the notion that there may be a possible relationship between β-adrenoceptors and polyploidy expression (Lee et al., 1992b; Conyers et al., 1995). In fact, a positive correlation exists between the expression of SHR and WKY cultured smooth muscle cell polyploidy and β-adrenoceptor density with age of the animals from which the cells were derived and the passage of smooth muscle cell cultures, suggesting that β-adrenoceptors may influence the expression of polyploid cells. This aspect of polyploid smooth muscle cell development, that is, the role of the plasma membrane β-adrenoceptor in DNA synthesis, was investigated in this study.

In order to evaluate a possible causative relationship from the existing correlative relationship between β-adrenoceptors and polyploid cells, it was necessary to, firstly, evalute the functionality of the β-adrenoceptor binding sites and then, secondly, to evaluate the effects of activation of functional β-adrenoceptors on polyploidy cell development. These objectives became

the focus in delineating the possible mechanism(s) of polyploid smooth muscle cell development.

1.4 Functional expresssion of β-adrenoceptors as determined by β-adrenoceptor agonist-stimulated cAMP production in ageing hypertensive animals

The expression of β-adrenoceptor binding sites in any system does not necessarily indicate the functionality of these receptors. Such a determination requires the evaluation of the signal transduction pathway that is coupled to the β-adrenoceptors, that is, a measure of the changes in the second messenger, cAMP, associated with activation of the β-adrenoceptors. Using this approach, it was found that stimulation of cultured smooth muscle cell β-adrenoceptors with isoproterenol, a β-adrenoceptor agonist, resulted in increased levels of cAMP in both SHR and WKY at all three age-groups (Conyers et al., 1996a). This elevation in cAMP was inhibited by *dl*-propranolol, a β-adrenoceptor antagonist, thereby confirming that the increase in cAMP levels was indeed due to direct activation of the β-adrenoceptor binding sites. Furthermore, the β-adrenoceptor agonist-stimulated increases in cAMP in cultured smooth muscle cells from SHR and WKY at all three age-groups supports the notion

that the β-adrenoceptor binding sites are receptors functionally coupled to adenylate cyclase via G_s proteins.

Interestingly, despite our previous findings of increased β-adrenoceptor binding sites in all three age-groups of SHR and the older age-group of WKY smooth muscle cells, stimulation of the β-adrenoceptors with isoproterenol resulted in similar increases in both SHR and WKY smooth muscle cells at 3-4-, 10-12-, and 28-30-weeks of age, (Conyers et al., 1995). Thus, the observed trend of an age-related increase in β-adrenoceptor binding sites in cultured smooth muscle cells from SHR and WKY is not reflected in a similar age-related increase in the expression of functional β-adrenoceptors. As discussed in Conyers et al. (1996a) (Materials and Methods), there may be several explanations to account for this.

Our findings that forskolin stimulation, which activates adenylate cyclase directly, resulted in greater elevation of cAMP than those induced by β-adrenoceptor activation, supports the hypothesis of an inefficient coupling of β-adrenoceptor-G_s-adenylate cyclase system (Conyers et al., 1995). Further, the fact that forskolin directly activates the adenylate cyclase catalytic subunit, thereby bypassing the β-adrenoceptor-G_s components, suggests that the inefficiency may involve G-proteins (G_s), specifically, at the level of β-adrenoceptor-G_s coupling or the availability of G_s proteins. Further, an inefficient conformational change of an agonist (hormone)-receptor complex can also explain the inefficient coupling

(Helmreich et al., 1985). It should be noted, however, that β-adrenoceptors mediate their effects via complex signalling pathways with many components and thus alterations in the expression, or in the activities of any of the pathway components, or in the various receptor-mediated signalling pathways which eventually regulate cAMP levels may all play some role in desensitization.

An interesting finding from the measurements of intracellular cAMP levels in cultured smooth muscle cells from SHR and WKY is the observation that, in the presence of a cAMP-specific phosphodiesterase inhibitor, the basal cAMP levels from 10-12-week old SHR were significantly higher as compared to WKY (Conyers et al., 1996a). As discussed in Conyers et al. (1996a) (Materials and Methods), this elevation in basal cAMP levels may be due to any number of alterations in the cAMP signalling pathway. Despite the lack of knowledge for the exact mechanism(s) involved, the elevation in basal non-stimulated levels of cAMP in SHR smooth muscle cells as compared to WKY at 10-12-weeks of age is quite significant because it may potentially lead to increased cAMP-dependent phosphorylation of several intracellular target proteins which could possibly play a role in DNA synthesis and cell growth.

1.5 The role of the β-adrenoceptor mediated intracellular signalling pathway in the development of polyploid smooth muscles in hypertension

The exact role of the β-adrenoceptor-mediated increase in cAMP in smooth muscle cell growth and polyploidy development is unknown. However, our findings that agonist activation of cultured smooth muscle cell β-adrenoceptors from both 3-4-week old SHR and WKY and 10-12-week old WKY resulted in an increase of polyploid smooth muscle cells as compared to those of the unstimulated cells suggests a *potential* causative role for the functional expression of β-adrenoceptors in the development of polyploidy (Conyers et al., 1996b). In fact, elevation of intracellular cAMP levels, either by direct activation of adenylate cyclase with forskolin or by the use of 8-bromo-cAMP, a membrane permeable analog of cAMP, resulted in increases in polyploid smooth muscle cells in both 3-4-week old SHR and WKY and 10-12-week old WKY as compared to non-stimulated conditions (Conyers et al., 1996b). These findings are consistent with other studies where cAMP has been shown to be a promoter of cell growth (Franks et al., 1984; Kanbe et al., 1983; Rozengurt, 1986; Dumont et al., 1989; Printseva et al., 1992).

Additionally, I have found that there was a significant elevation of β-adrenoceptor density in SHR as compared to WKY at 3-4-, 10-12-, and 28-30-weeks of age (Conyers et al., 1995). Despite this increase in β-adrenoceptor density in SHR as compared to WKY, isoproterenol, forskolin and 8-bromo-cAMP all caused a greater increase in the number of polyploid smooth muscle cells in WKY as compared to SHR at both 3-4- and 10-12-weeks of age (Conyers et al., 1996b). This finding may reflect that the coupling efficiency of β-adrenoceptor-G_s-adenylate cyclase, and the activation of cAMP dependent protein kinases (PKA) is greater in WKY as compared to SHR. This, in fact, is consistent with the finding of a functional uncoupling of receptors from the adenylate cyclase complex seen in *in vivo* hypertensives and ageing (Feldman, 1987).

Further, the finding that the β-adrenoceptor antagonist, *dl*-propranolol, did not completely block the β-adrenoceptor agonist (isoproterenol) stimulated increases in polyploidy development in SHR and WKY, suggests that alternative pathways independent of the β-adrenoceptor signalling pathway may also be involved.

An interesting observation, is that in 10-12-week old SHR, the addition of isoproterenol, forskolin and 8-bromo-cAMP did not affect the development of smooth muscle cell polyploidy. In contrast, when cAMP levels were elevated by these treatments in the 3-4-week old SHR and WKY and 10-12-week old WKY smooth muscle cells, polyploid cells developed.

I speculate that the lack of effect on the 10-12-week old SHR may be due to a 'ceiling effect', that is, this population of cultured smooth muscle cells have attained the maximum level of polyploid cells that can be stimulated. Current studies evaluating the effects of β-adrenoceptor agonist activation on the development of cultured smooth muscle cell polyploidy in older (28-30-week) SHR and WKY should shed light on this hypothesis.

1.5.1 The mechanisms involved in the β-adrenoceptor agonist-activated increases in the development of smooth muscle cell polyploidy.

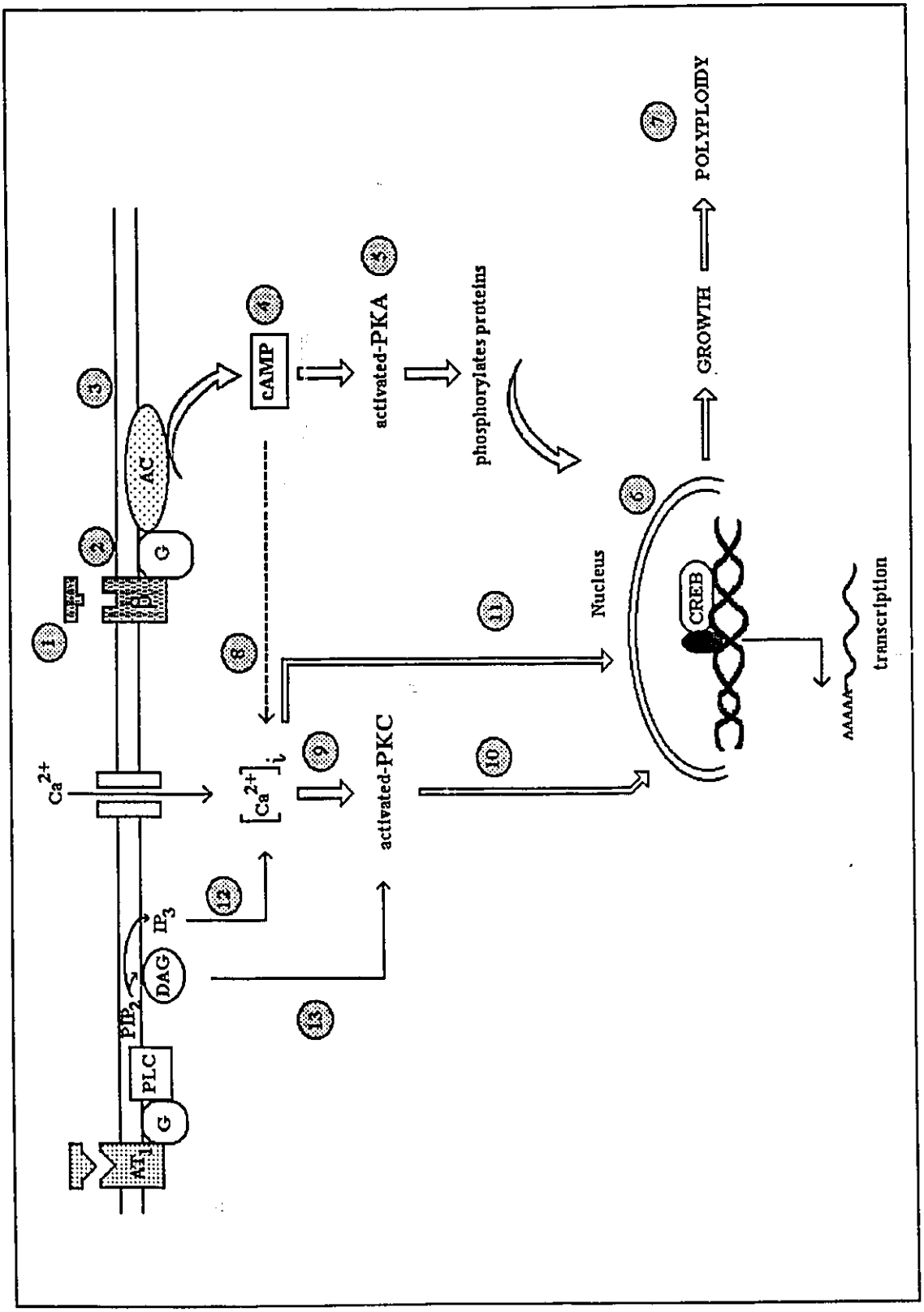
As previously mentioned, the exact role of β-adrenoceptors and cAMP in the development of polyploidy is unclear. I propose that one of the contributing mechanisms of cAMP mediated cell growth and hyperploidy may involve the activation of cAMP-dependent protein kinases (protein kinase A, PKA) and the regulation of a family of nuclear proteins, (cAMP response element binding protein, CREB) (Figure 1), (Karin et al., 1993; Spaulding, 1993; Herschman et al., 1991; Chrivia et al., 1993). Activation of β-adrenoceptors (for example, either by agonist binding, or by a constitutively active receptor (Lefkowitz et al., 1993)) results in the elevation of intracellular cAMP which in turn binds to the catalytic subunit of PKA thereby causing it to dissociate from PKA and translocate to the nucleus

where it phosphorylates CREB. Phosphorylated CREB binds to the cAMP response element (CRE) within the promoters of cAMP-inducible genes and mediates their induction (Figure 1) (Karin et al., 1993; Spaulding, 1993; Herschman et al., 1991; Chrivia et al., 1993). Smooth muscle cell polyploidy, in turn, develops in cells where DNA synthesis has occurred accompanied by failure of subsequent karyokinesis or cytokinesis.

Figure 1: Proposed mechanism for the role of β-adrenoceptor pathway in the synthesis of DNA (Modified from Collins et al., 1992; Dumont et al., 1987).

1. Binding of an agonist to SMC β-adrenoceptors results in,
2. activation of the α_s-subunit of a G_s protien
3. $G\alpha_s$ activates adenylate cyclase which,
4. leads to elevation of intracellular cAMP
5. cAMP activates PKA by binding to the PKA regulatory subunit
6. dissociation of the PKA catalytic subunit translocates to the nucleus where it phosphorylates the nuclear transcription factor, CREB. Phosphorylated CREB binds to cAMP response element within promoters of cAMP-inducible genes and mediates their induction
7. Polyploidy of SMC develops in cells where DNA synthesis has occurred with failure of subsequent karyokinesis and/or cytokinesis

8-11. Other mediators are involved in the process of DNA synthesis, such as Ca^{2+} and PKC


AT1
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As discussed in Conyers et al. (1996b), there are other possible mechanisms, involving vascular β-adrenoceptors, that affect DNA synthesis. These proposed intracellular pathways do not necessarly function in isolation. There is significant intercommunication or cross-talk among these various pathways (Minneman et al., 1993; Brodde et al., 1992). Further, stimulation of DNA synthesis is affected by many other pathways which do not involve β-adrenoceptors. Our findings that the β-adrenoceptor antagonist, *dl*-propranolol, did not completely block the β-adrenoceptor agonist (isoproterenol) stimulated increases in polyploidy in SHR and WKY supports the activation and involvement of other pathways, independent of the β-adrenoceptor mediated signalling mechansim (Conyers et al., 1996b).

1.6 Smooth muscle cell β-adrenoceptors and polyploidy: *correlation or causation*?

The studies and discussions, so far, have focused on the events pertaining to the β-adrenoceptor activated intracellular signalling pathway and to events, occurring in the cell nucleus, that eventually lead to gene transcription. The results obtained have provided support for a role of the β-adrenoceptor signalling pathway in smooth muscle cell DNA synthesis, and in so doing, have provided *correlative* evidence for the alteration of β-adrenoceptors in the development of smooth muscle cell polyploidy. These

results, however, do not provide conclusive *causative* evidence for the alteration of β-adrenoceptor density in the development of polyploidy. It is important to make this distinction because the studies conducted did not specifically investigate the stimulus or cellular mechanism or abnormality that effects smooth muscle cell polyploidy, namely the failure of karyokinesis and/or cytokinesis. In fact, it is quite conceivable that in both SHR and WKY, the β-adrenoceptor-cAMP-PKA-DNA synthesis pathway is equally efficient, that is, the nuclear cellular mechanisms that lead to DNA synthesis may be equally effective in both SHR and WKY. However, the abnormality that leads to the increased development of polyploidy in SHR may lie at events distal to the nuclear cellular mechanism, that is, at cellular abnormalities that specifically affect karyokinesis (nuclear division) and/or cytokinesis (cytoplasmic division). This cellular abnormality may be inhibitory in nature, for example, the presence of a factor that inhibits karyckinesis or cytokinesis. Alternatively, the cellular abnormaliy may be due to the absence of a specific stimulus or factor. Thus, events distal to DNA synthesis are equally important in resolving the mechanism of polyploidy cell development.

1.7 Study limitations

The experiments conducted in this study focused on the use of the cell culture technique. Findings from this *in vitro* approach may not necessarily parallel similar findings in the *in vivo* state. For example, the findings on β-adrenoceptor density and polyploidy alterations in cultured smooth muscle cells may not reflect the same magnitude of change in the *in vivo* state, that is, at the tissue level.

Alterations in polyploid smooth muscle cells were evaluated using cells cultured from an elastic artery (aorta). However, the resistance vessels play a primary role in the development of hypertension. Thus, there may be limited application of any findings associating aortic polyploid smooth muscle cells with hypertension development.

Further, this study focused on one mechanism that may be involved in DNA synthesis, namely the β-adrenoceptor-G_s-cAMP intracellular pathway. However, DNA synthesis and cell growth *in vivo* involves complex interactions among several growth factors and their respective intracellular pathways. Thus, our *in vitro* studies are not influenced by the potential stimulatory and/or inhibitory effects of other intracellular pathways.

Additionally, in investigating the mechanism of polyploid smooth muscle cell development, this study did not focus on the factors controlling mitosis. This is important because abnormal mitosis where cytokinesis

and/or karyokinesis do not occurr are crucial steps in the development of polyploid cells. Thus, caution should be excercised in interpretating the β-adrenoceptor mediated increases in polyploid smooth muscle cells.

Furthermore, this study did not investigate the functional consequences of *in vivo* polyploid smooth muscle cells. This is important in order to validate the results and interpretations of the *in vitro* findings and will serve as the basis for future studies.

1.8 The significance/implications of smooth muscle cell polyploidy development in hypertension

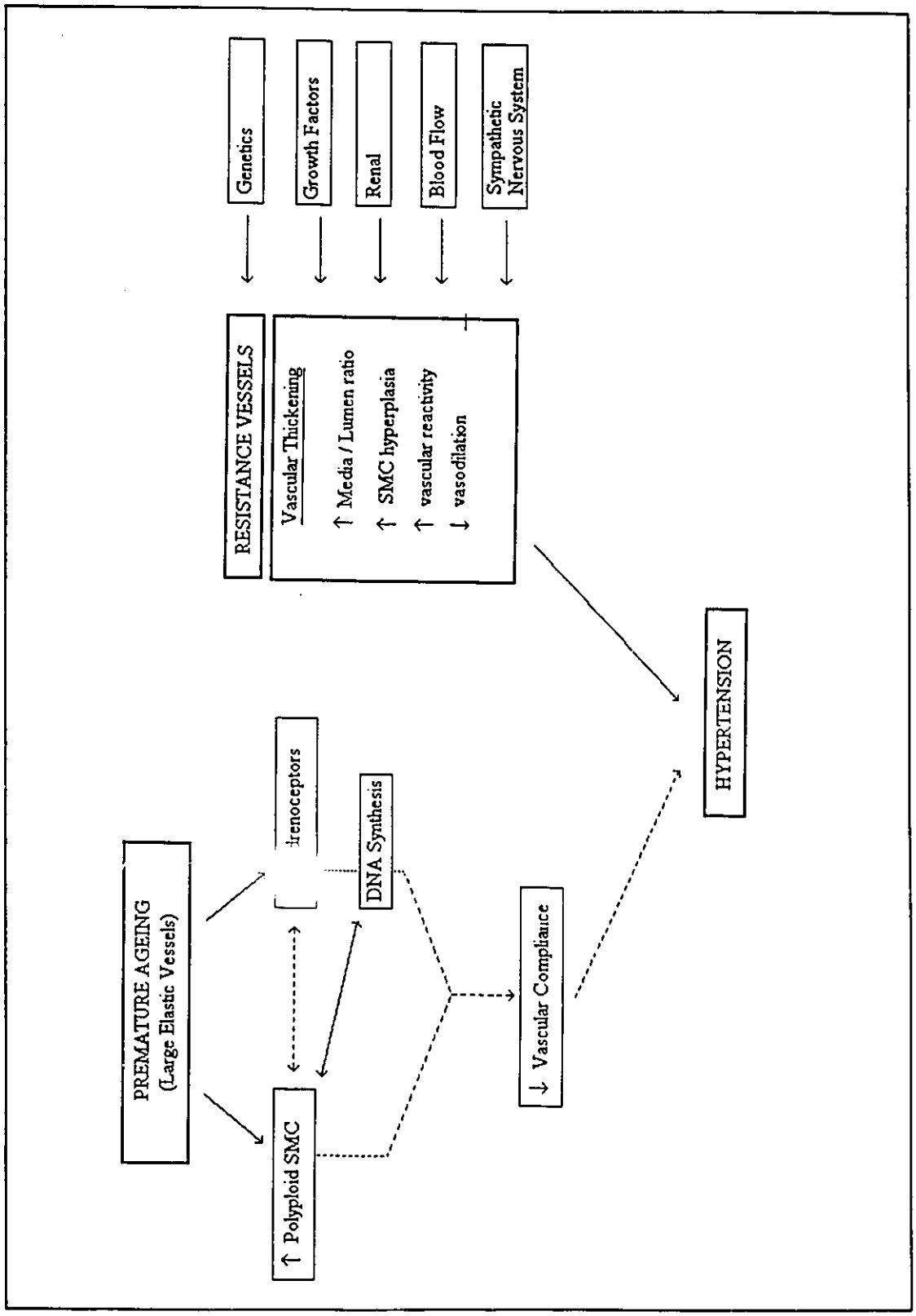
It is possible to speculate on the relative contribution of polyploid smooth muscle cells to the development of hypertension. In our studies, polyploid cells were evaluated in smooth muscle cells from the aorta, a large elastic artery. Structural alterations in these arteries, however, are secondary adaptative changes to blood pressure elevation (Lee et al., 1985; Kwan et al., 1987; Lee, 1987). Resistance vessels (such as the small mesenteric arteries and arterioles), on the other hand, are involved in the development and maintenance of hypertension. These resistance arteries, however, show no significant accumulation of polyploid smooth muscle cells with age and/or duration of hypertension (Black et al., 1988b). Thus, since it is the resistance vessels and not the elastic arteries which play a crucial

role in the development of hypertension, it can be concluded that there is little evidence that premature ageing, as defined by the presence of polyploid smooth muscle cells, play any role in the development of hypertension (Figure 2).

Figure 2: Potential mechanisms and factors leading to the development of hypertension (Modified from Lee, 1993).

Dottled lines represent speculative mechanisms.

Solid lines represent well established mechanisms.


PREMATURE AGEING
(Large Elastic Vessels)
↑ Polyploid SMC
Irenoceptors
DNA Synthesis
↓ Vascular Compliance
RESISTANCE VESSELS
Vascular Thickening
↑ Media / Lumen ratio
↑ SMC hyperplasia
↑ vascular reactivity
↓ vasodilation
Genetics
Growth Factors
Renal
Blood Flow
Sympathetic Nervous System
HYPERTENSION

The significant elevation of polyploid smooth muscle cells in the aorta of hypertensive animals with age, however, leads to the speculation that there may indeed be a functional consequence of vessels with polyploid cells. Ageing vessels, which are structurally distinguished by the presence of polyploid smooth muscle cells, are functionally characterized by decreased compliance (Avolio, 1992; Cooper et al., 1994). It is conceivable that the polyploid smooth muscle cells present in these arteries may play a role in this increased stiffness of vessels (Figure 2). Results from studies proposed (see Future Studies) should shed some light on the functional consequences of polypolid smooth muscle cells.

This study showed vascular β-adrenoceptor signalling pathway may potentially play a role in the development of polyploid smooth muscle cells and that blood pressure is not a necessary factor for this process. The functional consequences, however, of the increase in polyploid smooth muscle cells in hypertension and ageing are yet to be investigated.

2. Future Directions

Future studies should aim to resolve clearly the general hypotheses that the β-adrenoceptor-activated intracellular messengers have an important role in the regulation of the development of polyploid smooth muscle cells in hypertensive animals, and that polyploid smooth muscle cells alter the functional responses of the vasculature in hypertension. These studies should be focused on both *in vitro* and *in vivo* models.

2.1 *In vitro* studies

To investigate the roles of cAMP-activation in the development of polyploidy, smooth muscle cells from SHR and WKY should be cultured in the presence of a PKA activator (such as adenosine 3',5'-cyclic monophosphothioate, Sp-isomer (Sp-cAMPS)) and/or a PKA inhibitor (such as adenosine 3',5'-monophosphothioate, Rp-isomer (Rp-cAMPS)) with subsequent evaluation on their effects on developmnet of polyploid cells. If PKA plays a role in the development of polyploid smooth muscle cells in SHR and WKY, then activation of PKA would increase the number of polyploid smooth muscle cells while PKA inhibition would decrease the development of these cells. Further, comparison between young and old SHR and WKY would reveal any differences between ages or different strains.

To investigate the roles of cAMP-activated CREB in the development of polyploid cells, smooth muscle cells from SHR and WKY should be cultured in the presence of activators of the β-adrenoceptor pathway (such as isoproterenol, forskolin, Sp-cAMPS) and the level of phosphorylated CREB and polyploid smooth muscle cells determined. If phosphorylated CREB is a mediator of polyploid cell development then stimulatory conditions would result in elevated phosphorylated CREB and polyploid smooth muscle cells.

The use of the cell culture approach also allows investigation of the roles of other intracellular mediators such as PKC and Ca^{2+} in the development of smooth muscle polyploidy. Similarly to the above studies, agents that elevate intracellular Ca^{2+} and/or activate PKC could be used and their effects on polyploid development evaluated.

2.2 *In vivo* studies

An important aspect is the functional implications of polyploid smooth muscle cells *in vivo*. It has been shown that when hypertensive animals (SHR, a genetic model; DOCA/NaCl, an experimental model) are chronically treated with a β-adrenoceptor antagonist (nadolol or propranolol), the development of polyploid smooth muscles is inhibited (Lee et al., 1992b; Agnati et al., 1983). With these findings in mind, both SHR

and WKY rats should be chronically treated with nadolol with eventual evaluation of the functional properties of the arteries (contraction and relaxation). As previously discussed (Introduction 1.3), ageing and hypertensive vessels exhibit a decrease in the relaxation response. If polyploid smooth muscles play any role in this altered relaxation response then this would be confirmed by a comparison of the functional responses of arteries from animals with increased or decreased polyploid smooth mucle cell content.

2.3 Aim of *in vitro* and *in vivo* studies

The studies proposed above, provide a direction for future investigations of the role of polyploid smooth muscle cells in hypertension and the mechanisms underlying these events. The combination of *in vitro* and *in vivo* studies is important because *in vitro* studies will help to clarify and to relate the intracellular mechanisms in smooth muscle cell polyploidy development whereas *in vivo* studies will help with the correlation of the functional consequences of the presence of increased polyploid smooth muscle cells in the arteries of WKY and SHR rats.

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