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RELATIONSHIP BETWEEN IMMUNE DYSFUNCTION AND HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS

BY

© JIN XIAO

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

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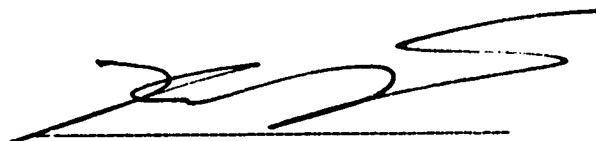

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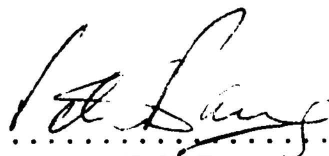

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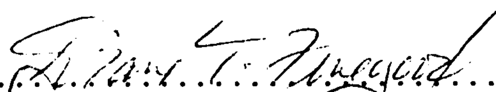

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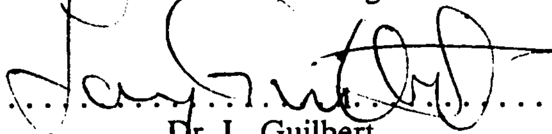

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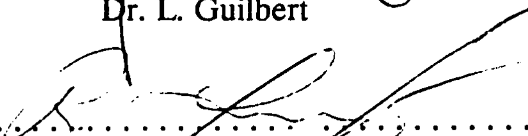

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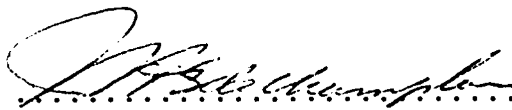

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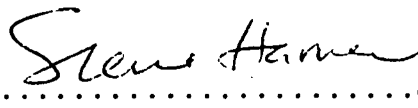

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To

My Parents

Fukun Xiao and Junping Li

and

My Husband

Jianshi Huang

Without Whose Love

This Thesis Would Not Have Been Written

blood pressure.

SHR VSMC significantly inhibited the proliferation response of SHR and WKY lymphocytes. SHR VSMC also produced significantly higher amounts of NO. The increase in NO synthesis in VSMC was significantly correlated with the rise in blood pressure in SHR. VSMC of rats with salt-induced hypertension also exhibited elevated NO production. These findings suggested that high blood pressure may influence the expression of inducible NO synthase in VSMC in an attempt to compensate for elevated blood pressure.

Elevated NO synthesis in SHR macrophages and VSMC suggested that a general activation of the inducible NO synthesis system may exist in SHR. The lymphocyte depression was the result of this activated NO synthesis, especially NO synthesis by macrophages. This abnormality in immune system, therefore, is not causally associated with hypertension or *vice versa* in SHR.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BNP	brain natriuretic peptide
BHR	borderline hypertensive rats
BSA	bovine serum albumin
Ca	calcium
CC	combine cytokines
CD2	cluster differentiation antigen 2
CD5	cluster differentiation antigen 5
cGMP	cyclic guanosine monophosphate
cGRP	calcitonin gene-related peptide
CNS	central nervous system
CO	cardiac output
Con A	Concanavalin A
CPM	count per minute
DMEM	Dulbecco's modified Eagle medium
D-NMMA	^{NG}-monomethyl-D-arginine
DOCA	deoxycorticosterone acetate
EDCF	endothelium-derived contracting factor
EDHF	endothelium-derived hyperpolarizing factor

EDRF	endothelium-derived relaxing factor
FCS	fetal calf serum
HBSS	Hank's balanced salt solution
IFNr	interferon gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-1β	interleukin-1 beta
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
L-NMMA	^{NG}-monomethyl-L-arginine
LPS	lipopolysaccharide
MAP	mean arterial blood pressure
NaCl	sodium chloride
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate-buffered saline
PKA	protein kinase A
PKC	protein kinase C
PDB	phorbol 12, 13 debutyrate
PG	prostaglandin
PHA	phytohemagglutinin
PHF	parathyroid hypertensive factor

SHR	spontaneously hypertensive rats
TCR	T cell receptor
TGF β	transforming growth factor beta
TNF	tumour necrosis factor
TPR	total peripheral resistance
TXA_2	thromboxane A_2
VIP	vasoactive intestinal peptide
VSMC	vascular smooth muscle cells
WKY	Wistar Kyoto rats

CHAPTER I. INTRODUCTION

I. INTRODUCTION

Essential hypertension is a disease that afflicts 20-25% of North Americans (Rowland and Robert, 1982). While many factors have been postulated to contribute to hypertension, the pathogenesis of this complex disorder is not fully understood. During the last fifteen years, a large body of evidence has come to suggest that altered immunological activity may contribute to both the initiation and the maintenance of the hypertensive state in human hypertension as well as in experimental animal models (Dzielak, 1992). It has also been documented that spontaneously hypertensive rats (SHR), a widely used experimental animal model of essential hypertension, exhibit abnormal immune responses (Takeichi *et al.*, 1988).

The substantial number of studies concerning the interaction between the immune and vascular systems point to two possibilities in this relationship. One is that hypertension may be intimately related to immune dysfunction. This possibility is supported by the evidence that more advanced lymphocyte abnormalities could be correlated with increasing age and severity of hypertension (Takeichi *et al.*, 1980; Pascual *et al.*, 1992). Thymic transplants and other immunological manipulations alleviated immune abnormalities and lowered blood pressure in SHR (Ba *et al.*, 1982; Strausser, 1983; Khraibi *et al.*, 1984; Norman *et al.*, 1985). Interleukin-2 (IL-2) administration has been shown to prevent the development of hypertension in young

SHR and reduce blood pressure in adult SHR with established hypertension (Tuttle and Boppana, 1990). It has also been shown that both food restriction and/or physical exercise reduced blood pressure and restored lymphocyte function in SHR (Fernandes *et al.*, 1986). The second possibility is that hypertension and immune dysfunction may not be related, but may, instead, be co-occurring defects. This hypothesis is supported by observations showing that immune abnormalities were present in prehypertensive rats, and did not change as the hypertensive state progressed (Fannon *et al.*, 1992). Hilme and co-workers did not observe a relationship between the severity of hypertension and immune abnormalities (Hilme *et al.*, 1993). Correction of the lymphocyte defect by IL-2 administration in SHR did not result in a reduction in blood pressure (Ofosu-Appiah *et al.*, 1993). Despite a decade of effort, the precise relationship between hypertension and immune abnormalities remains unclear.

Therefore, the overall objective of this thesis is to investigate the relationship between immune abnormalities and hypertension. Immune function in SHR and Wistar Kyoto (WKY) rats, their genetic normotensive control, will first be defined and compared. Because lymphocytes are the major effector cells in the immune system and also because of the complexity of the immune system, only the lymphocyte proliferation response and its relevant aspects will be studied and discussed. The role of nitric oxide (NO) in the mechanism of the lymphocyte proliferation defect in SHR will also be elucidated. These studies constitute chapters III and IV of this thesis.

Hypertension is characterized by an increase in the peripheral vascular resistance in the presence of normal cardiac output. The increased resistance may be caused by either excessive vascular smooth muscle contraction mediated by an altered regulatory system or by morphological and functional changes in the vascular wall. It has been suggested that vascular smooth muscle cells (VSMC) can influence the immune response by antigen presentation and cytokine production (Warner and Libby, 1989; Fabry *et al.*, 1990a; Loppnow and Libby, 1990; Ikeda *et al.*, 1993). The immune system can also influence VSMC and the vascular wall, especially in pathological conditions, by producing antibodies, cytokines and subsequent immune responses (Beasley *et al.*, 1989; Chen and Schachter, 1993; Hilme *et al.*, 1993). Therefore, it is of great interest to see if in hypertension there is any interaction between lymphocytes and VSMC, the major effector cells in the immune system and the vascular system, respectively. No direct evidence is available concerning this relationship. In chapter V of this thesis, the influence of VSMC on lymphocytes in the hypertensive state will be presented. The mechanism responsible for the effect of VSMC will also be discussed.

The final question that the present study addresses concerns the relationship between immune dysfunction and hypertension in SHR. The last two chapters will present the interrelationship of immune dysfunction and hypertension, and the involvement of NO in this relationship.

The main focus of this thesis is on immune abnormalities in relation to hypertension in SHR and not a study on the immune system in SHR *per se.*

Therefore, an extensive literature review of the immune system will not be included. The following section reviews relevant fields which this study attempts to address.

II. BLOOD PRESSURE CONTROL AND HYPERTENSION

A. GENERAL MECHANISMS

The overall goal of the blood pressure control system is to provide a steady pressure to ensure proper blood flow through each organ. There are two major parameters of blood pressure control: total peripheral resistance and cardiac output (MAP = CO x TPR). There is no single simple arterial pressure regulatory mechanism. Instead, arterial blood pressure is regulated by a fine control complex. It consists of a rapid feedback control which is vested almost entirely in the nervous control of circulation acting through reflexes and direct signals from the central nervous system. This short-term regulation depends on changes in the strength of contraction of the heart, the capacity of the blood vessels and the total peripheral resistance. The pressure controls that act with intermediate rapidity include capillary fluid shift between the circulation and the interstitial fluids and hormonal control involving angiotensin, vasopressin and other substances. The long-term pressure control system is primarily vested in the structural changes in the cardiovascular tissues and in the kidney and its related systems acting through the control of blood volume and extracellular fluid volume. Through these well designed mechanisms, the

cardiovascular system maintains a constant pressure that is high enough so that any tissue can receive an appropriate blood flow depending on circumstances.

Hypertension is a manifestation of disease processes which are the result of a derangement of the blood pressure control system. Many factors have been postulated to contribute to the hypertensive state, including alteration in renal function, enhanced vascular smooth muscle reactivity, increased sympathetic tone, alteration in the renin-angiotensin system, alteration in the endocrine system, diet and genetic factors. Despite continuing intense effort, the pathogenesis of essential hypertension is still poorly understood. The overall complexity of mechanisms is well illustrated by "Page' s mosaic concept of hypertension", which elucidated that hypertension was the result of these interrelated mechanisms (Page, 1987).

B. CONTROL AND CHANGES OF VASCULAR RESISTANCE IN HYPERTENSION

Arteries with diameters less than 300 μM play a key role in the determination of peripheral resistance and, thereby, blood pressure. Vascular resistance is under the control of multiple vasodilators and/or constrictors acting on VSMC. The hallmark of hypertension is an increase in peripheral vascular resistance. This increase is considered to be related to an increase in tone of the resistance arteries as well as sometimes to structural changes in these blood vessels.

1. Neural Mechanisms

It has been well documented that neuromechanisms are involved in the pathogenesis of hypertension (de Champlain, 1990; Dickinson, 1991a). Since a complete review of this subject is beyond the scope of this thesis, only neurotransmitters will be discussed. Norepinephrine is released by the adrenergic vasoconstrictor fibers of the sympathetic nervous system at the site of the blood vessels. It acts on α-adrenergic receptors eliciting vasoconstriction. Neural regulation of vascular resistance is accomplished primarily by alteration of the number of impulses passing down through these nerve fibers to the blood vessels. Acetylcholine is released by sympathetic cholinergic fibers innervating the resistance vessels of skeletal muscle and skin. It acts on muscarinic receptors causing vasodilation. Active sympathetic vasodilation can be observed in the resistance vessels that have β-adrenergic receptors. A small proportion of the resistance vessels receive parasympathetic fibers. Stimulation of these fibers induces vasodilation. However, the effect of these cholinergic fibers on total vascular resistance is small.

A large number of studies have reported that essential hypertension is frequently characterized by sympathetic activation and that this is more evident in the early phases of hypertension development (Floras and Hara, 1993; Mancia *et al.*, 1993). There are also reports demonstrating that the release or metabolism of certain neurotransmitters is altered in hypertension (Michel *et al.*, 1990). Increased norepinephrine has been reported in human essential hypertension (de Champlain *et al.*, 1991; Ferrier *et al.*, 1993). SHR exhibit increased sympathetic neural input to

a number of organs, including the vasculature, spleen and thymus (Donohue *et al.*, 1988; Burnstock, 1990; Gattone *et al.*, 1990; Purcell and Gatton, 1992). SHR show an increase in directly recorded sympathetic nerve activity (Thoren and Ricksten, 1979; Schramm and Choronboy, 1982) as well as augmented release of norepinephrine (Tsuda *et al.*, 1987; Westfall *et al.*, 1987). Recently, Pacak and co-workers observed that norepinephrine release and catecholamine synthesis in the posterolateral hypothalamus of SHR were elevated (Pacak *et al.*, 1993). They also observed that the α_2-adrenergic receptor inhibition of both norepinephrine release and catecholamine synthesis was augmented in juvenile SHR (Pacak *et al.*, 1993). Alterations in adrenergic receptors, such as α_1 and β receptors, have been reported in SHR (Castellano *et al.*, 1993). Renal denervation (Norman *et al.*, 1985) and systemic sympathectomy (Yamori *et al.*, 1985) prevented the onset of hypertension. Another regulatory function of adrenergic nerves is probably a long-term trophic effect. The evidence for this hypothesis comes mainly from experiments in which VSMC proliferation and wall-to-lumen ratio are attenuated and decreased, respectively, by ganglionectomy (Bevan, 1984) and by neonatal central and peripheral catecholaminergic lesions (Slotkin *et al.*, 1988). It has been suggested that due to the trophic effect, sympathetic hyperinnervation may protect smooth muscle cells from necrosis caused by the greater tangential wall stress associated with chronic hypertension (Tenkova *et al.*, 1993). However, the established blood pressure elevation is supported mainly by other means, many of which involve structural changes, especially in the systemic arterioles, kidney and heart.

Many autonomic vascular nerves release other transmitters, such as ATP, vasoactive intestinal peptide (VIP), substance P, calcitonin gene-related peptide (cGRP), serotonin, dopamine, neuropeptide-Y, somatostatin, etc. It has been suggested that many of these transmitters are co-stored and, presumably, co-released (Dickinson, 1991a). The amount of overspill of most of these neurotransmitters from nerve ending is small and difficult to measure. The involvement of these transmitters in hypertension is, therefore, not clear.

2. Humoral Factors

Genetic and environmental factors participate in the regulation of blood pressure and in the etiology of hypertension via intermediary phenotypes which control cardiac output and total peripheral resistance and, thus, blood pressure. Vasoactive humoral factors are important components of these intermediary phenotypes and blood pressure is the result of a balance between vasoconstrictors and vasodilators. Alteration of this equilibrium may result in hypertension. Humoral factors may affect vascular resistance by either altering vascular smooth muscle reactivity or by influencing cell growth.

a. Renin-angiotensin system

Over 30 years ago the renin-angiotensin system was revealed as a hormonal axis functioning as a major regulator of blood pressure and electrolyte homeostasis (Laragh, 1960). The enzyme renin, normally secreted by juxtaglomerular cells in response to the local perception of reduced perfusion, sets off a blood borne chain

reaction that yields angiotensin II which by its potent vasoconstrictor properties and its release of aldosterone, elevates blood pressure to the point where the local reduced renal perfusion is eliminated and the secretion of renin stops (Laragh *et al.*, 1972; Lynch and Peach, 1991). Accumulated evidence suggests the existence of a local renin-angiotensin system in various tissues, including those involved in cardiovascular regulation, such as heart, vascular wall, kidney, adrenal gland and brain (Gould *et al.*, 1964; Dzav, 1988; Mulrow, 1989; Soubrier *et al.*, 1993). The efficiency of angiotensin converting enzyme inhibitors in reducing blood pressure in SHR is thought to be due to inhibition of angiotensin II formation (Clough *et al.*, 1982) and inhibition of bradykinin degradation (Gohlke *et al.*, 1994). In essential hypertension, plasma renin concentrations are variable. On the average, the values are not notably different but the spread of values is wider (Brunner *et al.*, 1972). As a group, hypertensive patients appear to exhibit an impaired ability to turn off their renin secretion (Laragh and Brenner, 1990). Recent studies have demonstrated that plasma renin activity was increased in hypertensive subjects (Licata *et al.*, 1994) and the SHR VSMC contained a higher number of angiotensin binding sites (Jaiswal *et al.*, 1993). It has been shown that SHR have a genetically determined enhanced responsiveness to angiotensin II mediated by the AT1 receptor (Kost and Jackson, 1993). It has been reported that intracellular free calcium concentration is increased and intracellular free magnesium concentration is decreased in VSMC of hypertension and that the angiotensin II stimulated calcium response may be related to the simultaneously decreased intracellular magnesium

concentration (Tonyz and Schiffrin, 1993). Angiotensin II has also been demonstrated to increase VSMC growth rate and cell size (Campbell-Boswell and Robertson, 1981; Black *et al.*, 1993). It has been suggested that angiotensin II may act as a growth factor to promote structural changes in the vasculature in hypertension (Lever *et al.*, 1992; Oddie *et al.*, 1993). In addition, linkage analysis and transgenic studies suggest that abnormally elevated expression of the angiotensinogen gene may contribute to the development of hypertension (Jeunemaitre *et al.*, 1992; Kimura *et al.*, 1992).

b. Parathyroid hypertensive factor (PHF)

PHF, the most recent arrival in the family of circulating pressor factors, was described by Lewanczuk and Pang in 1989. PHF is isolated from plasma of SHR and of patients with essential hypertension (Benishin *et al.*, 1991). PHF appears to originate primarily in the parathyroid gland (Pang and Lewanczuk, 1989). This secretion was inhibited by an increase in dietary calcium (Lewanczuk *et al.*, 1990) It is believed that PHF directly influences the calcium balance in VSMC thereby increasing vascular resistance (Shan *et al.*, 1994). Since PHF has also been linked to a characteristic pattern of abnormalities in overall calcium regulation, it may, therefore, serve as a marker indicating the effectiveness of calcium channel blockade. In human hypertensive patients, the presence of PHF has been shown to predict a favourable therapeutic response to calcium channel blockade (Pang *et al.*, 1994). PHF has been strongly implicated as a causative factor in low-renin and salt-sensitive forms of rat and human hypertension (Pang *et al.*, 1994).

c. Vasopressin

Vasopressin is a powerful arterial vasoconstrictor at concentrations higher than those which reduce water excretion (Verney, 1947). In recent years, there has been considerable interest in the possible role of vasopressin in the pathogenesis of hypertension. Numerous studies have attempted to demonstrate the contribution of vasopressin to several forms of hypertension, particularly in deoxycorticosterone acetate (DOCA)-salt hypertensive rats and in SHR. Although it is likely that vasopressin is essential for the production of DOCA-salt hypertension (Zicha *et al.*, 1989), the contribution of vasopressin to hypertension in SHR is still a subject of controversy. Plasma vasopressin concentration, posterior pituitary vasopressin content, and urinary vasopressin excretion were found to be elevated in SHR (Crofton *et al.*, 1978; Morris, 1982). However, it has also been reported that the endogenous level of renal activity of vasopressin was suppressed in SHR (Li and Bukoski, 1993). Enhanced pressor responsiveness to AVP was observed in SHR (Ashida *et al.*, 1983). It has been reported that the AVP-induced increase in intracellular sodium concentration is augmented in SHR VSMC (Okada *et al.*, 1993). Treatment with a vasopressin antagonist significantly attenuated the development of hypertension in SHR (Sladek *et al.*, 1988; Yamada *et al.*, 1994) and in Dahl salt-sensitive hypertension (Crofton *et al.*, 1993) suggesting that vasopressin may play a role through V_1 receptors (vascular receptors) in the pathogenesis of hypertension.

d. Ouabain-like factor

Several laboratories have presented evidence that plasma from experimental

animals or patients with hypertension contains a factor that inhibits the electrogenic sodium-potassium pump (Poston, 1987; Kramer *et al.*, 1991; Ferrandi *et al.*, 1993). It was suggested that this inhibitory factor increases vascular resistance by turning off the electrogenic pump and causing membrane depolarization thereby opening voltage operated calcium channels, and by permitting sodium to accumulate intracellularly thereby causing a decrease in calcium extrusion (Bohr *et al.*, 1991; Meyer-Lehnert *et al.*, 1993). Recently, it has been shown that ouabain enhances the mitogenic effect of serum in VSMC (Golomb *et al.*, 1994) and induces the transcription of proto-oncogenes in different cell types (Nakagawa *et al.*, 1992). In addition, long-term ouabain administration has been shown to produce hypertension in rats (Yuan *et al.*, 1993).

e. Insulin

A growing number of studies in the last few years suggest an association between hyperinsulinemia or insulin resistance and hypertension (Reaven, 1990; Dengel *et al.*, 1994). It has been proposed that insulin plays a role in the pathogenesis of hypertension (Modan *et al.*, 1985), possibly by stimulating the sympathetic nervous system (Modan and Halkin, 1991), promoting kidney sodium retention (Defronzo *et al.*, 1976; Gupta *et al.*, 1992), and by affecting Na/K-ATPase, pH, calcium and other potential cellular functions (Ferrari and Weidman, 1990). Recently, it has been shown that a reduction in insulin sensitivity precedes the development of hypertension and may also be coupled to low physical fitness (Endre *et al.*, 1994). Blockade of insulin secretion with octreotide prevents fructose-induced

hypertension (Reaven *et al.*, 1989). VSMC have been shown to possess receptors for both insulin and insulin-like growth factor-1 (Pfeifle and Ditschuneit, 1983) implying that VSMC is an important insulin-sensitive tissue (Standley *et al.*, 1994). Insulin has been demonstrated to stimulate vascular smooth muscle cell growth (Pfeifle *et al.*, 1980; Banskota *et al.*, 1989). It has been suggested recently that insulin sensitivity was more closely related to blood pressure, serum triglycerides and HDL cholesterol than to hyperinsulinemia. Thus, insulin resistance may be more important than hyperinsulinemia as a determinant of the risk factors for cardiovascular diseases including hypertension (Lind *et al.*, 1993).

On the other hand, it has also been reported that insulin concentration was not positively related to hypertension and blood pressure in some races such as Asian Indian or Chinese (Tappy *et al.*, 1991; Dowse *et al.*, 1993). Adipocytes from young normotensive SHR showed resistance to insulin-stimulated glucose uptake. Chronic infusion of insulin resulted in lower blood pressure and total peripheral resistance in the dog (Brands *et al.*, 1991) and no change in blood pressure in rats (Bursztyn *et al.*, 1993). Renovascular hypertension was not associated with insulin resistance (Reaven and Chang, 1992). There was no significant difference in insulin stimulated calcium influx in VSMC of SHR or WKY (Zhu *et al.*, 1993). There is a lack of hypertension in patients with polycystic ovary syndrome despite profound insulin resistance (Zimmermann *et al.*, 1992). Clearly, much more investigation is needed to assess the possible role of hyperinsulinemia and insulin resistance in hypertension.

f. Serotonin

Serotonin (5HT) can cause either vasoconstriction or vasodilation depending upon the circumstances. The $5HT_2$ receptor mediates the vasoconstricting actions of serotonin. This receptor is also responsible for the amplifying effect of serotonin on other vasoconstrictor agents such as angiotensin II, norepinephrine and endothelin (Van-Hueten *et al.*, 1982; Yang, 1992). In addition, serotonin also promotes platelet aggregation. It has been suggested that these mechanisms might augment or cause hypertension (Vanhoutte, 1987). In hypertension, the vasoconstrictor effects of locally released serotonin are increased (Vanhoutte, 1987). Pressor responses to the serotonin receptor agonist α-Me-5HT were significantly greater in SHR than in WKY suggesting the possibility of $5HT_2$-receptor hypersensitivity in SHR (Balasubramaniam *et al.*, 1994). Furthermore, the serotonin antagonist ketanserin lowered blood pressure in hypertensive patients (Vanhoutte *et al.*, 1988) and in SHR (Balasubramaniam *et al.*, 1993). This antihypertensive action of ketanserin has been suggested to be due to a synergistic effect of combined peripheral $5HT_2$ and α_1-receptor blockade.

g. Kallikrein-kinin system

The kallikrein-kinin system is an important component of the vasodepressor side of the vasoconstrictor and vasodilator systems. Thus, a deficiency in the kallikrein-kinin system may result in hypertension, especially where the vasoconstrictor system is over expressed. Kallikreins are serine proteases which are divided into two groups: tissue or glandular kallikrein and plasma kallikrein (Bhoola

et al., 1992). The common link is that both plasma kallikrein and tissue kallikrein release kinins from kininogens. Plasma kallikrein is synthesized in the liver, circulates in the plasma as enzymogen and releases bradykinin from light molecular weight kininogen (Seidah *et al.*, 1990). Interacting with the Hageman factor, this kininogen participates in the intrinsic blood clotting and fibrinolysis cascade, local vascular resistance and inflammation (Kaplan and Silverberg, 1987). The glandular or tissue kallikrein gene is expressed in tissue such as the salivary glands and pancreas and in various tissues involved in the regulation of cardiovascular function, such as the kidney, arteries, veins, heart, brain and adrenal glands. This enzyme has strong kininogenase activity and releases bradykinin (Carretero *et al.*, 1993).

Kinins act mainly as local hormones (autocrine and paracrine) via two different types of receptors: B1 and B2 (Regoli *et al.*, 1989). B1 receptors appear to primarily mediate the response in inflammation. Most of the known cardiovascular effects of kinins are mediated by B2 receptors. NO and eicosanoids may mediate some of the effects of kinins (Carretero *et al.*, 1993). There are numerous indications that the kallikrein-kinin system is under-expressed in various forms of hypertension. In SHR, urinary kallikrein excretion was decreased (Carretero *et al.*, 1978). Hypertension itself may alter the expression of the kallikrein-kinin system. For example, in renovascular hypertension arterial kallikrein and urinary kallikrein excretion was decreased (Margolius *et al.*, 1972). It has been reported that a restriction of fragment length polymorphism (RFLP) of the kallikrein gene in SHR is linked to high blood pressure (Pravence *et al.*, 1991). RFLP in the kininase II or

angiotensin converting enzyme (ACE) gene is also linked to the development of hypertension (Hilbert *et al.*, 1991). Over-expression of ACE may result in a decrease in vasodepressor kinins and an increase in the vasopressor angiotensin II (Carretero *et al.*, 1993). There is significant evidence that genetic alterations of the various components of the kallikrein-kinin system may be important in the pathogenesis of hypertension. Recently, a role for the brain kallikrein system in the central regulation of blood pressure and also in alterations in blood pressure in SHR has been reported (Maddu *et al.*, 1990; Khan *et al.*, 1993).

h. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)

ANP is a naturally occurring hypotensive agent which probably antagonizes end organ responses to vasoconstrictors such as angiotensin II (Laragh, 1986). It has been shown that a longer period of ANP infusion was required for significant effects on blood pressure to be observed in hypertensive subjects (Cusson *et al.*, 1990). Deficiency of ANP might thus theoretically raise blood pressure. However, plasma ANP levels are either increased or normal in essential hypertension (Sagnella *et al.*, 1986; Schiffrin, 1989). It is most unlikely that ANP deficiency plays a part in raising blood pressure.

BNP is a recently identified member of the atrial peptide family. Initially isolated from porcine brain (Sudih *et al.*, 1988), it was later demonstrated to be present in other species including the rat (Kambayashi *et al.*, 1989) and human (Mukoyama *et al.*, 1991). BNP appears to be constitutively released from cardiac

ventricular tissue (Mukoyama *et al.*, 1991). Plasma concentrations of BNP are increased in heart failure and in acute myocardial infarction (Mukoyama *et al.*, 1991; Morita *et al.*, 1993). In essential hypertension, pathophysiological plasma concentrations of BNP had significant acute effects in promoting natriuresis and suppressing plasma aldosterone (Richards *et al.*, 1993). These effects are similar to the action of ANP. BNP and ANP may play separate but complementary roles in fluid volume and blood pressure control (Richards *et al.*, 1993).

i. Medullipin II

Medullipin II was initially described by Muirhead and co-workers (Muirhead, 1990). Renomedullary interstitial cells secrete medullipin I, which is conveyed to the liver where it is converted to medullipin II. This factor is a vasodilator that suppresses sympathetic tone, causes natriuresis and affects the central nervous system (Muirhead, 1993). It has been suggested that medullipin has actions that counter the major actions of the renin-angiotensin system (Muirhead, 1993). A deficiency of medullipin is considered to contribute to the pathogenesis of various hypertensive states. Medullipin lowered the blood pressure of SHR (Muirhead *et al.*, 1991), raising the possibility of using medullipin as a therapeutic agent in human hypertension.

3. Endothelium

The endothelium, an important tissue in the regulation of both contraction and growth of vascular smooth muscle, produces and releases relaxing and

contracting factors (Furchgott and Vanhoutte, 1989). It also synthesizes factors that keep the surface nonadhesive and nonthrombogenic for circulating blood cells as well as inhibiting proliferation of vascular smooth muscle cells (Vane, 1990). In hypertension, it has been proposed that the endothelium is abnormal in all of these aspects (Vanhoutte, 1989). The most important factor, endothelium-derived relaxing factor (EDRF or nitric oxide) will be reviewed in a separate section.

a. Endothelin

Endothelin is a linear 21-amino acid peptide (Yanagisawa *et al.*, 1988). There are three structurally and pharmacologically separate endothelin isopeptides, named endothelin-1,-2,and -3 (Yanagisawa *et al.*, 1989). Activation of endothelin-1 receptors on the cell membrane is coupled to phospholipase C, increase of inositol phosphates and diacylglycerol and elevation of intracellular free calcium levels (Resink *et al.*, 1988). The most striking property of endothelin-1 is its long-lasting vasoconstrictive and hypertensive action (Vane, 1990). It has been reported that the plasma endothelin level was increased in essential hypertension (Naruse *et al.*, 1991; Fernandez-Cruz *et al.*, 1993). The plasma levels of endothelin and the reactivity to endothelin are altered in SHR (Miyamori *et al.*, 1991) and in diabetic patients with hypertension (Haak *et al.*, 1992). However, contradictory results have also been reported (Suzuki *et al.*, 1990). Most forms of vascular diseases as well as congestive heart failure and renal insufficiency are associated with increased circulating levels of endothelin. The discrepancies in plasma endothelin concentration in hypertension may be related to the presence or absence of these conditions. Endothelin may be

a useful marker of end-organ damage, but its pathogenic role in hypertension remains unclear at the present time (Luscher *et al.*, 1993; Neild *et al.*, 1994).

b. Cyclooxygenase products

Other vasoconstrictors released from endothelial cells are cyclooxygenase products of arachidonic acid metabolism including thromboxane A_2, prostaglandin H_2, superoxide anions and unidentified factors. Abnormal prostaglandin synthesis has been implicated in the pathophysiology of hypertension (Dunn and Grone, 1985).

i. Prostacyclin (prostaglandin I_2 [PGI_2])

PGI_2 is a potent vasodilator, stimulates natriuresis, and is a potent inhibitor of platelet aggregation (Dusting, 1982). PGI_2 activates the adenylate cyclase pathway leading to stimulation of protein kinase A (PKA). PKA phosphorylates proteins from the motile apparatus and cellular structure elements, and phosphorylates proteins in signal transduction pathways resulting in inhibition of phospholipase C and activation of phosphodiesterase. PKA also phosphorylates the proteins that regulate gene transcription (Thierauch *et al.*, 1994). Vascular tissue has a large capacity to generate PGI_2. Moreover, PGI_2 attenuates vasoconstrictor responses to vasoactive stimuli such as angiotensin II (Moncada and Vane, 1979). In turn, angiotensin II augments the release of PGI_2 from a variety of organs (Jaiswal *et al.*, 1993). Recently, it has been reported that the basal and stimulated levels of PGI_2 were markedly reduced in SHR (Jaiswal *et al.*, 1993). The ability of PGI_2 to attenuate angiotensin II-induced vasoconstriction is also reduced in SHR (Jackson and Herzer, 1993).

ii. Thromboxane A_2 (TXA_2)

TXA_2 is produced by activated platelet aggregation and causes vasoconstriction and platelet aggregation (Hamberg *et al.*, 1975). TXA_2 activates the phospholipase C pathway resulting in the release of calcium from intracellular stores and the opening of calcium channels leading to increased intracellular free calcium (Thierauch *et al.*, 1994). It has been shown that when stimulated with acetylcholinethe, endothelium of arteries from hypertensive rats releases this vasoconstrictor which was not observed in normotensive rats (Luscher and Vanhoutte, 1986). In SHR the endothelium-dependent vasoconstriction induced by acetylcholine can be prevented by inhibitors of cyclooxygenase and antagonists of endoperoxide-thromboxane receptors (Auch-Schwelk and Vanhoutte, 1992). It has been suggested that the reduced relaxing response to acetylcholine in SHR resistance arteries may result from the release of TXA_2 or prostaglandin H_2, a prostaglandin precursor, which opposes endothelium-derived NO mediated relaxation (Dai *et al.*, 1992).

iii. Superoxide anions

Recently, it has been demonstrated that endothelium-dependent relaxation in SHR is impaired and that this endothelium-dependent vasoconstriction was selectively prevented by inhibitors of superoxide production. This suggests that superoxide anions may be one of the endothelium-derived contracting factors (Katusic *et al.*, 1989; Jameson *et al.*, 1993). The generation of oxygen derived free radicals including superoxide anions is significantly higher, and is positively correlated

with blood pressure, in essential hypertension (Sagar *et al.*, 1992). Superoxide anions are potent chemical inactivators of NO and inhibitors of PGI_2 synthesis (Gryglewski *et al.*, 1986). In contrast, they do not inhibit production of contractile prostanoids (Katusic and Vanhoutte, 1989). Superoxide anions cause endothelium-dependent contraction by preventing endothelium-dependent relaxation. Thus, increased production of superoxide anions may impair the balance between relaxing and contracting factors released from the endothelium and may lead to contraction of underlying VSMC (Cosentino *et al.*, 1994). This mechanism is probably responsible for the observation that endothelium-dependent contraction in response to acetylcholine and arachidonic acid was seen only in SHR and not in WKY aorta (Boulange and Luscher, 1993; Jameson *et al.*, 1993).

c. Endothelium-derived hyperpolarizing factor (EDHF)

Bolton and colleagues first reported that acetylcholine can cause endothelium-dependent hyperpolarization (Hoeffner *et al.*, 1989). Most studies indicated that NO does not mediate endothelium-dependent hyperpolarization (Vanhoutte, 1993). It appears likely that EDHF acts on vascular smooth muscle by opening K channels (Chen *et al.*, 1991). However, the exact type of K channel involved is still unclear.

At present, an attractive hypothesis has been advanced that an imbalance in endothelium-derived constricting factors outweighs the action of endothelium-derived dilating factors, leading to increased peripheral vascular resistance (Junquero *et al.*, 1992). The damage to the endothelium may also serve as a stimulus for abnormal vascular smooth muscle growth in hypertension (Chobanian, 1990).

4. Vascular smooth muscle cells

Vascular smooth muscle is responsible for controlling the lumen diameter of resistance vessels and, thus, controlling the vascular resistance. A common finding in hypertensive individuals and animals is an increase in the wall to lumen ratio of the arteries (Folkow, 1982). Several studies have shown that the thickening of the wall of small resistance arteries in SHR is due to an increase in VSMC number, *i.e.* hyperplasia (Lee, 1985; Mulvany, 1992). In contrast, in the large conduit arteries an increase in the size of smooth muscle cells accounts for much of the increase in vessel wall thickness, *i.e.* hypertrophy (Owens and Schwartz, 1982). It has been shown that VSMC from young SHR exhibited enhanced proliferation and that there was an age-dependent, differential and specific up-regulation of growth rate mechanisms in SHR VSMC (Saltis *et al.*, 1993a). The inhibitory effect of transforming growth factor-β1 (TGF-β1) on VSMC proliferation were absent in SHR (Agrotis *et al.*, 1993). The enhanced proliferative ability of VSMC from SHR appears to be not only an intrinsic property of this smooth muscle but also of growth factors to which VSMC are exposed (Saltis *et al.*, 1993b; Zhu *et al.*, 1994). A large number of molecules are mitogenic for smooth muscle *in vitro*. These molecules include catecholamines, angiotensin II, prostaglandins, LDL, lipoprotein, IL-1, neuropepetides and polypeptides, such as PDGF, FGF, and EGF (Schwartz 1990; Sachinidis *et al.*, 1993; Bjorkerud and Bjorkerud, 1994). It has been shown that SHR developed vascular hypertrophy before the rise of blood pressure (Rizzoni *et al.*, 1994), suggesting that a genetic factor may play a major role in the pathogenesis of

vascular hypertrophy.

In some vascular beds of hypertensive individuals, however, it has been reported that the ratio of wall-to-lumen is increased while the total mass of cells is the same. This phenomenon has been termed remodelling (Baumbach and Heistad, 1989). The alteration of vascular structure by remodelling has been suggested to play a more important role in essential hypertension than does vascular growth (Mulvany, 1992).

The intrinsic properties of the VSMC that are different in hypertension have been described both in the resting state and in response to stimuli (Bohr *et al.*, 1991; de Champlain *et al.*, 1991). VSMC in hypertension are more sensitive than normal VSMC to many constrictor agonists including epinephrine, serotonin, ouabain, Bay K 8644, and phorbol ester (Raval *et al.*, 1989; Storm *et al.*, 1990). It has been suggested that the plasma membrane of the VSMC has a primary defect causing dysfunction of many membrane transport systems including channels for sodium, potassium and calcium, exchangers for sodium-hydrogen, for sodium-calcium, and sodium-potassium (Bohr *et al.*, 1991; Aviv, 1994; Ellstrom *et al.*, 1994). These multiple abnormalities of diverse transporter proteins might be related to an alteration in the lipid bilayer (Carruthers and Melchior, 1986). It has also been observed that the number and affinity of various membrane receptors (α, β, serotoninergic, etc) are changed (Michel *et al.*, 1990). However, it appears that the cellular events following receptor activation also contribute significantly to abnormal vascular responsiveness (Bohr *et al.*, 1991). Phosphoinositide metabolism has been

reported to be augmented in SHR VSMC (Turla and Webb, 1990; Hamilton, 1994). Phospholipase D activity in VSMC from SHR was also enhanced (Kondo, 1994). Impaired calcium transport and handling associated with enhanced free calcium content has been reported in various cell types of hypertensive individuals and animals (Sharme and Bhalla, 1988; Lograno *et al.*, 1993; Neusser *et al.*, 1993; Bukoski *et al.*, 1994; Higashino, 1994). The increase in calcium sensitivity of VSMC contractile machinery in SHR may be linked to the increase in PKC activity (Soloviev and Bershtein, 1992) or to signal transduction events distal to PKC activation (Silver *et al.*, 1992; Zhu *et al.*, 1992). There are a number of possible mechanisms underlying the relationship between abnormal calcium metabolism and hypertension, and no single explanation can be used to clarify this association. There is also evidence of other factors that may cause parallel changes in both blood pressure and calcium metabolism (Hvarfner, 1991; Storm *et al.*, 1992).

C. NITRIC OXIDE

In 1980, Furchgott and Zawadzki demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium. Their investigations provided evidence that this effect was mediated by a labile humoral factor, later known as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Moncada and his associates subsequently provided evidence that EDRF was identical to nitric oxide (NO) (Palmer *et al.*, 1987). Since then it has

been revealed that the generation of NO by nitric oxide synthase (NOS) is an important autocrine and paracrine signalling pathway in the regulation of various cell functions and communications (Culotta and Koshland, 1992).

1. NO Synthase

In mammalian cells, NO is formed from a terminal guanidino-nitrogen of L-arginine (Schmidt *et al.*, 1988; Palmer *et al.*, 1988) by a gene family of NOS (Marletta, 1993). The expanding family of NOS isoforms generally falls into two categories: i. a constitutive form, and ii. a cytokine-inducible form (Moncada *et al.*, 1991)

a. Isoforms

Constitutive NOS is expressed in vascular endothelial cells and the brain (Lowenstein *et al.*, 1992; Marsden *et al.*, 1992; Nishida *et al.*, 1992). The constitutive NOS isolated from rat and porcine cerebella have been reported to be cytosolic proteins (Mr=150,000 - 160,000) that are dimeric in the native state (Bredt and Snyder, 1990; Mayer 1990). An endothelial constitutive NOS isoform was purified from bovine aortic endothelial cells and found to be a membrane-bound protein with a Mr=135,000 (Pollock *et al.*, 1991).

Inducible NOS purified from lipopolysaccharides (LPS) or cytokine-treated murine macrophages (Hevel *et al.*, 1991; Stuehr *et al.*, 1991) is also a cytosolic protein that has a Mr=130,000 and is a dimer under native conditions. After stimulation by cytokines, the inducible NOS has been found in VSMC (Kanno *et al.*, 1993;

Nunokawa *et al.*, 1993; Koide *et al.*, 1994), hepatocytes (Geller *et al.*, 1993a) and insulin-producing cells (Eizirik *et al.*, 1992). Recently, it has been shown that even without stimulation NO is released by VSMC, but to a lesser extent (Zehetgruber *et al.*, 1993). The significance of this NO generation is, however, unknown.

b. Amino acid sequences

The amino acid sequences derived from the isolated cDNA for constitutive NOS from rat cerebellum and bovine aortic endothelial cells have now been reported (Bredt *et al.*, 1991; Lamas *et al.*, 1992; Ogura *et al.*, 1993). The inducible NOS genes have also been cloned in macrophages (Xie *et al.*, 1992), VSMC (Nunokawa *et al.*, 1993) and hepatocytes (Geller *et al.*, 1993b). It was found that the derived sequence from rat brain had a significant homology to NADPH cytochrome P-450 reductase (Bredt *et al.*, 1991). The nucleotide binding sequence as well as those sequences associated with FAD, NADPH and FMN binding were highly conserved when compared with P-450 reductase from rat liver. Overall, approximately a 50% homology has been observed in all the reported NOS sequences. The N terminus in all sequences shows a great deal of similarity suggesting a common functional role. Such a role is most likely related to the arginine binding site and catalysis. Further analysis of sequences suggests that NOS isoforms from endothelial cell, neurons, macrophages and hepatocytes are the products of distinct genes (Sessa *et al.*, 1993), while a comparison of rat and bovine brain sequences is consistent with their being derived from the same gene (Marletta, 1993).

c. Regulation of NO

When Bredt and his co-workers initially purified constitutive NOS from rat brain, they found that the activity of this NOS was calmodulin-dependent (Bredt and Snyder, 1991). Subsequently, all constitutive NOS have been shown to require calcium and calmodulin. Intracellular calcium levels, therefore, can strictly regulate constitutive NOS activity in cells that express this isoform (Schmidt *et al.*, 1993). The activation of constitutive NOS occurs within seconds or, at most, a few minutes. This activation occurs by diverse substances including acetylcholine, bradykinin and agonists that are known to elicit prompt increases in intracellular calcium, such as arginine vasopressin, norepinephrine, histamine, thrombin and shear stress (Shepherd and Katusic, 1991). Endothelial cells, neurons, neutrophils and mast cells produce NO in this manner and are unaffected by inhibitors of transcription and translation (Schmidt *et al.*, 1993).

The inducible NOS are activated by cytokines or LPS within hours and are sensitive to inhibitors of DNA transcription or mRNA translation and to inhibitors of protein synthesis (Moncada *et al.*, 1991). Once induced, this inducible NOS isoform irreversibly binds calmodulin independent of calcium (Moncada *et al.*, 1991). NO released by inducible NOS may persist for days. What terminates this high output of NO is not known. Interleukin-1 β (IL-1β), tumor necrosis factor (TNF), interferon-γ and LPS have all been shown to induce this form of NOS in macrophages, VSMC, hepatocytes and β-cells (Xie *et al.*, 1992; Geller *et al.*, 1993a; Nunokawa *et al.*, 1993; Koide *et al.*, 1994). On the other hand, interleukin-4 (IL-4) and TGF-β1 have been observed to down-regulate inducible NOS gene expression

(Nelson *et al.*, 1991; Bogdan *et al.*, 1994). It has been reported that TGF-β1 inhibited cytokine-induced NO production by blocking the post-transcriptional synthesis of inducible NOS (Koide *et al.*, 1994). TNF has been shown to down-regulate an endothelial NOS (Yoshizumi *et al.*, 1993). It has been reported that although endothelial cells constitutively express NOS, they can also be induced by cytokines to express inducible NOS (Gross *et al.*, 1991).

2. NO Functions

Recently, work in several disciplines has converged to establish NO as a major messenger molecule regulating immune function and blood vessel dilatation and serving as a neurotransmitter in the brain and peripheral nervous system. Once synthesized, NO diffuses within the cell or to adjacent cells where it stimulates soluble guanylate cyclase or other heme-containing proteins. The resultant increase in cyclic guanosine monophosphate (cGMP) in the target cell produces the physiological effects (Culotta and Koshland, 1992). NO also attacks susceptible iron groups in certain enzymes, including those that synthesize DNA and help cells to respire (Moncada *et al.*, 1991).

a. Immune system

There is strong evidence that NO contributes to immune function and, in particular, to the phenomenon previously labelled as "non specific host defence". Macrophages can kill tumor cells when activated to generate NO (Moncada *et al.*, 1991). NO has been shown to be involved in cytokine-induced killing of microbes

(Nathan and Hibbs, 1991). NO may also be involved in the tissue damage associated with rejection of transplanted organs (Langrehr *et al.*, 1993). In addition to mediating cell killing, NO may play a role in cell-cell communication within the immune system (Langrehr *et al.*, 1993).

b. Nervous system

NO is a central and peripheral neuronal messenger. It is involved in classical anterograde neuronal signalling and also has unique properties as a retrograde transmitter. Within the CNS, NO is increased in response to the increase in intracellular calcium that follows stimulation of excitatory amino acid receptors (NMDA receptors). NO may be a mediator of long-term synaptic depression (LDT) and long-term potentiation of synaptic transmission (LTP). Therefore, NO may be involved in learning and memory (Shibuki and Okada, 1991; Nowak, 1992).

Recent evidence suggests that NO may also serve as a mediator of nonadrenergic noncholinergic (NANC) nerve neurotransmission. NANC nerves have been observed to be widely distributed in the vascular system, GI tract (Fang and Christensen, 1994) and genital area (Grozdanovic *et al.*, 1994).

c. Cardiovascular system

NO is a powerful endogenous vasodilator influencing blood pressure and organ perfusion. Constitutive NO synthesis in the endothelial cells provides a rapid-responding physiological control mechanism. Basal generation of NO is enhanced for short periods in response to physical and chemical stimulation. Changes in vessel wall shear stress affect basal NO release and this may be the most important

mechanism by which blood flow through vascular networks is controlled (Moncada *et al.*, 1991). NO also influences blood pressure and heart rate via baroreceptors (Silva *et al.*, 1994) and the central nervous system (Dinerman *et al.*, 1993). Therefore, it appears that when the cardiovascular system is in a normal resting state, there is an active vasodilatation mediated by NO (Moncada, 1994). Inflammatory stimuli, including cytokines, induce the expression of inducible NOS in VSMC, cardiac myocytes, and many other cell types, causing prolonged release of large amounts of NO (Warren *et al.*, 1994). This overproduction of NO by inducible NOS contributes to the profound hypotension and resistance to vasoconstrictor agents that characterise endotoxic shock (Nava *et al.*, 1992).

In biological systems, NO is metabolised within seconds because it is a free radical, reacting readily with sulfhydryl groups in amino acids or protein, superoxide and oxygen (Star, 1993). In aqueous solution, NO reacts rapidly with oxygen and accumulates as nitrite and nitrate ions, which can be easily measured.

It has been revealed that NO increases the intracellular levels of cGMP in VSMC (Holzmann, 1982). Several mechanisms have been proposed to explain cGMP-evoked vascular relaxation: (1) inhibition of inositol triphosphate (IP_3) generation, (2) stimulation of intracellular calcium sequestration, (3) increase in the dephosphorylation of the myosin light chain, (4) inhibition of receptor operated calcium channels, (5) activation of cGMP-dependent protein kinase, (6) stimulation of membrane calcium-ATPase, and (7) increase of K permeability through K channels causing membrane hyperpolarization (Marin and Sanchez-Ferrer, 1990;

Blatter and Wier, 1994). It is not known whether the relaxation is only produced by an increase in cGMP or whether other intracellular mechanisms of NO could also contribute to vasodilatation (Beasley and McGuiggin, 1994).

In addition to its vasodilating effect, other effects of NO have now been identified. These include synergistic action with prostacyclin to contribute to the inhibition of adhesion and aggregation of platelets (Moncada *et al.*, 1991), inhibition of leukocyte adhesion (Kubes *et al.*, 1991), endothelin generation (Boulanger and Luscher, 1990, Yokokawa *et al.*, 1993) and smooth muscle cell proliferation (Nakaki *et al.*, 1990). Each of these effects has the potential to play an important physiological or pathophysiological role.

3. Nitric oxide and hypertension

A considerable number of studies have provided evidence that alteration of NO synthesis may be involved in the pathogenesis of hypertension. Persistent hypertension following inhibition of NO formation has been reported in animal models (Manning *et al.*, 1993; Morton *et al.*, 1993). It has been proposed that the NO pathway is abnormal in patients with essential hypertension. The endothelium-dependent NO mediated vasodilatation appears to be impaired (Calver *et al.*, 1992; Luscher, 1992; Panza *et al.*, 1993), suggesting that the reduced endothelium-dependent vasodilatation in hypertension may be associated with decreased release of endothelium-derived NO (Luscher *et al.*, 1992; Deng *et al.*, 1993; Malinski *et al.*, 1993). It has been demonstrated that administration of L-arginine lowered blood

pressure in patients with essential hypertension. The plasma concentration of aldosterone was also decreased significantly, suggesting that the NO synthesis pathway may play an important role in the pathogenesis of hypertension, not only by controlling vascular tone directly, but also by modulating the endocrine and central nervous systems (Hishikawa *et al.*, 1993). It has been reported that in essential hypertension the sensitivity to NO may be reduced (Woods, 1993) while the generation of superoxide anions and hydrogen peroxide, substances that inactivate NO, are increased (Kumar and Das, 1993).

Although there are many reports concerning the involvement of NO in the pathogenesis of hypertension, the results are not clear. Several groups have shown that the release of NO in arteries is decreased in SHR (Lockette *et al.*, 1986; Diederich *et al.*, 1990; Koller and Huang, 1994). However, other investigators reported that the release of NO in SHR is not reduced (Fozard and Part, 1991; Arnal *et al.*, 1993; Li and Bukoski, 1993). It was also reported that an NOS inhibitor produced a greater inhibition of the arterial relaxation response to acetylcholine (Lee and Webb, 1992) and an exaggerated hypertension in SHR (Lacolley *et al.*, 1991). L-arginine has been demonstrated to induce a greater fall in blood pressure in SHR than in WKY (Schleiffer *et al.*, 1991). Furthermore, the cGMP content of SHR blood vessel wall was either increased (Mourlon-Le Grand *et al.*, 1992; Legrand *et al.*, 1993) or exhibited no change compared with that in WKY (Arnal *et al.*, 1993) Recently, it was reported that the cGMP content was increased in cultured VSMC and aortic ring of SHR compared to these cells and tissues of WKY, suggesting that

NOS activity is probably increased in SHR. A very recent report showed that the cGMP content was increased in cultured VSMC and aortic rings of SHR and the mRNA for the $\beta 1$ subunit of soluble guanylate cyclase was enhanced in response to both endogenous and exogenous NO (Papapetropoulos *et al.*, 1994). Therefore, the enhanced NOS activity or increased NOS protein may serve as a major counter-regulatory vasodilator mechanism to balance the elevated vasoconstriction seen in SHR.

It has been observed recently that pregnancy is associated with increased NO production (Chu and Beilin, 1993; Conrad *et al.*, 1993). It is tempting to suggest that NO mediates the vascular responses in pregnancy, including the fall in systemic blood pressure, vasodilatation and increased circulating blood volume. Endogenous NO may contribute to vasodilation in pregnancy by directly controlling vascular tone and modulating vascular responses to sympathetic nerve activity and circulating catecholamines (Beilin and Chu, 1993). Pre-eclampsia is associated with an increase in blood pressure and this may be secondary to a fall in NO production (Warren *et al.*, 1994). Inhibition of NO synthesis in rats during pregnancy produced symptoms similar to those of pre-eclampsia (Yallampalli and Garfield, 1993). Recently, it has been demonstrated that estrogen can induce NOS mRNA in the vascular wall and activate constitutive NOS. This may explain the gender difference in terms of decreased frequency of vascular diseases in pre-menopausal women (Moncada, 1994).

Thus, it appears that the involvement of NO in hypertension is complicated. In some forms of hypertension, there is a deficiency of NO production or effect

leading to an increase in vasoconstriction. In other situations, NO synthesis is increased to compensate for the primary increase in vasoconstriction. This, however, is still not enough to counter-balance the vasoconstriction. It may also be possible that the alterations of NO synthesis in different vascular beds or in different cells of the same vascular bed are not the same. Furthermore, the mechanisms of NO synthesis alteration in hypertension remain unclear.

Understanding the nature of the NO pathway, especially the alteration of the NO system in hypertension is important. Manipulating this system in the right direction at the right time may provide a new approach to the therapeutic management of hypertension.

III. IMMUNE SYSTEM AND HYPERTENSION

While many factors have been postulated to contribute to the hypertensive state, a large body of evidence has come to suggest that in many forms of experimental and human essential hypertension altered immune function may contribute to both the initiation and the maintenance of hypertension.

A. HUMAN ESSENTIAL HYPERTENSION

Over 20 years ago, Ebringer and his co-workers reported that serum immunoglobulin G (IgG) levels were significantly higher in hypertensive patients

compared with normal subjects (Ebringer and Doyle, 1970). This finding was subsequently confirmed by other investigators (Olsen *et al.*, 1973; Kristensen, 1978). The immunoglobulin level was significantly elevated in 20-40% of patients with essential hypertension (Suryaprobha *et al.*, 1984). There is a positive correlation between the level of serum IgG and standing blood pressure in untreated hypertensive patients and in patients with poorly controlled hypertension (Kristensen, 1978). Why serum immunoglobulin levels are elevated in patients with essential hypertension is not known. It is also unclear whether the elevation in serum immunoglobulin levels is related to a primary pathological process or is secondary to vascular damage caused by the elevated arterial pressure.

Another finding in patients with essential hypertension was the presence of an elevated level of autoantibodies to a number of cell structures and smooth muscle (Kristensen, 1978; Wilson *et al.*, 1978). It has been shown recently that hypertensive patients had increased frequency of antinuclear antibodies (Hilme *et al.*, 1993). There was also an association between antinuclear antibodies and blood pressure in untreated hypertensive patients (Kristensen, 1978). However, some antihypertensive agents can induce autoantibody formation (Booth *et al.*, 1982). Although these data suggest a possible involvement of these antibodies in the pathogenesis of hypertension, no information is available to support the involvement of autoantibodies in the etiology of hypertension.

There is evidence to suggest that cellular immune response may also be altered in patients with essential hypertension. It has been shown that the number

of T lymphocytes was decreased in malignant hypertension. The lymphocyte proliferation response to Concanavalin-A (Con A), a T-cell mitogen, is also depressed (Hilme *et al.*, 1993). The human leucocyte antigen (HLA) B15 tended to occur more frequently in hypertensive patients than in control subjects, especially if the family history of hypertension was taken into consideration (Kristensen, 1979; Hilme *et al.*, 1993). Hypertensive patients with positive B15 were 3-4 times more prone to vascular complications than patients without this antigen (Hilme *et al.*, 1993). A study on the association between essential hypertension and histocompatibility antigens revealed that there was increased frequency of HLA-DR4 (Gerbase-Delima *et al.*, 1992) and HLA-DR7 (Sengar *et al.*, 1985) suggesting that these antigens may act as genetic markers for the development of essential hypertension.

The complement system is a system of functionally linked proteins that interact with one another in a highly regulated manner to provide many of the effector functions of humoral immunity and inflammation. C_3F, a component of the complement proteins, was found expressed in 64% of patients with essential hypertension (Schaadt *et al.*, 1981). Expression of this protein increases the risk of cardiovascular complications in hypertension (Kristensen, 1978). The mechanism for this association is also unknown.

Because of the complexity of the pathophysiology of essential hypertension, a definitive connection between immunologic factors and the hypertensive state is difficult to prove. It is possible that elevated arterial pressure in itself leads to

arterial damage and the subsequent release of vascular antigens which trigger the immune responses. Humoral immune components directed against a vascular antigen could then perpetuate the pathology. Alternatively, primary activation of immunologic mechanisms directed against renal or vascular tissue could induce the hypertensive state. Clarification of these problem awaits further investigation.

B. THE SPONTANEOUSLY HYPERTENSIVE RAT (SHR)

The SHR was developed as a model of hypertension by Okamoto and Aoki 30 years ago using subsequent selective breeding of litter-mates with high blood pressure (Okamoto and Aoki, 1963). The increased arterial pressure eventually results in cardiovascular complications and premature death (Khraibi *et al.*, 1984)). The SHR has since been accepted as an animal model for human essential hypertension by many investigators (Trippodo and Frohlich, 1981). Although the etiology of hypertension in SHR has been a subject of intense investigation for many years, the primary cause remains unexplained.

There is a growing body of evidence suggesting that alterations in the immune system may be related to hypertension in SHR. It was shown that SHR had a reduced number of rosette-forming cells in thymus tissue indicating a reduction in the number of T-lymphocytes (Takeichi and Boone, 1976). The T non-helper cell population was found to be depressed from 2 weeks of age onward. This change persisted throughout the 4 month study period (Fannon *et al.*, 1992) and was also

found in adult SHR (Norman *et al.*, 1985). It has also been reported that the blastogenic responses of spleen cells, lymph node cells, and peripheral blood lymphocytes were depressed in SHR (Takeichi *et al.*, 1980; Strausser, 1983; Takeichi *et al.*, 1988). The proliferation response of SHR spleen cells was depressed in mixed lymphocyte reactions and in response to T cell receptor monoclonal antibody or interleukin-2 (IL-2) (Purcell *et al.*, 1993). Morphological investigations revealed that the medullary/cortical ratio in SHR thymus is reduced although thymic weight was not significantly different from that observed for WKY. The volume density of white pulp, composed of dense lymphoid tissues, in spleen was also reduced, suggesting decreased immunologic responsiveness *in vivo* (Fannon *et al.*, 1992).

Other immune abnormalities have also been identified in the SHR. These include a reduced delayed-type hypersensitivity, a delayed allograft rejection time, and the inability of T-thymocytes to cooperate with B-lymphocytes in the production of antibodies (Takeichi *et al.*, 1980, 1981). It has been reported that a thymotoxic autoantibody was present in SHR (Takeichi *et al.*, 1981) and that the production of natural thymotoxic autoantibody was enhanced by natural infection by microorganisms (Takeichi *et al.*, 1988). Recently, it was reported that plasma immunoglobulin A (IgA) and circulating IgA autoantibodies to DNA and thyroglobulin were increased in SHR (Chen and Schachter, 1993).

Immunological interventions including thymus transplantation (Norman *et al.*, 1985), and treatment with thymic hormone (Ba *et al.*, 1982; Strausser, 1983), immunosuppressant drugs such as cyclophosphamide and cylosporine A (Khraibi *et*

al., 1984) showed antihypertensive and/or immunological modulatory effects. It has been reported that a single injection of human recombinant IL-2 can lower blood pressure in adult SHR and prevent the development of hypertension in young SHR (Tuttle and Boppana, 1990). However, this finding has not been confirmed by other investigators (Pascual *et al.*, 1990; Dzielak, 1991; Ofosu-Appiah *et al.*, 1993). Recently, it was reported that the leucocyte count in SHR was 10-100% above that found in WKY and the number of spontaneously activated granulocytes was also higher (Schmid-Schonbein *et al.*, 1991; Arndt *et al.*, 1993). These findings suggested that an elevated leucocyte count and an increased number of activated leucocytes may be closely associated with the enhanced level of vascular injury, altered vascular smooth muscle contractility and elevated blood pressure in hypertension.

Several possible mechanisms responsible for immune dysfunction in SHR have been considered. It has been reported that experimental infection with Sendai virus induced T cell depression and increased autoantibody production in SHR (Takeichi *et al.*, 1988). Microbial, particularly viral infection may be able to suppress immune response through destruction of lymphocytes. There is evidence of a close relationship between the sympathetic nervous system and the immune system (Hadden *et al.*, 1970). It has been shown that norepinephrine inhibits lymphocyte proliferation *in vitro* and neonatal sympathectomy increases *in vivo* immune responsiveness (Besedovsky *et al.*, 1979). Several investigators studied the involvement of the sympathetic nervous system in immune dysfunction in SHR. Increased norepinephrine concentration was seen in SHR spleen and kidney

(Donohue *et al.*, 1988). The innervation of thymus, spleen (Purcell and Gatton, 1992) and kidney (Gattone *et al.*, 1990) was increased in SHR, suggesting that abnormal sympathetic innervation may be contributing to the immune deficiency. SHR also exhibit an increased AVP secretion (Crofton *et al.*, 1978). The increased AVP secretion may influence the immune system in SHR through a variety of mechanisms (Fannon *et al.*, 1992). It was reported that renal IL-6 mRNA was extremely low in SHR and the regulation of IL-6 mRNA expression is different from that of WKY (Nakamura *et al.*, 1993). However, the way in which this change may be related to immune dysfunction is not clear. Although these studies are suggestive, both the mechanism underlying immune dysfunction in SHR and the relationship between immune dysfunction and hypertension are largely unknown.

C. HYPERTENSION AFTER PARTIAL RENAL INFARCTION

Partial infarction of the kidney produced by ligation of two of the three branches of the renal artery results in the development of a sustained hypertension in the rat (Loomis, 1946). It was shown that if the partially infarcted kidney was removed within 1-2 days after induction of the infarction, the hypertension was not sustained. However, if the infarcted kidney remained in the animal for longer than 1 week, the hypertension was sustained (Sokabe and Grollman, 1963). In addition, the same investigator reported that injection of extracts of normal or infarcted renal tissue resulted in a sustained hypertension that was similar to the hypertension which

occurred after partial infarction.

The immune system has been suggested to play an important role in this type of hypertension. Autoantibodies directed against renal tissue were found in animals with partial renal infarct hypertension (White and Grollman, 1964). The hypertension could be transferred by means of viable lymph node cells from animals with established renal infarct hypertension into normotensive recipient rats and this hypertension was partly prevented by splenectomy and thymectomy (Okuda and Grollman, 1967). Chronic immunosuppressive therapy with cyclophosphamide prevented the later maintenance phase of hypertension and could also completely reverse established infarct hypertension (Norman *et al.*, 1988). It has been suggested that the infarcted kidney releases an antigenic substance, and, subsequently, induces an immune reaction that not only acts against damaged tissue but also against viable renal tissue (Norman *et al.*, 1988). It has been shown that plasma renin activity was elevated 3-6 fold after partial renal infarction. After 4 weeks, as plasma renin activity returned to pre-infarct levels, arterial pressure remained elevated. In contrast, in animals treated with immunosuppressive agents, arterial pressure paralleled plasma renin activity. As plasma renin activity returned to pre-infarct levels, so did arterial pressure (Norman *et al.*, 1988). These data suggest that activation of the renin-angiotensin system is responsible for the initial rise in blood pressure, whereas immunological reaction appears to be responsible for the maintenance of partial renal infarct hypertension. However, the question of which immunological mechanisms are involved in the perpetuation of hypertension remains

to be answered.

D. MINERALOCORTICOID SALT HYPERTENSION

Mineralocorticoid salt hypertension is induced by the simultaneous administration of a salt-retaining hormone (deoxycorticosterone) and a salt load in rat (Friedman and Friedman, 1949). This DOCA-salt hypertension has been characterized as a form of volume-expansion hypertension. The hypertension persisted after removal of the mineralocorticoid hormone and the salt (post DOCA-salt hypertension) (Friedman *et al.*, 1953). It has been proposed that persistence of hypertension may result from damage to resistance vessels and the renal vasculature caused by the high blood pressure itself (Beilin and Ziekas, 1972), and that it may cause irreversible changes in the renal handling of salt and water(Anderson *et al.*, 1985). There is evidence to suggest that tissue and organ damage caused by DOCA-salt administration may initiate an immunological response that can sustain the post-DOCA-salt hypertension. It was reported that induction of this type of hypertension is dependent on the presence of the thymus (Svendsen, 1976) and that the hypertensive state could be transferred to untreated control animals by an intravenous injection of spleen cells from the hypertensive rats (Olsen, 1980). It has been reported that after exposure to DOCA and salt, glomerulopathy was significantly less in mice that were complement-deficient than in normal mice, suggesting that activation of the complement cascade may play an important role in

this type of hypertension (Raij *et al.*, 1989). It seems that a dysregulation of immunological function is a factor in the pathogenesis of hypertension that persists after exposure to mineralocorticoid hormone and a salt load. Whether the elevated blood pressure initiates the vascular damage which then activates an immunologic response or whether the immunologic mechanism is primary to the hypertension is unclear.

IV. OBJECTIVES

As mentioned earlier, a substantial body of evidence suggests that immune abnormalities may be associated with hypertension. The present study investigates the relationship between immune abnormalities and hypertension. The objectives of this work are as follows:

1. characterization of immune abnormalities in SHR.
2. delineation of the mechanism responsible for immune dysfunction.
3. study of the interaction between the immune system and the vascular system.
4. investigation of the relationship between immune abnormalities and hypertension.

CHAPTER II. MATERIALS AND METHODS

I. ANIMALS

A. SHR and WKY

Male SHR and WKY controls matched for age were obtained from Taconic Farms (Germantown, NY). The animals had been randomly bred in a closed colony from breeding stock obtained at the F_{35} (SHR) or F_{10} (WKY) generations from the National Institutes of Health (Bethesda, MD). In some experiments, male SHR and age matched WKY were obtained from Charles River Breeding Laboratories (Wilmington, MA). These rats had been continuously inbred from stock obtained from the National Institutes of Health at the F_{32} (SHR) or F_{11} (WKY) generations. The rats were fed with a rodent laboratory chow (Purina No. 5001, Purina Mills, Richmond, IN) and given tap water. The rats were housed two to three in a cage in a temperature controlled room with a 12 hour light, 12 hour dark cycle. All the rats were housed for at least one week after arrival before experiments.

B. Borderline Hypertensive Rats (BHR) and WKY

Male WKY and BHR, which are the first generation offspring of SHR females and WKY males (Lawler *et al.*, 1987; DiBona and Jones, 1993), were purchased from Taconic Farms. The rats were weaned at 4 weeks of age. Standard rat chow (Purina

No. 5001) and tap water were available to all rats until the dietary regimens were instituted. At 5 weeks of age, the animals were housed two in a cage and randomly assigned to two groups. One group received the standard rat chow containing 1% NaCl. Another group received a chow containing 8% NaCl (Purina No. 5001C-2) for 8 weeks (DiBona and Jones, 1991; Melby *et al.*, 1991). All animals had free access to tap water. Because the rats which were fed with a high NaCl diet urinated more frequently, the cages were changed twice a week to keep them dry and clean. At 13 weeks of age, blood pressures were measured in both BHR and WKY rats and tissues were collected for further experiments.

C. Newborn SHR and WKY

Pregnant SHR and WKY dams (fifteen to seventeen days) were purchased from Taconic Farms. The dams were housed in individual cages with a soft bedding material in a temperature controlled room with a 12 hour light, 12 hour dark cycle. On the 20th or 21th day of pregnancy, the dams gave birth. One day after birth, the newborn rats were mixed in a clean cage. Nine to eleven newborns were randomly put back to a dam in a clean cage and housed with the dam for 4 weeks. The dams did not reject the newborns during the entire experimental period (described later in this chapter).

II. BLOOD PRESSURE MEASUREMENT

Male SHR or WKY rats were injected with sodium pentobarbital (65 mg/Kg, i.p.). While the rat was fully anaesthetized, a polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ), filled with heparinized saline (100 unit/ml) (Sigma Chemical Co., St. Louis, MO) was inserted into the right carotid artery. The arterial cannula was connected to a Statham pressure transducer and the arterial pressure was continuously recorded with a Dynograph (Beckman Instrument Inc., Fullerton, CA) for at least 5 minutes or until the blood pressure was stable. Mean arterial blood pressure (MAP) was calculated as the diastolic blood pressure plus one-third of the pulse pressure. The tissues were then collected and the rats were sacrificed by pentobarbital overdose.

III. CELL PREPARATION

A. Spleen cells and thymocytes

Lymphocytes from rat spleen and thymocytes were prepared using a method modified from that described in *Current Protocols in Immunology* (Kruisbeek, 1991). The spleen and thymus were removed from anaesthetized animals. The tissues were place in 90 x 20 mm petri dishes containing 10 ml Hanks' Balance Salt Solution (HBSS, Gibco Laboratories, Grand Island, NY). The tissues were gently ground through fine steel meshes with the plunger of a 10-ml syringe until mostly fibrous

tissue remained. The cell suspension was transferred into a centrifuge tube and centrifuged at 200 x g for 10 minutes. The cells were re-suspended with HBSS and distributed into centrifuge tubes at 0.5-1 x 10^8 cells/5 ml (15-ml tube) or 1-5 x 10^8 cells/10 ml (50-ml tube). Four ml (for 15-ml tubes) or 10 ml (for 50-ml tubes) of high-density histopaque solution (Sigma Chemical Co.) was layered under the cell suspension by drawing the high-density solution into a pipet, placing the tip of the pipet at the bottom of the tube, and slowing letting the solution flow under the cell suspension. The tubes were then centrifuged at 800 x g at room temperature for 20 minutes. The cells floating on the top of the histopaque, from which red blood cells and dead lymphocytes had been removed, were collected and washed twice with HBSS. The lymphocytes were re-suspended in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories Inc, Logan, Utah), 2 mM L-glutamine, 0.1 mM MEN non-essential amino acids, 100 unit/ml penicillin and 50 μg/ml streptomycin (Gibco Laboratories), complete RPMI-1640 medium). Cell viability was usually more than 98% as tested by trypan blue exclusion.

B. T-enriched lymphocytes

Different adherence properties of T cells, B cells, and accessory cells such as macrophages can be employed for T cell enrichment (Kruisbeek, 1991). A rat T immunocolumns kit (Biotex Laboratories, Edmonton, Alberta, Canada) is a rapid affinity chromatography tool for rat T cell enrichment. By a process of negative

selection, virtually all rat B cells were removed by immunocolumns coated with polyclonal goat-anti-rat IgG (H+L) antibody (anti-B cell antibody). Macrophages and monocytes were also largely removed due to their non-specific binding properties. During the experimental procedure, the columns were activated by adding the antibody and then allowing the columns to remain at room temperature for at least one hour. The activated columns were washed with HBSS before they were loaded with the cells. One to 1.5×10^8 spleen cells from SHR and WKY were applied to the column. The T-enriched lymphocytes were then eluted by slowly washing the column with HBSS. The effluent cell suspension was centrifuged at 200 x g for 10 minutes. The cell pellets were re-suspended with complete RPMI-1640 medium. Flow cytometric analyses showed more than 85% T cell enrichment after passage through the column according to the information provided by Biotex Laboratories.

C. Macrophage-depleted lymphocytes

Spleen cells from SHR and WKY ($2\text{-}5 \times 10^7$ in 10 ml 5% FCS RPMI-1640) were incubated in a 90 x 20-mm plastic tissue culture dish for 60 minutes at 37°C. The nonadherent lymphocyte suspension was transferred into a new plastic tissue dish and incubated for another 60 minutes at 37°C. Macrophages were removed because they adhered to the plastic surface (Mills, 1991). The cell suspension was centrifuged at 200 x g for 10 minutes. The cell pellet, which contained macrophage-depleted lymphocytes, was re-suspended with complete RPMI-1640 medium. The

contamination of macrophages was examined by immunocytochemical staining for macrophage marker ED1 and ED2 (Beelen *et al.*, 1987). The results showed that there were 0% ($ED2^+$) or less than 2% ($ED1^+$) of positive cells.

D. Macrophages

Spleen cells from SHR and WKY were incubated in 60 x 15 mm plastic dishes (5 x 10^6 cells/dish) or in 96 well tissue culture plates (0.5-1.5 x 10^6 cells/well) in complete RPMI-1640 medium for 90 minutes at 37℃ (Wahl and Smith, 1991). The media which contained nonadherent lymphocytes were decanted. The dishes or plates were washed twice with HBSS to remove any residual nonadherent cells. The majority of adherent cells expressed macrophage marker ED1 or ED2, examined by immunocytochemical staining. Therefore, this cell population was considered to be composed of mainly macrophages (Beelen *et al.*, 1987).

E. VSMC

VSMC were isolated from SHR or WKY rat aortic artery according to a standard method established in our laboratory (Pang *et al.*, 1990). The aortic artery was dissected out and cleaned free of connective tissues in cold calcium-free HBSS. The artery was cut open longitudinally. The inner layer of artery was gently scraped with a forceps to remove the endothelium. The muscle layer was torn off from the artery and left at 4℃ in calcium-free HBSS for 30 minutes. The medium was then changed to enzyme solution 1 [HBSS containing 1.5 mg/ml collagenase/dispase

(Boehringer Mannheim, Laval, Quebec), 0.5 mg/ml elastase, 1 mg/ml trypsin inhibitor, 2 mg/ml bovine serum albumin (BSA), 0.2 mM $CaCl_2$ and 10 mM HEPES (Sigma Chemical Co., St. Louis, MO)]. After 60 to 90 minutes, the arterial tissue was washed with cold calcium-free HBSS and incubated with enzyme solution 2 [HBSS containing 1 mg/ml collagenase (Sigma Chemical Co.), 0.3 mg/ml trypsin inhibitor and 2 mg/ml BSA] for another 60 minutes. All incubations were carried out under 5% CO_2 in air at 37℃. The arterial tissues were then triturated with a pipet in order to disperse them into single cells. The VSMC suspension was centrifuged at 200 x g for 8 minutes and washed twice with HBSS. The VSMC were subsequently planted in a 50 x 15 mm culture dish in 10% FCS DMEM medium with 100 units/ml penicillin and 50 μg/ml streptomycin (Gibco Laboratories), at 37℃ in a humidified atmosphere of 5% CO_2 in air. The primary VSMC proliferated sufficiently to be passed in 2-3 weeks. The subcultured VSMC were passed every week.

VI. LYMPHOCYTE PROLIFERATION STUDY

Measurement of the proliferation response of lymphocytes is a fundamental technique for the assessment of their biological responses to various stimuli. This involves the measurement of the number of cells present in a culture before and after the addition of a stimulating agent. However, this can be both laborious and difficult inasmuch as the proliferating cells under investigation may constitute only

a small component of the total cell population. In practice, therefore, cell proliferation is determined by estimating incorporation of tritiated thymidine (^{3}H-thymidine) into DNA, a process which is closely related to underlying changes in cell number.

Spleen cells, thymocytes, T-enriched lymphocytes or macrophage-depleted lymphocytes were counted using a hemocytometer. The cell concentration was adjusted to 1 x 10^6 cells/ml with complete RPMI-1640 medium. 200 μl of the cell suspension was dispensed into each well of a 96 flat-bottom well tissue culture plate. Triplicate or quadruplicate wells were prepared for each experimental condition, i.e. each concentration of stimuli to be tested, including wells with no stimuli to measure background response. Con A and PHA, which primarily stimulate T cell proliferation, or other activators were added into the culture in a volume of no more than 20 μl. The plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for three days. ^{3}H-thymidine (sp act 2 Ci/mmol, Dupont, Boston, MA) was added to the culture (0.5 μCi/10 μl/well) for the final 18 hours. The cells were harvested using a semiautomatic multiwell cell harvester (Skatron, Sterling, NJ) that aspirates cells, lyses cells and transfers cell debris including DNA onto filter paper while allowing unincorporated ^{3}H-thymidine to be washed out. The filter discs for each well were transferred to scintillation vials. Scintillation fluid was added to each vial. The vials were then placed in a liquid scintillation counter (1217 Rackbeta, LKB Wallac). The amount of radioactivity on each disc was represented as CPM (Takeichi *et al.*, 1980).

V. VSMC GROWTH AND PROLIFERATION STUDY

A. VSMC culture

Isolated aortic VSMC from SHR and WKY were grown in 10% FCS DMEM medium using 90 x 20 mm tissue culture dishes, and were passed every week. Only VSMC below passage 20 were used in experiments. When VSMC were grown to near confluence (approximately 1×10^5 cells/ml) in dishes, the mediun was decanted. The dishes were washed twice with calcium-free HBSS. Each dish received 1.5 ml of 0.25% trypsin (Gibco Laboratories). After 10 to 15 minutes at room temperature, 10 ml HBSS was added into dishes and the cell suspension obtained was centrifuged at 200 x g for 8 minutes. The cell pellet was washed once more with HBSS and re-suspended with 10% FCS DMEM medium. Cell viability was tested by trypan blue exclusion. The VSMC were either used in experiments or split for continuous culture.

B. VSMC proliferation study

VSMC (1×10^4 cells/well) were dispensed into 96 flat-bottom well tissue culture plates in 10% FCS DMEM medium and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hours to allow VSMC attachment to the bottom of the plate. The medium was changed to 0.4% FCS DMEM medium to render the VSMC quiescent (Rao and Berk, 1992) for 4 days. The test substances re-suspended in 5 or 10% FCS DMEM medium were added to quiescent VSMC. After a 24 hour

incubation, the VSMC were pulsed with ^{3}H-thymidine 1 μCi/ml (0.2 μ Ci/well) and incubated for another 18-40 hours. The medium was then removed and the VSMC were washed twice with HBSS. Each well of the plates received 100 μl 0.5% trypsin followed by a 30 minute incubation at room temperature. The VSMC were then harvested onto filter paper by the cell harvester. The amount of radioactivity incorporated into cells was determined using a liquid scintillation counter (Saltis *et al.*, 1993).

C. Protein determination

VSMC (1.5 ml of 1x10^5 cells/ml) from SHR and WKY were incubated in 24 well tissue culture plates in 10% FCS DMEM medium for 36-40 hours. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. The medium was then replaced by 10% FCS DMEM in the presence of ligands. At various times, the VSMC were washed twice with HBSS. The VSMC were solubilized by addition of 200 μl 1 M NaOH to each well followed by a 30 minute incubation at room temperature (Beasley *et al.*, 1991). The protein concentrations were determined by the Pierce BCA protein assay (Pierce, Rockford, IL). This reagent system combines the well known reaction of protein with Cu^{2+} in an alkaline medium (yielding Cu^{1+}) with a highly sensitive and selective detection reagent for Cu^{1+}, namely bicinchoninic acid. The purple reaction product is water soluble and exhibits a strong absorbance at 562 nM (Brown *et al.*, 1989). The protein concentration was determined relative to a standard curve obtained with aqueous

solution of BSA.

VI. IL-2 PRODUCTION AND QUANTIFICATION

IL-2, first described as T cell growth factor (TCGF), is a cytokine produced by activated T cells, and was originally identified by its ability to promote and maintain *in vitro* long-term T cell cultures (Gillis and Smith, 1977). Mitogenic or antigenic stimulation of resting lymphocytes induces IL-2 production from the activated lymphocytes and expression of the IL-2 receptor on the lymphocytes. IL-2 acts as both an autocrine and a paracrine growth factor to activate lymphocytes producing IL-2 and to induce lymphocyte proliferation. To determine the capacity of lymphocytes to produce IL-2, spleen cells from SHR and WKY were cultured in 12 well tissue culture plates at a concentration of 4 x 10^6 cells/well in 4 ml of complete RPMI-1640 medium in the presence of Con A (2.5 or 5 μg/ml) at 37℃ in a humidified atmosphere of 5% CO_2 in air for 48 hours. The cell suspensions were centrifuged at 800 x g, 4℃ for 15 minutes. The supernatant was collected and stored at -20℃ until tested.

The amount of IL-2 in a culture supernatant can be determined by its ability to stimulate the growth of an IL-2-dependent murine cell line, CTLL-2. The CTLL-2 line was derived from murine spleen cells that were stimulated by allogeneic cells and propagated with crude T cell supernatant. These cells transformed spontaneously, but remained dependent on exogenous IL-2. CTLL-2 proliferation

can be determined by a standard ^3H-thymidine incorporation method (Gillis and Smith, 1977). Briefly, 1×10^4 CTLL-2 cells (obtained from late Dr. Wegmann, Department of Immunology, University of Alberta, Edmonton, AB) were added in triplicate to 96 flat-bottom tissue culture plates with different dilutions of culture supernatant and IL-2 standards including a negative control of medium alone. After 24 hours incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, ^3H-thymidine was added to the culture (0.5 μ Ci/well) for 4 hours. The cells were harvested onto filter paper and counted (Xiao and Brahmi, 1989). One unit of IL-2 was defined as the amount of IL-2 that was required to support half-maximal ^3H-thymidine incorporation into CTLL-2 cells.

Continuously proliferating CTLL-2 cells were maintained in complete RPMI-1640 medium containing 1 unit/ml rat IL-2 at a density between 0.5×10^5 to 1×10^6 cells/ml in a flask. When the cell density reached 1×10^6 cells/ml, the culture was split 1:5 or 1:10 with the same culture conditions. The cells were used at least 2 days after they were split to avoid high background and reduced sensitivity. In addition, the cells were washed twice with HBSS before use to wash out any residual IL-2.

VII. IMMUNOCYTOCHEMICAL STAINING

The expression of rat macrophage markers, ED1 and ED2, on cells was examined using an immunoperoxidase staining kit, Histostain-SP Kit (Zymed Lab. Inc., South San Francisco, CA). The method was adopted from Dr. Larry Guilbert

(Department of Immunology, University of Alberta).

A. Coating slide

Microscope slides (Fisher Scientific Co., Pittsburgh, PA) were soaked in 10% detergent overnight, and were then washed thoroughly with running water and dried in an oven at 60°C. The slides were dipped in each of the following solutions for 2 minutes: acetone, 2% 3-aminopropyl triethoxy silane (Aptex) (Sigma Chemical Co.) in acetone, acetone, water and water. The treated slides were dried at 37°C and stored until use.

B. Cell preparation

Spleen cells or macrophage-depleted lymphocytes from SHR and WKY were fixed with 4% phosphate-buffered paraformaldehyde (paraformaldehyde 40g/l, $NaH_2PO_4.H_2O$ 16.8g/l, NaOH 3.85g/l and glucose 5.4g/l, pH 7.4) for 10 minutes (Sander *et al.*, 1991). The cells were washed 3 times with phosphate-buffered saline (PBS). An aliquot of cell suspension (3-5 x 10^6 cells in 20 μl) was added to a circle drawn on an aptex coated slide. The cells were dried at room temperature and stored at -70°C until use.

Spleen cells from SHR and WKY were incubated in a 96 well tissue culture plate (0.5-1.5x 10^6 cells/well) in 10% FCS RPMI-1640 for 4 hours. The nonadherent cells were discarded and the remaining adherent cells were washed 3 times with PBS. The adhered cells in the wells were then fixed with 4% phosphate-buffered

paraformaldehyde for 10 minutes. The fixed cells were washed 2 times with PBS and stained for the expression of ED1 or ED2 the next day.

C. Immunochemical staining

The slides or plates containing fixed cells were washed with 3% H_2O_2 for 10 minutes. Because ED1 antigen was predominantly located intracellularly (Dijkstra *et al.*, 1985), the cells were permeabilized with 0.1% saponin (Sigma Chemical. Co.) in PBS for 10 minutes (Sander *et al.*, 1991) followed by a 30 minute incubation with 3% H_2O_2 in 0.1% saponin PBS. Non-immune mouse serum (10%) plus 0.1% saponin was added to the circles or wells containing fixed cells and incubated at room temperature for 1 hour to reduce non-specific binding. The cells were then incubated with mouse anti-rat ED1 (1:1000 dilution) or anti-rat ED2 (1:800 dilution) antibody plus 0.1% saponin at room temperature for 1 hour. Normal mouse IgG_1, the same isotype as ED1 and ED2 antibody, was used as a negative control. The cells were then washed thoroughly with PBS. The Biotinylated goat anti-mouse IgG antibody plus 2% rat serum (to reduce non-specific binding) was added to the cells and incubated for 15 minutes. The cells were then washed 3 times with PBS. Peroxidase conjugated streptavidin was added to the cells and incubated for 15 minutes followed by 3 washings with PBS. The substrate chromagen was added to cells and incubated for 5 to 10 minutes and then rinsed with water. The cells in 96 well plate were kept in PBS without counterstaining. The cells on slides were counterstained with 50% hematoxylin.

IIX. MIXED CELL CULTURE

A. Macrophage-lymphocyte

The spleen cells from SHR or WKY (2×10^5 cells/well) were incubated in 96 flat-bottom well tissue culture plates at 37℃ in a humidified atmosphere of 5% CO_2 in air for 90 minutes. The medium which contained nonadherent lymphocytes was decanted. The plates were washed twice with cold HBSS to remove any residual nonadherent cells. The majority of adherent cells were macrophages (Wahl and Smith, 1991). Macrophage-depleted lymphocytes (2×10^5 cells/well) were added into the plates in quadruplicate and incubated in the presence of 2.5 or 5 μg/ml Con A at 37℃ in a humidified atmosphere of 5% CO_2 in air for 72 hours. ^{3}H-thymidine was added to the culture for the last 18 hours and the cells were harvested. The amount of radioactivity incorporated into cells was counted using a liquid scintillation counter.

B. Lymphocyte-lymphocyte

The spleen cells from SHR or WKY were mixed with one another. The control 1 group contained only SHR or WKY spleen cells (2×10^5cells/well). The control 2 group also contained only SHR or WKY spleen cells but only 80% of the total cell number (1.6×10^5cells/well). The mixture group contained 80% (1.6×10^5cells/well) of one type of cells plus 20% (0.4×10^5 cells/well) of another type of cells. The proliferation response was induced by 2.5 μg/ml Con A and determined by ^{3}H-thymidine uptake. The amount of radioactivity incorporated into cells was

counted using a liquid scintillation counter.

C. VSMC-lymphocyte

VSMC (10-20 passages) were planted in 96 well tissue culture plates at various cell concentrations in 10% FCS DMEM medium for 36-40 hours. The medium was then replaced by fresh 10% FCS DMEM medium. For some experiments, the VSMC in the plates were irradiated using a ^{137}Cs r-irradiator at a dose of 4000 rad to prevent VSMC proliferation (Kruisbeek and Shevach, 1991). After irradiation, the cells were washed twice with HBSS. The efficiency of irradiation was examined by the amount of ^{3}H-thymidine incorporated into VSMC as described previously. The lymphocytes from SHR or WKY (2 x 10^5 cells/well) were co-cultured in quadruplicate with VSMC at a ratio of VSMC : lymphocytes 1:40, 1:20 or 1:10 in complete RPMI-1640 medium in the presence of 2.5 or 5 μg/ml of Con A at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 hours. ^{3}H-Thymidine was added to the culture for the last 18 hours and the cells were harvested. The amount of radioactivity incorporated into cells was counted using a liquid scintillation counter (Xiao and Pang 1993).

IX. NO PRODUCTION AND MEASUREMENT

A. NO production by spleen cells

Five ml of 1×10^6 cells/ml spleen cells were incubated in each of the 12 wells

in tissue culture plates in complete RPMI-1640 medium for 72 hours in the presence of 2.5 μg/ml Con A or 40 μg/ml LPS. The supernatant was collected by centrifuging at 4℃, 800 x g for 10 minutes, and kept at 4℃ until tested.

B. NO production by macrophages

Five ml of 1×10^6/ml spleen cells were incubated in 60 x 15 mm plastic dishes in complete RPMI-1640 medium for 90 minutes. The media which contained nonadherent lymphocytes were decanted. The dishes were washed twice with HBSS to remove any residual nonadherent cells and 5 ml of fresh medium was added. The majority of adherent cells were macrophages. The macrophages were then incubated for 72 hours in complete RPMI-1640 medium in the presence of 2.5 μg/ml of Con A or 40 μg/ml of LPS. The supernatant was collected as described above.

C. NO production by VSMC

VSMC (1.5 ml of 1×10^5 cells/ml) were incubated in 24 well tissue culture plates in 10% FCS DMEM medium for 36-40 hours. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. To induce NO production, the VSMC were incubated with LPS or a cytokine mixture containing 5 units/ml interleukin-1 β (IL-1β); 5 nM tumor necrosis factor α (TNFα); 200 units/ml interferon γ (IFNγ) and 10 μg/ml LPS in 10% FCS DMEM medium for 48 hours (Geller *et al.*, 1993a). The supernatant was collected as described above.

D. NO measurement

Although the complex nature of the biosynthesis or metabolism of NO is not completely clear, it is widely accepted that in aqueous solution NO reacts rapidly with O_2 and accumulates in the culture medium as nitrite and nitrate ions (Hibbs *et al.*, 1987a). Nitrite was measured by a standard method (Green *et al.*, 1982; Junquero *et al.*, 1992). An aliquot of cell culture supernatant was mixed with an equal volume of Greiss reagent (prepared by adding 1 part 0.1% napthylethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 minutes. The absorbance at 550 nm was measured and nitrite concentration was determined by reference to a standard curve obtained by using different concentrations of sodium nitrite diluted in the stock culture medium. The background value of the medium was calibrated to zero.

X. T CELL ABLATION AND ASSESSMENT

A. Antibody treatment

Anti-T cell monoclonal antibodies have been used *in vivo* to prolong allograft and xenograft survival time by depletion of certain T cells (Chavin *et al.*, 1992; Teramoto *et al.*, 1992). It is believed that anti-T cell antibodies bind to specific surface antigens on T cells. This antigen antibody complex triggers a cascade of events which leads to activation of the complement system and other mechanisms causing depletion of these specific T cells. Since CD2 and CD5 antigens are

expressed on most T cells, anti-CD2 and anti-CD5 monoclonal antibodies were used to ablate T cells in this study.

Three groups of one week old SHR or WKY were injected with mouse anti-rat CD2, mouse anti-rat CD5 monoclonal antibodies (ascites) or mouse control ascitic fluid. The rats were injected three times a week for two weeks. Three days after the last injection, the rats were sacrificed and the spleen were collected. Single antibody treatment is not usually sufficient to deplete all T cells. Previous studies have shown that six treatments in a period of two weeks should achieve an optimal depletion. This multiple treatment schedule has been successfully used by others (Barlow and Like, 1992). It has also been shown that this schedule was well tolerated by animals and did not cause morbidity or mortality. A control mouse ascites fluid was used to ensure that there was no non-specific antibody effect.

B. Assessment of T cell ablation

Since it is important to ensure that anti-CD2 and anti-CD5 monoclonal antibodies can induce T cell depletion in SHR, a proliferation response of spleen cells to T cell mitogens was carried out. If T cells are ablated by these antibodies, the number of proliferative cells will be minimal. Since SHR already have a reduced lymphocyte proliferation response, WKY which have normal lymphocyte proliferation are used as the control group to assess the level of T cell depletion.

XI. REAGENTS

Con A, LPS, PDB, ionomycin, napthylethylenediamine dihydrochloride and sulfanilamide were obtained from Sigma Chemical Co. L-NMMA and D-NMMA were purchased from Calbiochem, La Jolla, CA. Rat IL-2 was purchased from Collaborative Research Inc.,Bedford, MA. Recombinant IL-1β, Recombinant TNFα, Recombinant IL-6 and Recombinant IFNγ were purchased from Boehringer Mannheim. Mouse anti-rat CD2, mouse anti-rat CD5, mouse control ascitic fluid, mouse anti-rat ED1, mouse anti-rat ED2 and Mouse IgG_1 were purchased from Cedarlane, Hornby, Ontario.

XII. STATISTICAL ANALYSIS

Data are presented as mean $\pm$ SD when original values are used. The results of lymphocyte proliferation studies are summarized as mean $\pm$ SE from the mean values of 4 to 6 original results. The paired or non-paired Student 's t test is used for the comparisons between two groups. The analysis of variance or Newman-Keul 's analysis is used for multiple comparisons. The analysis of the regression line is used to estimate a correlation relationship between two variances. When a third variance is involved, the analysis of covariance is performed. In all the statistical analyses, results were considered to be significant at $p < 0.05$. Correlation is considered to be significant when the correlation coefficient (r) > 0.5 and $p < 0.05$.

CHAPTER III. CHARACTERIZATION OF IMMUNE FUNCTION IN SHR

I. INTRODUCTION

Lymphocytes are major effector cells in the immune system. Activation of lymphocytes initiated by antigen recognition is the biological event that enables lymphocytes to mount a useful immune response to foreign antigens. Lymphocyte activation includes a series of interrelated steps: an early signal transduction event, transcriptional activation of a variety of genes, expression of new cell surface molecules, secretion of cytokines, and the end result of proliferation or clonal expansion of the antigen specific lymphocytes. Naturally, the number of individual cells with a particular antigen specificity represents a very small fraction of the total cell number and, thus, it is not possible to measure antigen-specific responses. Functional responses of lymphocytes can be more easily studied by the use of polyclonal activators, which bind to many or all TCR:CD3 complexes or bypass this event, regardless of their antigen specificity, and mimic large scale activation events, which may occur normally *in vivo*.

In this study, the following activators were used to study the function of SHR lymphocytes compared with that of WKY lymphocytes. Con A and PHA are polymeric plant proteins called lectins which are strong mitogens. They bind specifically to certain sugar residues on T cell surface glycoproteins (Goldstein and Poretz, 1986), including the TCR:CD3 complex. They thereby stimulate the T cells

and induce lymphocyte proliferation and release of cytokines (Lis and Sharon, 1986). Con A and PHA have been widely used to study the function of lymphocytes.

IL-2, a T cell-derived cytokine with several immunoregulatory functions, is known to be the principal cytokine responsible for clonal expansion of antigen or mitogen activated T cells (Gillis and Smith, 1977; Shaw *et al.*, 1978). IL-2 functions as an autocrine and paracrine growth factor. Because IL-2 stimulates lymphocyte growth, the IL-2-T cell system provides the means to investigate fundamental questions in immunology as well as in other fields. These include the understanding of lymphocyte regulation and immune function and also regulation of both normal and neoplastic cell growth.

PKC is presumably activated, as a result of an increase in diacylglycerol and calcium. The relevance of PKC activation to the functional activation of T cells is supported by the fact that PKC activators and calcium ionophores act synergistically to promote the later differentiation and proliferation of T cells (Goldsmith *et al.*, 1989). The fact that neither PKC activators nor calcium ionophores alone are sufficient for T cell activation has been interpreted as evidence for a two-signal model of T cell activation in which T cells require at least two different signals in order to generate a full response. One signal may be provided by the binding of MHC-associated antigen to TCR or the binding of a mitogen to cell surface sugar residues. The second signal may act through accessory molecules or unidentified co-stimulatory factors (Abbas *et al.*, 1991a).

A substantial number of reports have shown that hypertension in SHR may

result, in part, from immune dysfunction. In this thesis, the proliferation response of lymphocytes to activators and IL-2 production were first investigated in order to better understand the nature of immunological dysfunction in SHR.

II. EXPERIMENTAL DESIGN

A. Proliferation responses of three populations of lymphocytes: a) spleen cells which are a mixture of cells containing T cells, B cells, macrophages, and others, b) thymocytes which are mature and developing T cells isolated from thymus and c) T-enriched lymphocytes which are spleen cells from which adherent cells including macrophages have been removed by adherence onto a plastic surface. To study function of immune system, the proliferation responses of these cells were carried out as described in the Materials and Methods chapter. Briefly, the spleen cells, thymocyte and T enriched lymphocytes were cultured in a 96 well tissue plate. Different concentrations of Con A or IL-2, 20 μg/ml PHA or 1×10^{-7} M PDB plus 1×10^{-6}M ionomycin were added into the cultures and tested separately. Three days after incubation at 37℃ in a CO_2 incubator, the proliferation responses of these cells were determined by ^{3}H-thymidine incorporation.

B. Effect of exogenous IL-2 or IL-1β on the mitogen-induced proliferation response of spleen cells from SHR and WKY. Cytokines such as IL-2 and IL-1 play important role in lymphocyte activation and proliferation. A reduced lymphocyte proliferation response could result from a deficiency of these cytokines. To examine

this possibility, different concentrations of IL-2 or IL-1β were added into the spleen cell culture simultaneously with 2.5 μg/ml Con A for three days. The proliferation responses were determined by ^{3}H-thymidine uptake.

C. Effect of the supernatant from SHR or WKY spleen cell culture on the proliferation responses of SHR and WKY spleen cells. A reduced lymphocyte proliferation response might be due to the inhibition caused by substances which present in the culture medium. To test this possibility, the spleen cells isolated from SHR or WKY were cultured in 12 well tissue culture plates at the concentration of 1x10^6/ml in the presence of 2.5 μg/ml Con A for 72 hours. The culture supernatant was collected by centrifugation at 200 x g, at 4°C for 15 minutes. The supernatant was kept at -20°C until tested. Three preparations of supernatant (40 μl) from SHR and three from WKY were added into SHR or WKY spleen cell culture (200 μl in each well) in the presence of 2.5 μg/ml Con A, and incubated for 72 hours. The proliferation response of spleen cells was determined by ^{3}H-thymidine uptake.

D. Mixed culture of spleen cells from SHR and WKY. Spleen cells are a mixture of cells including the cells which have inhibitory function. A lymphocyte inhibition could be due to these inhibitory cells presented in spleen cell culture. To test this possibility, the spleen cells from SHR and WKY were mixed with one another. The group designated control 1 contained only SHR or WKY spleen cells (2 x 10^{5}cells/well). The group designated control 2 also contained SHR or WKY spleen cells but was only 80% of the total cell number of control 1 (1.6 x 10^{5}cells/well). The mixture group contained 80% (1.6 x 10^{5}cells/well) of one type

of cells plus 20% (0.4 x 10^5 cells/well) of another type of cells. The proliferation response was induced by 2.5 μg/ml Con A and determined by ^{3}H-thymidine uptake.

E. IL-2 production by spleen cells from SHR and WKY. The ability to produce IL-2 is an important index of T cell function. The spleen cells of SHR or WKY were stimulated with 2.5 or 5 μg/ml Con A for 48 hours. The amount of IL-2 produced by the spleen cells was determined by a bioassay as described in the Materials and Methods chapter.

III. RESULTS

A. SHR spleen cells showed a significantly attenuated response to Con A (*t* test), exhibiting only 10 - 20% of the response of WKY spleen cells (Figure III-1). The proliferation response of SHR spleen cells to IL-2 was also significantly reduced (Figure III-2). Decreased proliferation responses of SHR spleen cells were also observed after administration of PHA and phorbol 12,13-dibutyrate (PDB), a protein kinase C stimulator, plus ionomycin (Figure III-3).

B. When exogenous IL-2 was added to SHR spleen cell culture in the presence of 2.5 μg/ml Con A, the reduced proliferation response persisted (Figure III-4). Exogenous IL-1 also failed to restore the reduced proliferation response of SHR spleen cells, even at high doses (Figure III-5).

C. WKY spleen cell culture medium did not have any significant effect on the proliferation response of SHR spleen cells nor did SHR culture medium on WKY

spleen cell proliferation (Table III-1). When WKY spleen cells were co-cultured with SHR spleen cells (20%), the proliferation response of WKY was slightly, but not significantly, decreased. On the other hand, when SHR spleen cells were co-cultured with WKY spleen cells (20%), the proliferation response of SHR was slightly, but not significantly, increased (Figure III-6). The amount of proliferation of the added portion (20%) of lymphocytes was probably too small in both cases to produce any significant effect.

D. IL-2 production of spleen cells in response to Con A was tested in both SHR and WKY. The results show no significant difference in the production of IL-2 between SHR and WKY (Figure III-7).

E. However, SHR thymocytes and T-enriched lymphocytes had very similar proliferation responses to those from WKY. There was no statistically significant difference in proliferation between SHR and WKY in response to Con A (Figure III-8; Figure III-9) and IL-2 (Figure III-10; Figure III-11), except that SHR T-enriched lymphocytes had a greater response to IL-2 when compared to those of WKY (Figure III-11). SHR thymocytes and SHR T-enriched lymphocytes also had similar proliferation responses to PHA and PDB plus ionomycin compared with those of WKY (Figure III-12; Figure III-13).

F. After removing macrophages from SHR spleen cells, the proliferation response of SHR spleen cells increased dramatically and reached the same level as, or became even higher than, those from WKY (Figure III-14).

IV. DISCUSSION

It has been reported that the proliferation responses of peripheral lymphocytes, lymph node cells, and spleen cells obtained from SHR were significantly depressed when compared with those from WKY (Takeichi *et al.*, 1980; Bendich *et al.*, 1981; Strausser, 1983). The depressed proliferation response to T cell mitogens, Con A and PHA, were more substantial than the response to B cell mitogen. In addition, the number of rosette-forming cells was also decreased suggesting that the number of T cells is reduced in SHR. In a recent report, patients with malignant hypertension exhibited a reduced T cell count and a depressed lymphocyte proliferation response to Con A (Hilme *et al.*, 1993). Based on these observations, it has been suggested that SHR T cells had a proliferation defect. With a deficit in T cells, especially a deficit in the T suppressor subset, autoantibodies were generated as seen in hypertensive patients and SHR. Therefore, in this study, the proliferation responses of SHR spleen cells to Con A, PHA and IL-2 were first investigated. The results showed that the response of SHR spleen cells, which contain T cells, B cells and macrophages, was markedly decreased when compared with spleen cells from WKY.

To determine the mechanism responsible for this proliferation defect, the PKC pathway was studied using PDB plus ionomycin to stimulate PKC in SHR lymphocytes. The severely reduced proliferation response of SHR spleen cells suggested two possibilities. One was that the defect might occur at the level of PKC

or its downstream. An abnormal PKC distribution was found in spleen, thymus and aorta from SHR and gamma-interferon corrected aberrant PKC levels in SHR (Sauro and Hadden, 1992) suggesting that an abnormal PKC pathway may exist in SHR. Another possibility might be that the whole signal transduction pathway in SHR lymphocytes is intact and normal, and the reduced proliferation response is a result of a lack of cytokines, and/or a lack of the second signal or co-stimulator that is required to activate lymphocytes. It is also possible that this lymphocyte inhibition may be due to the presence of inhibitory substances or inhibitory cells in the spleen cell population. Since the signal transduction events are not clear beyond the level of PKC at the present time, the second possibility was investigated.

To study the mechanism of lymphocyte depression, the effects of IL-2 on the mitogen induced proliferation response of SHR spleen cells were tested. The results showed that IL-2 failed to restore the decreased Con A induced proliferation response in SHR. This result agreed with a recent report that IL-2 cannot restore the Con A activated suppressor T cell activity of SHR when administrated *in vivo* or *in vitro* (Ofosu-Appiah *et al.*, 1993). However, they showed that IL-2 restored suppressor T cell function in the syngeneic mixed lymphocyte reaction. Their explanation for these conflicting results was that either SHR lost precursors of Con A activatable suppressor cells or that the generation of Con A activated suppressor T cells may require other growth factors.

IL-2 is the major growth factor for T cells and other cells in the immune system. The quantity of IL-2 synthesized by activated helper T cells is an important

determinant of the magnitude of the immune response. Antigen or mitogen-driven T cell activation stimulates IL-2 production and the expression of high-affinity receptors for IL-2, which allows the cell to receive an IL-2 growth signal in either an autocrine or paracrine fashion (Kuziel and Greene, 1990). It has been demonstrated that the proliferation response of lymphocy[illegible] [illegible]duced in lupus-prone mice, in BB rats with autoimmune diabetes and in pa[illegible]ents [illegible] systemic lupus erythematosus, rheumatoid arthritis and active multiple sclerosis. In those pathological conditions, there was often a markedly decreased IL-2 production in cell culture (Kroemer and Wick, 1989). The incapacity of helper T cells to produce IL-2 was attributed to the decreased proliferation responses (Kroemer and Wick, 1989). Tuttle and Boppana reported that a bolus injection of IL-2 prevented the increase in blood pressure in youn[illegible] [illegible]HR (1990). However, other investigators have failed to confirm this o[illegible] (Dzielak 1991; Pascual *et al.*, 1992). Recently, it has been reported that IL-2 [illegible]ent lowered [illegible]lood pressure in Dahl salt-sensitive rats. This antihypertensive effect was associated with an in[illegible]ease in glomerular filtration rate and reduction in cardiac weight (Ishimitsu *et al.*, 1994). Therefore, it was of great interest to investigate the capability of SHR T cells to synthesize IL-2. The results showed that SHR spleen cells had the same capacity to produce IL-2 as did those from WKY, suggesting that the function of SHR T cells might be essentially normal.

The possibility of a lack of IL-1 in SHR spleen cell culture was tested by adding exogenous IL-1β to induced SH[illegible] [illegible]een cell culture in the presence of Con A. The results showed that IL-1β could not restore the depressed proliferation

response. Thus, the reduced proliferation response of SHR spleen cells does not appear to be caused by a lack of production of IL-2 or of IL-1β which is able to induce proliferation. Thus far, thirteen cytokines have been identified. The functions of most of them have been elucidated. Although the capacity of SHR lymphocytes to produce these cytokines could be altered, screening the whole cytokine profile in SHR would be both laborious and time consuming. It was considered not practical to be performed in this thesis. Therefore, other possibilities were studied.

The effect of culture medium from SHR or WKY spleen cells on lymphocyte proliferation response was examined for the presence of any released inhibitory or stimulatory substances. The results showed that SHR spleen cell culture medium did not have any stable inhibitory effect on the WKY proliferation response. In addition, the culture medium from WKY spleen cells did not have any stable stimulatory effect on the SHR proliferation response. However, this did not rule out the presence of an inhibitory substance in the culture medium that was not detected because of its instability. An experiment involving co-culture of SHR and WKY spleen cells was also carried out to determine whether SHR spleen cells had the ability to inhibit WKY spleen cells by cell-cell contact. The results of this experiment showed that SHR spleen cells slightly inhibited the proliferation response of WKY spleen cells while WKY spleen cells slightly increased the proliferation response of SHR spleen cells. This small decrement in WKY response and small increment in SHR response may be due to the small proportion of the added cells (20% of total

cell number). It is also possible that the effect of the added cells, i.e, the inhibitory effect of SHR spleen cells or the stimulatory effect of WKY spleen cells, was shielded by the response of the majority of the cells in the culture. The data from this experiment do not provide any conclusive answers to the question under investigation.

A striking result in this present study involved T cells which were purified from SHR spleen cells. The proliferation response of those T-enriched cells to all mitogens and activators tested was normal. The same result was observed in the proliferation response of SHR thymocytes. These results clearly indicated that SHR T cells are normal and that they have the same ability to proliferate as do those from WKY (Xiao *et al.*, 1991). This fact is supported by the finding that SHR spleen cells have the same capacity to produce IL-2. More than ten years ago it was reported that the proliferation response of SHR lymphocytes was depressed, and it has always been thought that this defect resided in the SHR T cells, especially suppressor T cells (Takeichi *et al.*, 1981; Norman *et al.*, 1985; Fannon *et al.*, 1992). In these investigations, however, only proliferation responses of mixed cells such as spleen cells, lymph node cells and peripheral blood lymphocytes were studied. The present study demonstrates for the first time that the proliferation defect of SHR lymphocytes does not reside in the T cells themselves because enriched T cells and thymocytes exhibit normal proliferation responses. This suggests that cells other than T cells may be responsible for this proliferation defect as seen in mixed lymphocyte populations.

To investigate the cell type that was involved in this proliferation defect in SHR, macrophages were removed from the spleen cell population. After removing the macrophages, the proliferation response of these macrophage-depleted spleen cells was increased dramatically and reached the same level as, or was even higher than, that in WKY (Xiao *et al.*, 1991). A similar result was reported later by Pascual and co-workers (1992) showing that SHR spleen cells had proliferation responses equal to or greater than those of WKY after removal of adherent cells. Since macrophages were removed from the SHR spleen cells, the remaining cells included T cells and B cells. These remaining cells showed a normal response not only to T cell mitogens but also to other activators, suggesting that B cells might not be involved in the SHR spleen cell proliferation defect. Indeed, it has been observed that the number of blood lymphocytes capable of producing Ig A (B cells) in response to B cell mitogens is increased in SHR (Chen and Sanders, 1993) suggesting that the proliferation response in SHR B cells may be increased. Therefore, the involvement of B cells in this defect can be ruled out.

In summary, the proliferation response of SHR spleen cells was depressed. However, the proliferation responses of SHR thymocytes and T-enriched lymphocytes were normal. In addition, SHR T cells had same capacity to produce IL-2 as those of WKY. Removing macrophages restored the depressed proliferation response of SHR spleen cells.

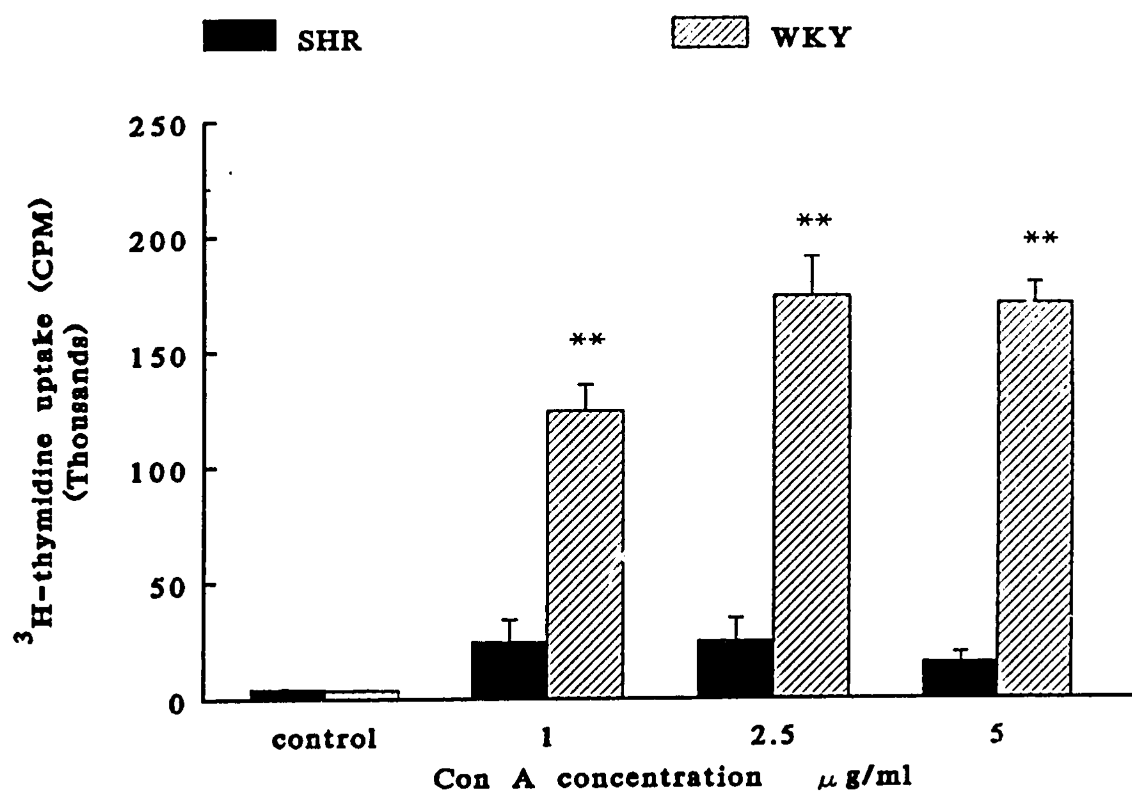


Figure III-1. The proliferation response of spleen cells from SHR and WKY in response to Con A. The spleen cells (2×10^5 cells/well) were dispensed in 96 well tissue culture plates in quadruplicate. The different concentrations of Con A were added into the culture and incubated for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The cells were then harvested and counted. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five rats. Significant difference: ** $P < 0.01$, compared with respective SHR spleen cells.

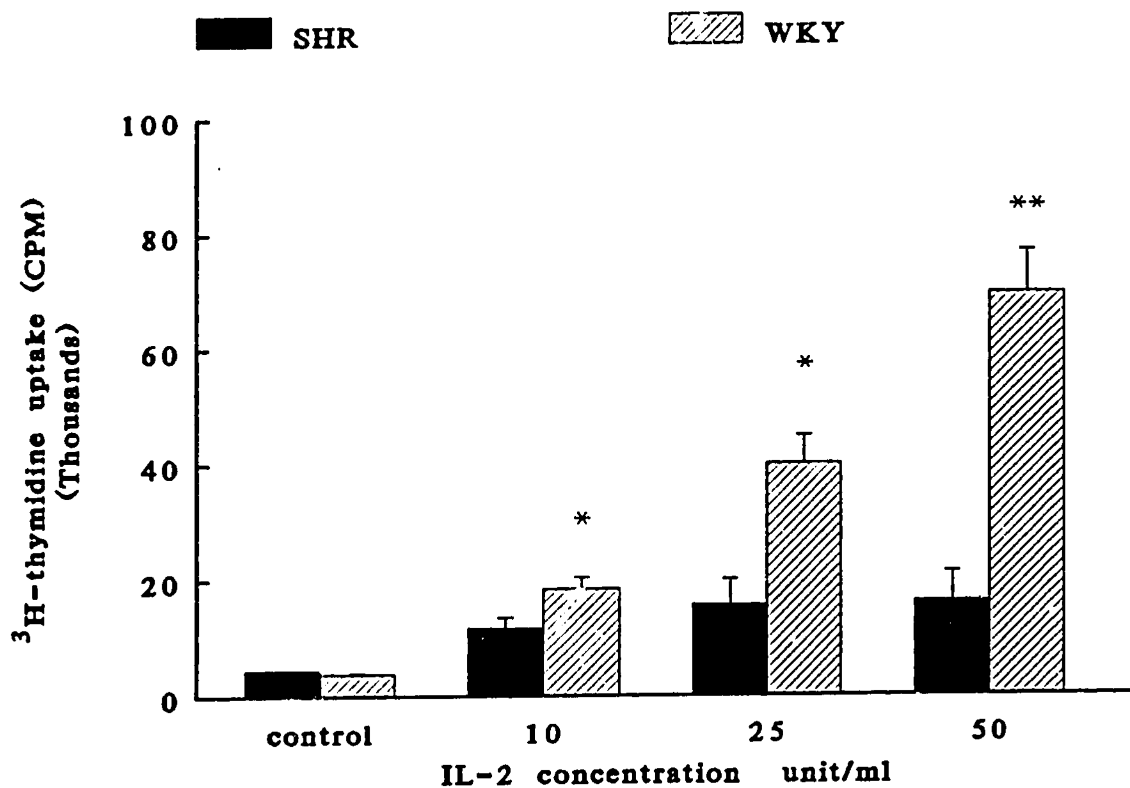


Figure III-2. **The proliferation response of spleen cells from SHR and WKY in response to IL-2.** Different concentrations of IL-2 were added into the spleen cell culture and incubated for 72 hours as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five rats. Significant difference: * $P < 0.05$; ** $P < 0.01$, compared with respective SHR spleen cells.

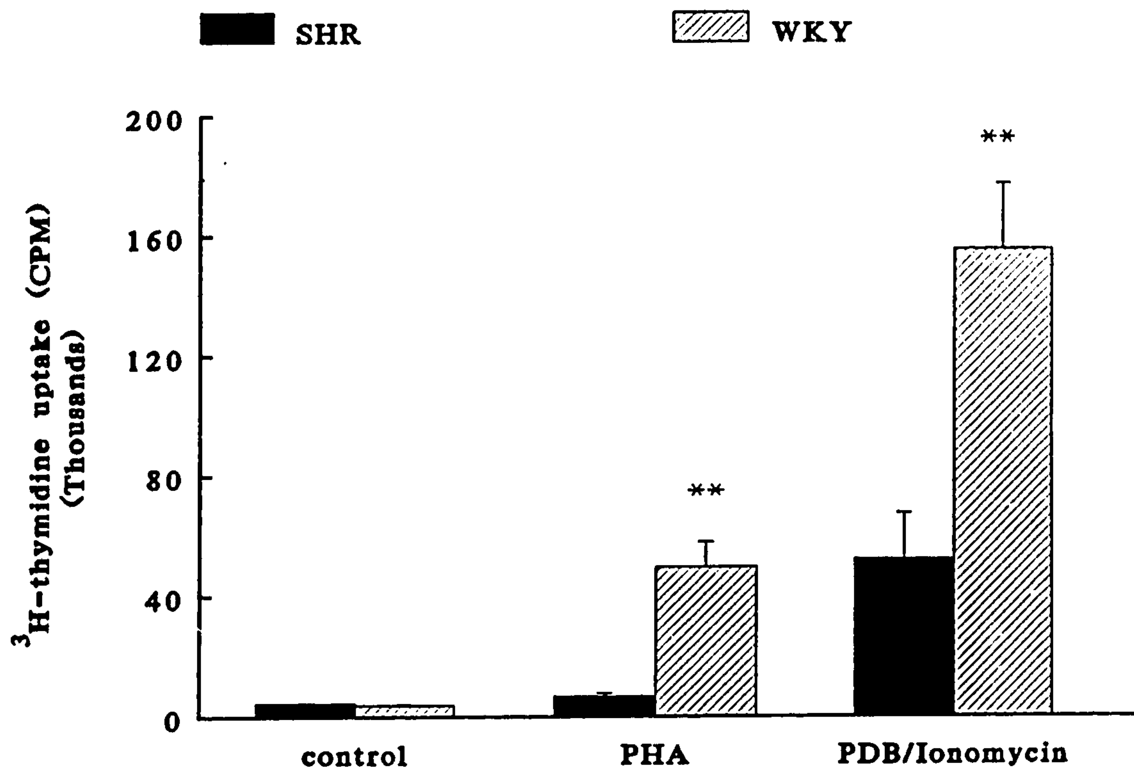


Figure III-3. **The proliferation response of spleen cells from SHR and WKY in response to PHA or PDB plus ionomycin.** PHA (20 μg/ml) or PDB ($1x10^{-7}$ M) plus ionomycin ($1x10^{-6}$ M) was added to the spleen cell culture. The culture was then incubated for 72 hours as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from four rats. Significant difference: ** $P < 0.01$, compared with respective SHR spleen cells.

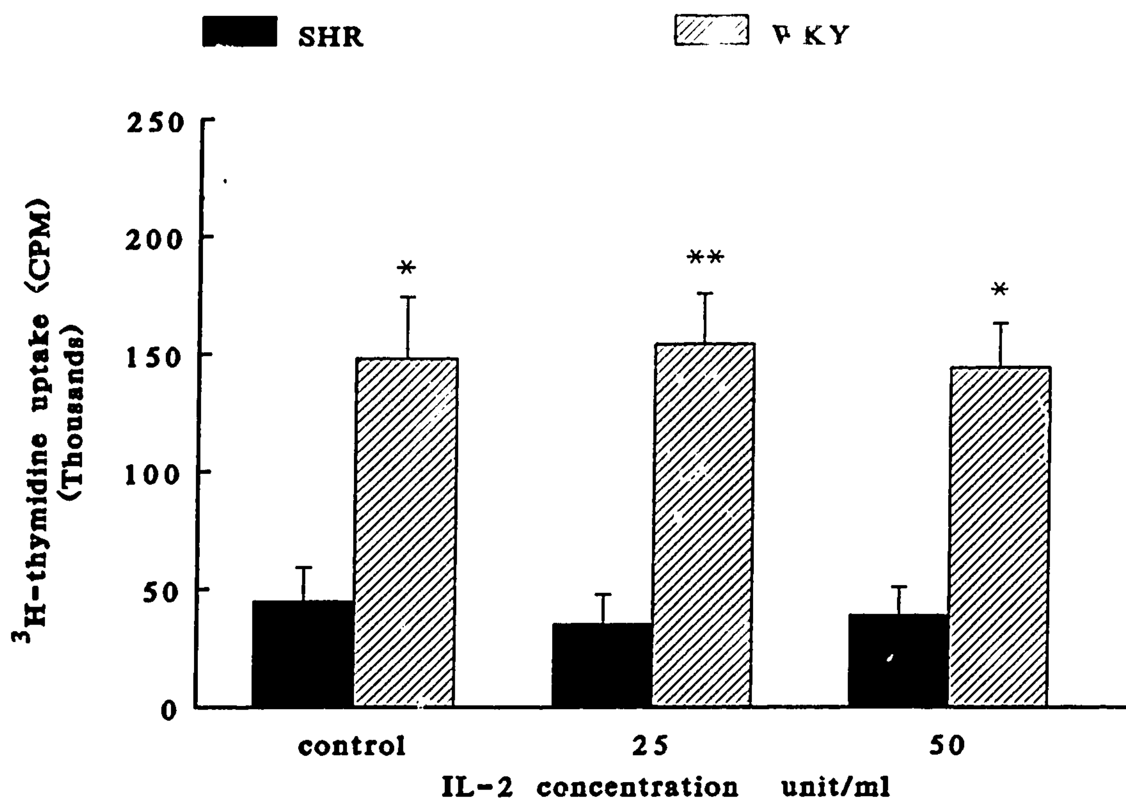


Figure III-4. **The effect of IL-2 on the proliferation response of spleen cells in response to Con A.** IL-2 (25 or 50 unit/ml) were added to the spleen cell culture in the presence of 2.5 μg/ml Con A and incubated for 72 hours. The data represent the means of ^{3}-thymidine uptake by cells (CPM) per well ± SE from four rats. Significant difference: * $P < 0.05$; ** $P < 0.01$, compared with respective SHR spleen cells.

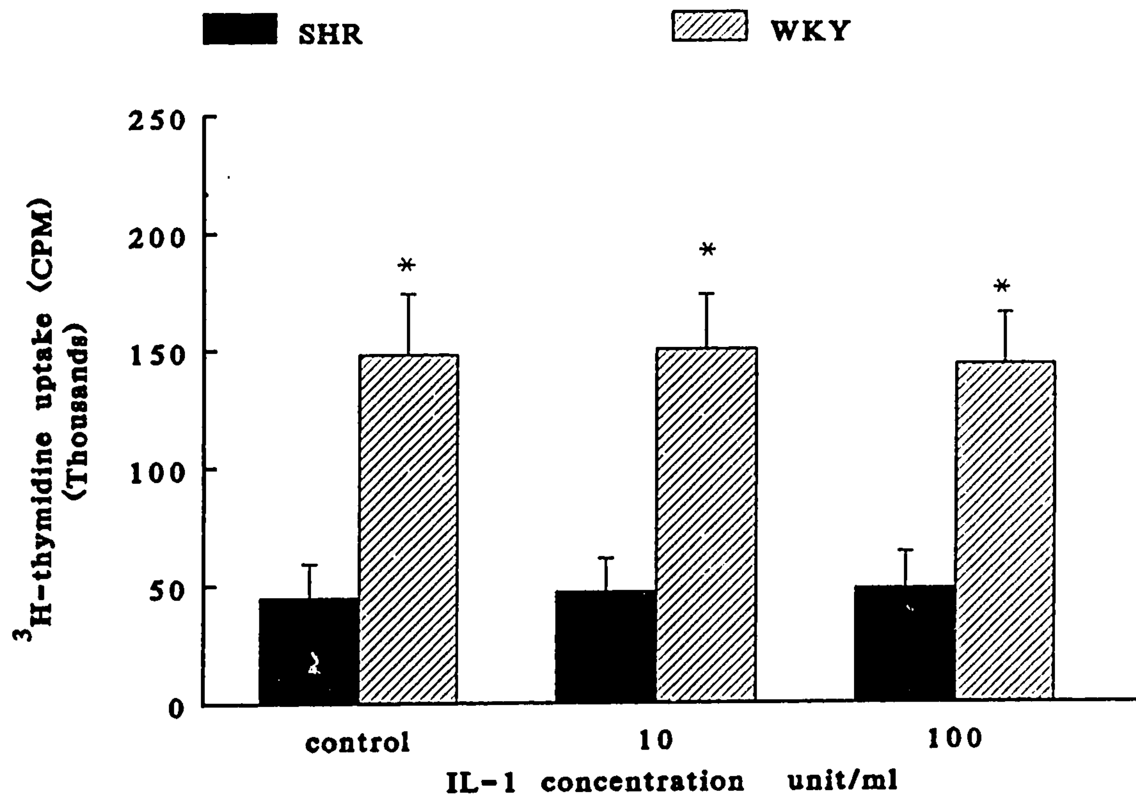


Figure III-5. **The effect of IL-1β on the proliferation response of spleen cells in response to Con A.** IL-1β (10 or 100 units/ml) were added to the spleen cell culture in the presence of 2.5 μg/ml Con A and incubated for 72 hours. The data represent the means of ^{3}H-thymidine uptake by cells (CPM) per well ± SE from four rats. Significant difference: * $P < 0.05$, compared with respective SHR spleen cells.

TABLE III-1

Effect of culture supernatant from SHR or WKY spleen cell culture on lymphocyte proliferation response

		% changes	
	control	WKY supernatant	SHR supernatant
WKY	100	100 ± 2.9	98 ± 4.1
SHR	100	103 ± 15	98.5 ± 7.9

The spleen cells from SHR and WKY (1 x 10^6 cells/ml in 5 ml) were cultured for 72 hours in the presence of 2.5 μg/ml Con A. The culture supernatant was collected. The culture supernatant (40 μl) was added into each well (200 μl) of spleen cell culture in the presence of 2.5 μg/ml Con A and incubated for 72 hours as described in Figure III-1. The values represent the % changes in uptake of ^{3}H-thymidine (CPM) by spleen cells from SHR or WKY.

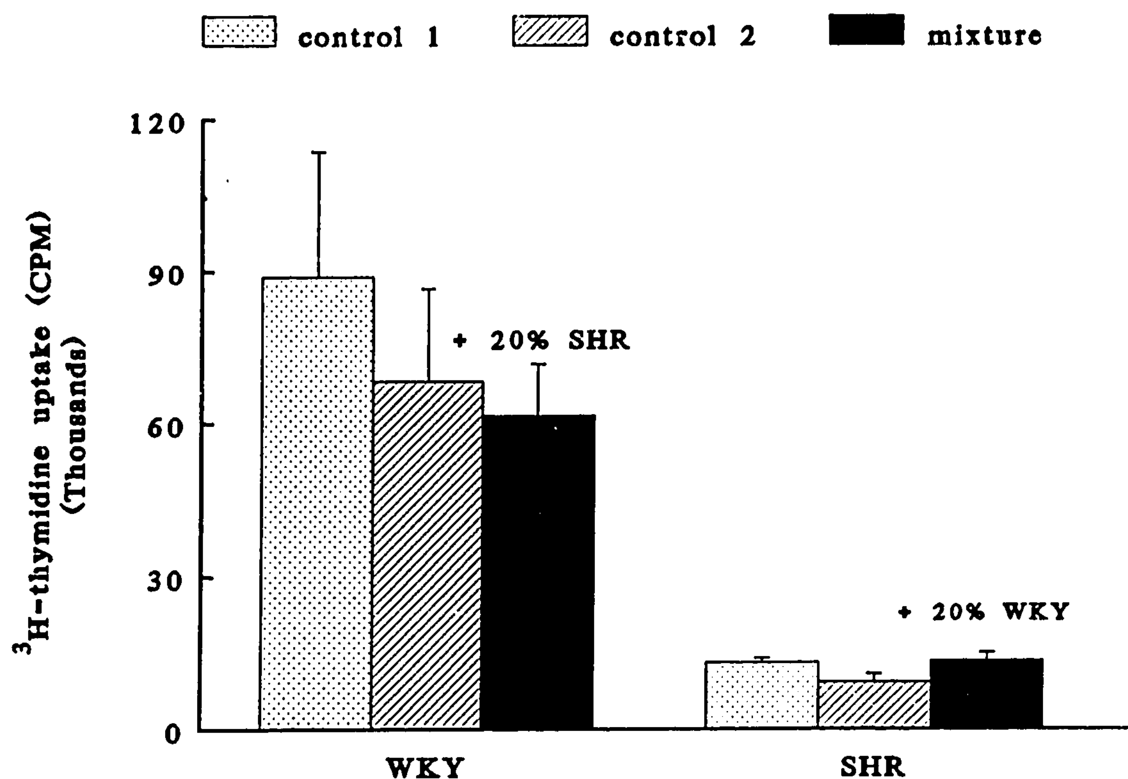


Figure III-6. **The mixed culture of spleen cells from SHR and WKY.** The spleen cells from SHR or WKY were mixed with one another. The group of control 1 contained only SHR or WKY spleen cells (2 x 10^5cells/well). The group of control 2 also contained SHR or WKY spleen cells but only 80% of the total cell number (1.6 x 10^5cells/well). The mixture group contained 80% (1.6 x 10^5cells/well) of one type of cells plus 20% (0.4 x 10^5 cells/well) of another type of cells. The proliferation response was induced by 2.5 μg/ml Con A and determined by ^{3}H-thymidine uptake. The data represent the means of ^{3}H-thymidine uptake by cells (CPM) per well ± SE from four experiments.

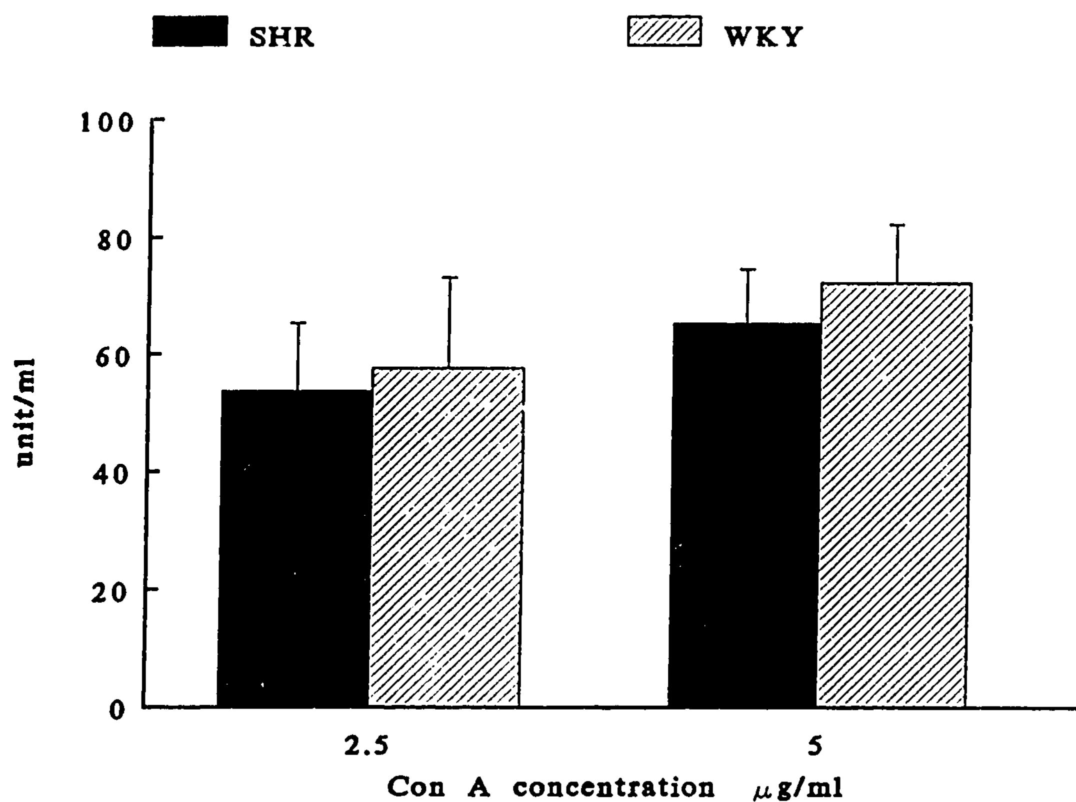


Figure III-7. **IL-2 production by spleen cells from SHR and WKY.** The spleen cells from SHR or WKY (1 x 10^6 cells/ml) were stimulated by 2.5 or 5 μg/ml Con A for 48 hours. The amount of IL-2 present in the culture supernatant was tested by a bioassay. One unit is defined as the amount of IL-2 that is required to support half-maximal ^{3}H-thymidine incorporation into CTLL-2 cells. N=14.

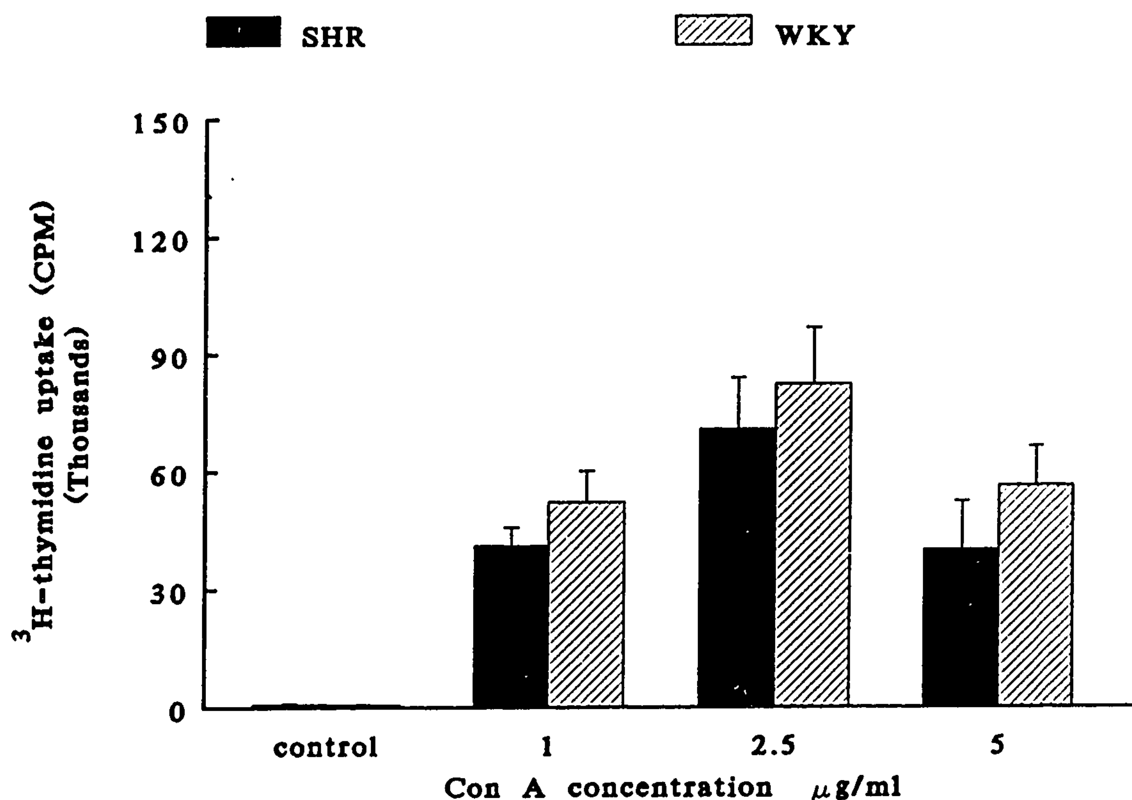


Figure III-8. **The proliferation response of thymocyte from SHR and WKY in response to Con A.** The thymocytes from SHR or WKY (2 x 10^5 cells/well) were dispensed in 96 well tissue culture plates in quadruplicate. The various concentrations of Con A were added into the culture and incubated for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The cells were then harvested and counted. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five rats.

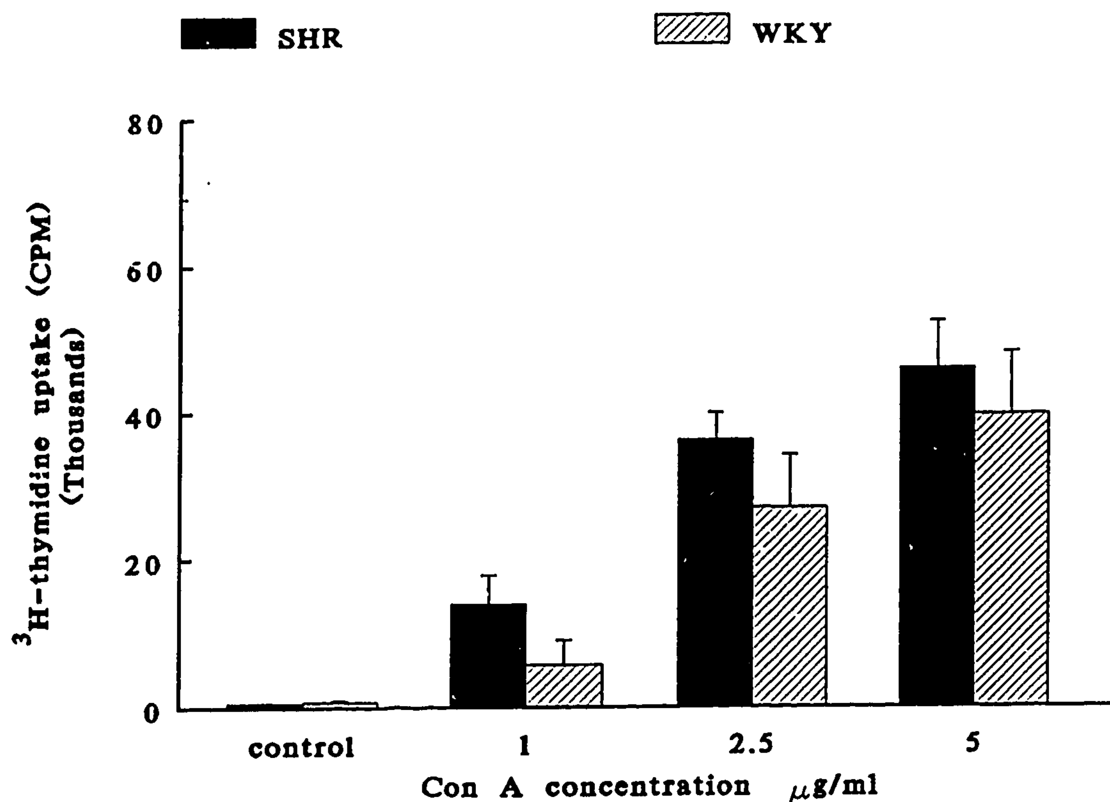


Figure III-9. **The proliferation response of T-enriched lymphocytes from SHR and WKY in response to Con A.** The T-enriched lymphocytes from SHR or WKY (2 x 10^5 cells/well) were dispensed in 96 well tissue culture plates in quadruplicate. The various concentrations of Con A were added to the culture and incubated for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The cells were then harvested and counted. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five rats.

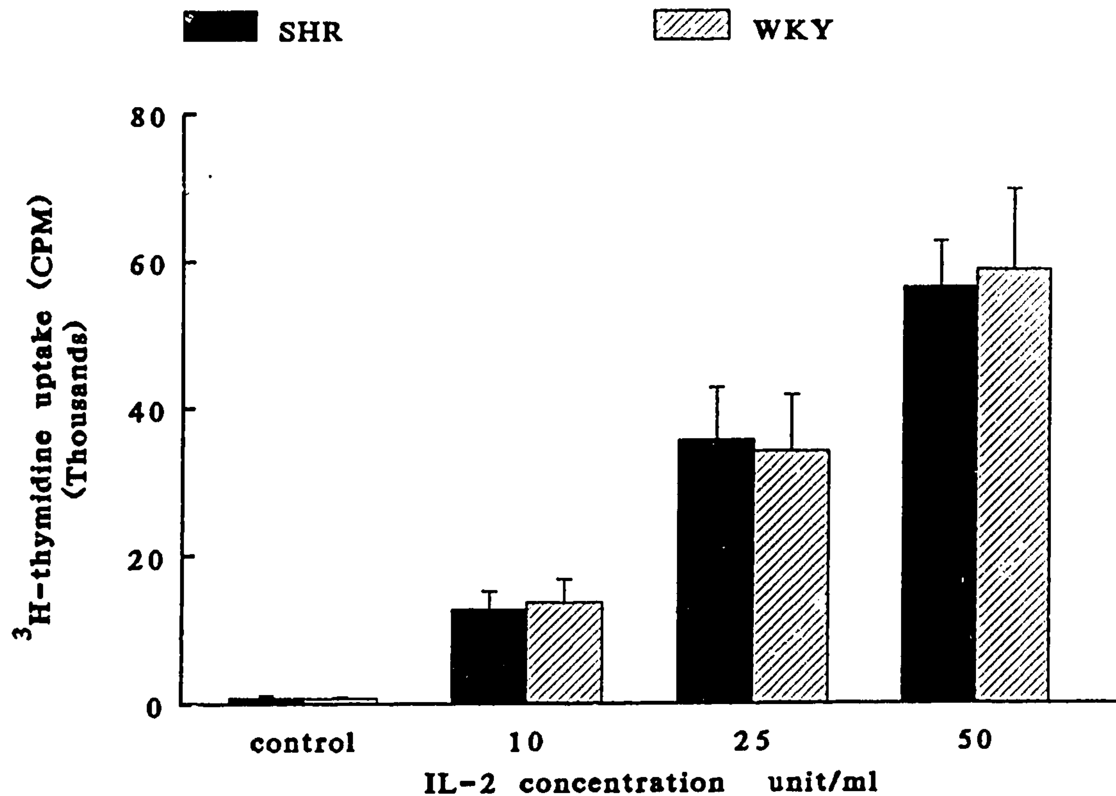


Figure III-10. **The proliferation response of thymocyte from SHR and WKY in response to IL-2.** Various concentrations of IL-2 were added to the thymocyte culture and incubated for 72 hours as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five rats.

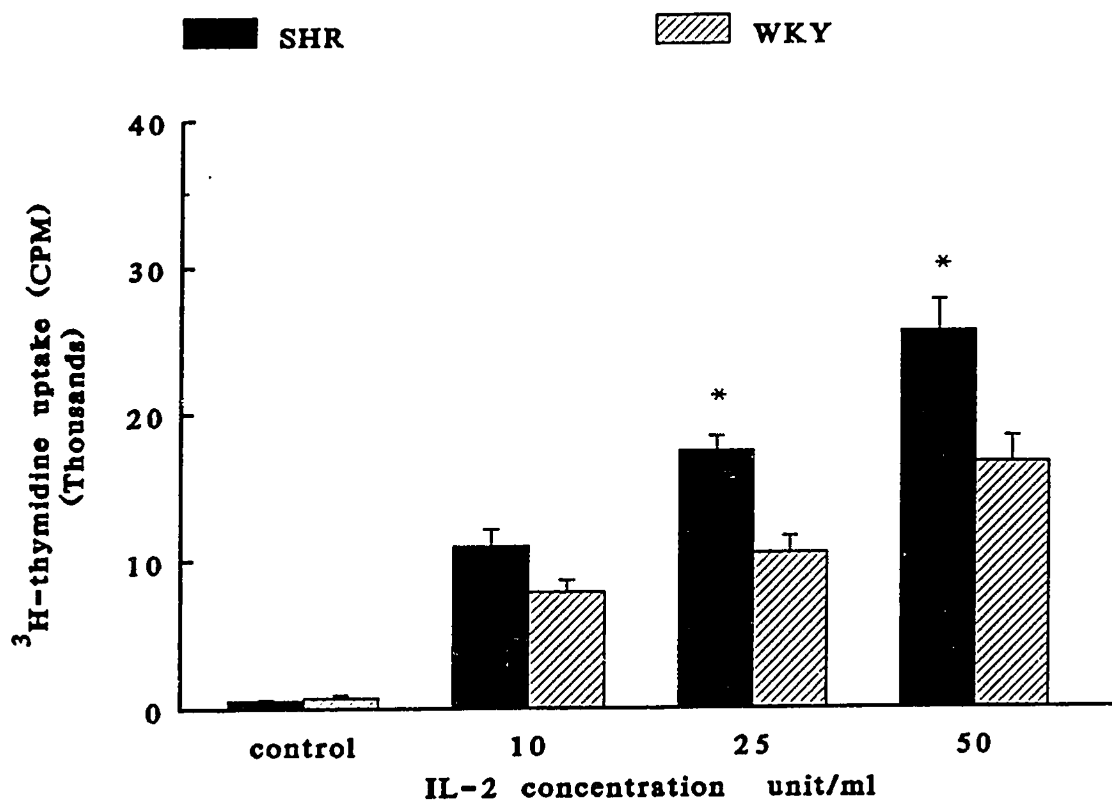


Figure III-11. **The proliferation response of T-enriched lymphocytes from SHR and WKY in response to IL-2.** Various concentrations of IL-2 were added into the T-enriched lymphocyte culture and incubated for 72 hours as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five rats. Significant difference: * $P < 0.05$, compared with respective WKY cells.

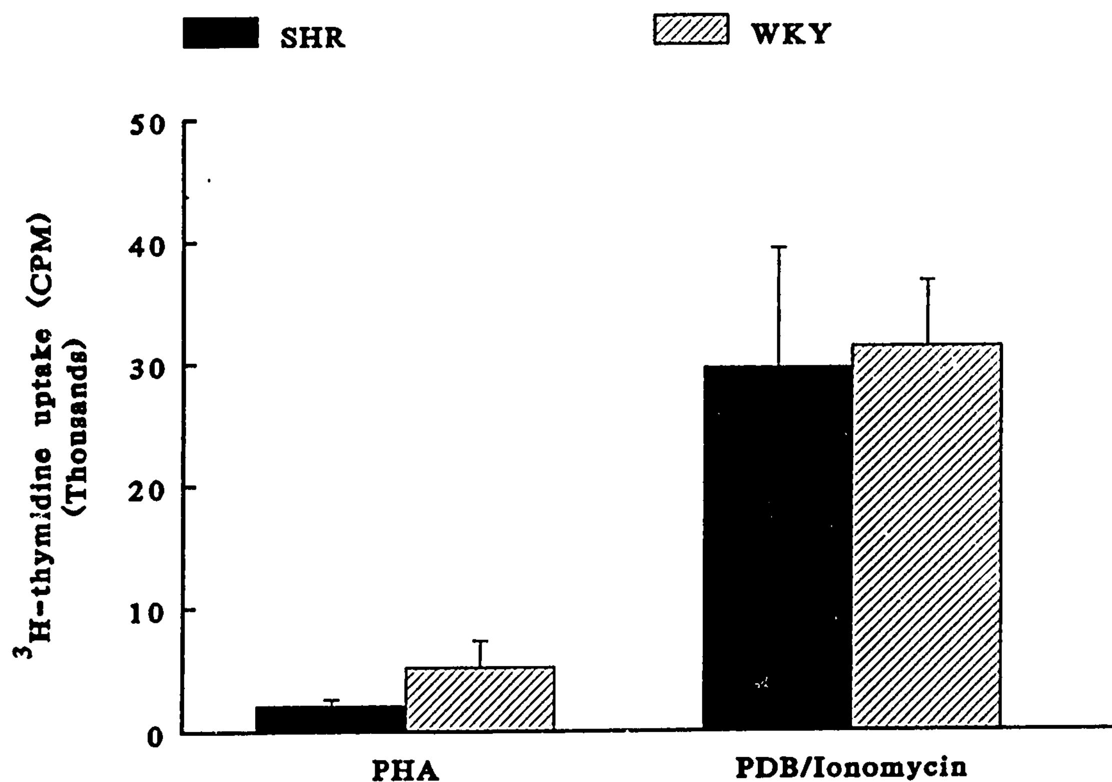


Figure III-12. The proliferation response of thymocytes from SHR and WKY in response to PHA or PDB plus ionomycin. PHA (20 μg/ml) or PDB ($1x10^{-7}$ M) plus ionomycin ($1x10^{-6}$ M) was added to the thymocyte culture and incubated for 72 hours as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from four rats.

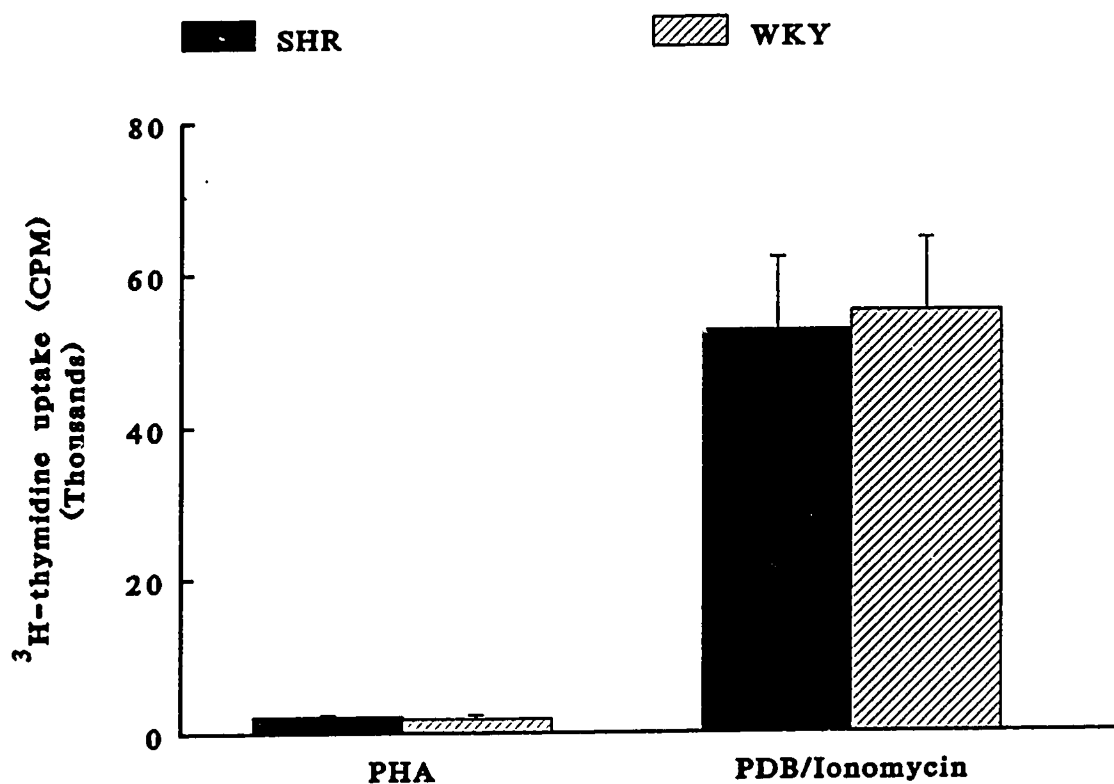


Figure III-13. **The proliferation response of T-enriched lymphocytes from SHR and WKY in response to PHA or PDB plus ionomycin.** PHA (20 μg/ml) or PDB ($1x10^{-7}$ M) plus ionomycin ($1x10^{-6}$ M) was added to the T-enriched lymphocyte culture and incubated for 72 hours as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from four rats.

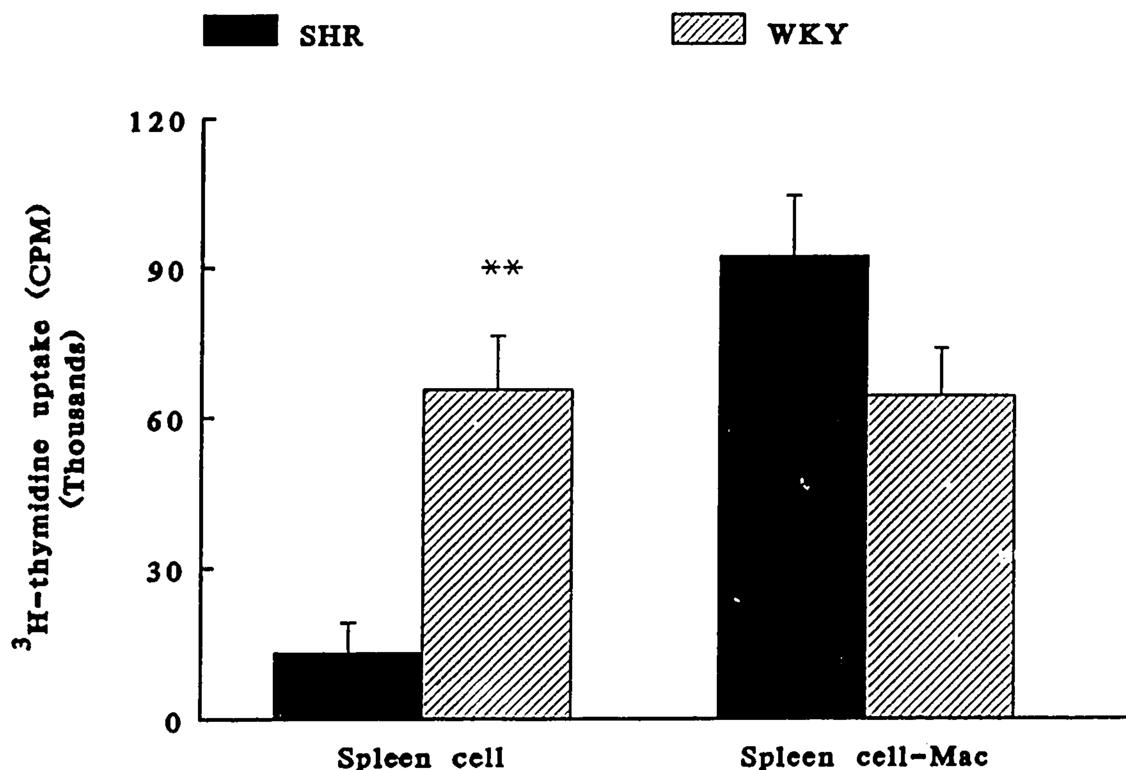


Figure III-14. **The effect of removal of macrophages on the proliferation response of spleen cells in response to Con A.** Spleen cells (2-5x10^7 in 10 ml of 5% FCS RPMI-1640) were incubated in a 90x20 mm plastic tissue culture dish for 60 minutes. The nonadherent lymphocyte suspension was transferred into a new dish and incubated for another 60 minutes. The cell suspension was centrifuged. The cell pellet, which contained macrophage-depleted lymphocytes (**Spleen cell-Mac**), was re-suspended with complete medium. The proliferation responses of the macrophage-depleted lymphocytes to 2.5 μg/ml Con A were examined as described in Figure III-1. The data represent the means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from five experiments. Significant difference: ** $P < 0.01$, compared with SHR spleen cells.

CHAPTER IV. MACROPHAGES AND NITRIC OXIDE PATHWAY IN LYMPHOCYTE ABNORMALITY OF SHR

I. INTRODUCTION

Macrophages play a key role in diverse aspects of immune responses, including the regulation of specific and nonspecific immunity, by virtue of their capacity to either augment or inhibit lymphocyte activation or proliferation (Unanue and Allen, 1987). Antigen processing and presentation, and the production of soluble factors such as IL-1 by macrophages are required for the development of T cell mediated immune responses (Gery and Handschumaker, 1974; Weaver and Unanue, 1990). On the other hand, macrophages have also been reported to act as "natural suppressor" cells that down-regulate lymphocyte-dependent immune responses (Metzger *et al.*, 1980; Denham and Rowland, 1992). Lymphocyte proliferation *in vitro* can be suppressed by the addition of excess macrophages to the culture system (Allison, 1978). Evidence has been presented suggesting that superoxide anion, prostaglandins, and other macrophage products may mediate this suppressive effect (Kung, 1977; Allison, 1978; Metzger *et al.*, 1980; Schultz, 1991). Recently, it was reported that the concentration of arginine available to macrophages, and the pathway utilized in its metabolism, play important roles in determining the functions of macrophages (Hibbs *et al.*, 1987a; Green *et al.*, 1990). In this regard, macrophages can convert arginine to NO and citrulline (Stuehr and Marletta, 1985; Mills, 1991)

and produce lymphocyte inhibition.

In the previous chapter, the removal of macrophages was shown to correct the depressed lymphocyte proliferation response in SHR. The expression of rat macrophage markers, ED1 or ED2 antigen, on different cell population was examined to prove that the cells removed by adhering process were macrophages. In order to confirm the involvement and the mechanism of action of these macrophages in lymphocyte proliferation inhibition, the effect of isolated SHR macrophages on the proliferation response of WKY spleen cells or SHR macrophage-depleted lymphocytes was tested. NO production in SHR macrophages in comparison with that in WKY macrophages was also investigated.

II. EXPERIMENTAL DESIGN

A. Examination of the expression of rat macrophage markers on spleen cells, macrophage-enriched adherent cells and macrophage-depleted lymphocytes from SHR and WKY. The details for the immunocytochemical staining of ED1 or ED2 antigen were described in the Materials and Methods chapter.

B. Effect of SHR or WKY macrophages on the lymphocyte proliferation response. Spleen cells from SHR or WKY (2×10^5cells/well) were incubated in 96 well tissue culture plates in a CO_2 incubator for 90 minutes. The medium, which contained non-adherent lymphocytes, was decanted. The plates were washed twice with HBSS to remove any residual non-adherent cells (Albina and Henry, 1991).

Most of the adherent cells were macrophages (Wahl and Smith, 1991). Macrophage-depleted lymphocytes (2 x 10^5 cells/well) were then added to these plates, to which the macrophages were already adhered, in quadruplicate and incubated in the presence of 2.5 or 5 μg/ml Con A for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours, and the cells were harvested. The amount of radioactivity incorporated into the cells was counted.

C. The effect of N^G-monomethy-L-arginine (L-NMMA) on lymphocyte proliferation responses. L-NMMA has been shown to inhibit the NO synthesis pathway specifically and to reverse the suppression of mouse lymphocyte proliferation brought about by NO (Albina *et al.*, 1991). In this study, various concentrations of L-NMMA were added to the lymphocyte culture simultaneously with 2.5 μg/ml Con A. The proliferation responses of SHR and WKY lymphocytes were carried out as described in the Materials and Methods chapter. In the study of the effect of L-NMMA exposure time on the SHR lymphocyte proliferation response, 0.05 mM L-NMMA was added to the culture of SHR spleen cells in the presence of 2.5 or 5 μg/ml Con A at various times during the 72 hours incubation period. The proliferation assay was carried out as described before.

D. Comparison of NO production in spleen cells and macrophages from SHR and WKY. Con A (2.5 μg/ml) or PHA (40 μg/ml) were used to induce NO synthesis in macrophages and spleen cells from SHR and WKY. The experimental procedures were described in the Materials and Methods chapter.

II. RESULTS

A. Figure IV-1 presents colour photos of the negative control, in which mouse IgG_1 (the same isotype as mouse anti-rat ED1 and ED2 antibody) was used as a primary antibody. Nuclei of the cells were stained by hematoxylin as blue colour dots. There was no dark red colour in the cytoplasm of the cells, indicating no nonspecific staining. The results of the expression of ED1 or ED2 antigen (rat macrophage marker) on spleen cells from SHR and WKY is shown in Figure IV-2. The cells with dark red colour deposits around the nuclei are the cells expressing ED1 or ED2 antigen. The number of ED1 positive cells in SHR spleen cells was not significantly different from that in WKY spleen cells. In addition, the number of ED2 positive cells in SHR spleen cells was very similar to that in WKY spleen cells. Figure IV-3 demonstrates the efficiency of the adherent process by which macrophages were removed from spleen cells. The numbers of ED1 positive ($ED1^+$) cells in the non-adherent cell population were minimal, indicating that the majority of macrophages had been removed. On the other hand, the adherent cells on the plastic surface of 96 well culture plates were mostly ED1 or ED2 positive (Figure IV-4). The results from cells in 96 well plates for negative staining are shown in Figure IV-5.

B. Figure IV-6 shows the effects of macrophages on lymphocyte proliferation responses. When SHR macrophages were co-cultured with WKY macrophage-depleted lymphocytes, the proliferation response of WKY macrophage-depleted

lymphocytes was significantly inhibited. As demonstrated before, removal of the macrophages from SHR spleen cells corrected the reduced SHR lymphocyte proliferation response. When SHR macrophages were added back to these SHR macrophage-depleted lymphocytes, the proliferation response to Con A was again significantly suppressed (examined by ANOVA). WKY macrophages had no significant effect on SHR or WKY lymphocyte proliferation responses.

C. The involvement of NO in the proliferation defect in SHR spleen cells was studied using L-NMMA. Figure IV-7 shows that L-NMMA at 0.05 mM can fully reverse the reduced proliferation response of SHR spleen cells in response to Con A. D-NMMA, a stereoisomer of L-NMMA, failed to correct the reduced SHR lymphocyte proliferation response (Figure IV-8).

D. Figure IV-9 shows the effects of different concentrations of L-NMMA on the spleen cells and macrophage-depleted lymphocytes obtained from SHR and WKY. L-NMMA markedly increased the proliferation response of SHR spleen cells in response to Con A. This effect occurred in a dose-dependent manner. L-NMMA had no significant effect on SHR macrophage-depleted lymphocytes nor on WKY spleen cells or WKY macrophage-depleted lymphocytes. In addition, L-NMMA had no significant effect on the proliferation response of thymocytes from either SHR or WKY (Figure IV-10).

E. The time course of the L-NMMA effect on the proliferation response of SHR spleen cells is shown in Figure IV-11. In order to completely reverse the suppressed SHR lymphocyte proliferation response, it was necessary to add L-

NMMA to the SHR spleen cell culture for at least 48 hours before termination of the cell culture at 72 hours. When L-NMMA was added to the culture for 24 hours before termination of the culture, the proliferation response of SHR spleen cells was only partially corrected.

F. Figure IV-12 shows the effect of L-NMMA on the inhibition of lymphocyte proliferation responses caused by SHR macrophages. L-NMMA at a concentration of 0.05 mM fully reversed the inhibition of the proliferation response of WKY macrophage-depleted lymphocytes or SHR macrophage-depleted lymphocytes brought about by SHR macrophages. Again, WKY macrophages had no significant effect on the lymphocyte proliferation response. L-NMMA had no effect on the proliferation response of lymphocytes co-cultured with WKY macrophages.

G. The results of NO synthesis by macrophages from SHR and WKY is shown in Figure IV-13. Without stimulation, a very small amount of NO was produced in either SHR or WKY macrophages. After being stimulated with Con A or LPS, SHR macrophages produced significantly higher levels of NO than did those of WKY. The production of NO by SHR spleen cells was also significantly higher than that of WKY spleen cells (Figure IV-14). In addition, L-NMMA at 0.05 mM, the same concentration used to reverse the inhibited lymphocyte proliferation response, inhibited the increased NO production in SHR spleen cells.

IV. DISCUSSION

The present study continues the investigation into the mechanism of immune abnormalities observed in SHR. In the previous chapter, it was demonstrated that the proliferation response of SHR spleen cells was severely decreased. This decreased response can be reversed by removing macrophages from the lymphocyte population, suggesting that SHR macrophages might be involved in this inhibition. In this study, the involvement of SHR macrophages in SHR lymphocyte depression was confirmed. SHR macrophages not only inhibited SHR lymphocytes but also inhibited WKY lymphocytes, suggesting that this inhibition brought about by SHR macrophages may be due to a soluble molecule released by SHR macrophages in the culture.

The inhibition of lymphocyte proliferation may also be due to an increased number of macrophages in SHR. It is known that ED1 antigen is expressed on most rat macrophages and monocytes but ED2 antigen is expressed on certain subpopulation of macrophages in rats (Dijkstra *et al.*, 1985; Westermann *et al.*, 1989). Therefore, a comparison of expression of these macrophage markers on the spleen cells between SHR and WKY was investigated. The staining of ED1 and ED2 antigens reveals that the percentages of cells expressing these antigen on SHR spleen cells were not significantly different from that in WKY spleen cells. Compared with an earlier study in which the percentage of ED1^{+} cells was 13% in spleen cells (Westermann *et al.*, 1989), the percentages of ED1^{+}cells were 18-23% in this study.

The reason for this difference is not known. That about 93% of adherent cells removed from spleen cell population and less than 3% of non-adherent spleen cells expressed ED1 antigen confirms that macrophages were the cells depleted from spleen cells and the macrophages are indeed the cells which induce lymphocyte inhibition in SHR.

Activated macrophages can produce a variety of substances. The list of these products is now quite long (more than 50), including clotting factors, neutral and acid proteinases, lipoproteins, TGF-β, toxic oxygen intermediators, prostaglandins and leukotrienes (Cohn, 1986; Nathan, 1987). Some of them such as superoxide anions, prostaglandins and TGF-β act as inhibitory substances to lymphocytes and immune responses. The release of these substances by SHR macrophages may potentially be the mechanism responsible for SHR lymphocyte inhibition. However, in recent years, NO has been identified as an intermediate molecule for tumor cytotoxicity and microbiostasis of activated macrophages (Hibbs *et al.*, 1988; Stuehr and Nathan, 1989). Recent studies demonstrated that NO was responsible for macrophage inhibition of normal T cell proliferation in response to alloantigens or mitogens (Hoffman *et al.*, 1990; Albina *et al.*, 1991). Although it is not known whether this mediator also plays a role in pathology-associated immunosuppression such as hypertension, this mechanism was first investigated here to see if NO may be involved in the inhibition of lymphocyte proliferation by SHR macrophages.

The present study provides evidence for the first time that the overproduction of NO by SHR macrophages is responsible for the lymphocyte proliferation defect

in SHR. This was accomplished by showing that L-NMMA, NOS inhibitor, fully reversed the inhibition of the SHR lymphocyte proliferation response. Furthermore, L-NMMA also reversed the inhibitory effect of added SHR macrophages on macrophage depleted lymphocytes. In addition, macrophages from SHR produced more NO upon stimulation (Xiao *et al.*, 1993). This finding agrees with a recent report that the lymphoid depression in SHR is mediated by a mononuclear subpopulation through NO (Pascual *et al.*, 1993). The fact that D-NMMA failed to correct the inhibition suggests that L-NMMA reversed the suppressed lymphocyte proliferation response by specifically inhibiting NO synthase in SHR macrophages. This study also shows that L-NMMA inhibited elevated NO production by SHR spleen macrophages. L-NMMA at the same concentration, however, did not significantly inhibit NO production by WKY macrophages. The explanation for this is not clear.

The present study also shows that without stimulation, only a small amount of NO was produced. After stimulation with Con A or LPS, large amounts of NO were present in the culture media. This is in agreement with the fact that NOS in macrophages is an inducible isoform. After the induction process, SHR produced significantly greater amounts of NO than did WKY in both isolated macrophages and total spleen cells including macrophages. The magnitude of increased NO production in isolated macrophages was lower than that in spleen cells in SHR. The reason might be due to the number of macrophages present in the test system. Although the isolation process started with the same number of spleen cells, some of the

macrophages may be lost by wash out from the dishes during the isolation process. It would be ideal if a known number of macrophages were studied. However, this was technically difficult because the removal of adherent macrophages for enrichment and counting from petri dishes would cause damage to the macrophages. The overproduction of NO could be the result of increased NOS activity in individual macrophages or the result of increased total number of macrophages in SHR spleen cell populations. However, the result from the staining study showed that the number of macrophages in SHR spleen was not significantly different from that in WKY spleen. This suggests that the overproduction in SHR spleen cells is not due to an increased total number of macrophages, but rather to changes in the macrophages themselves or in the regulation of NO synthase in macrophages.

It has been reported that the number of spontaneously activated monocytes and neutrophils in SHR was $>300\%$ above control values (Schmid-Schonbein *et al.*, 1991). In addition, young SHR already display an elevated circulating leukocyte count at the time when blood pressure is beginning to increase. In the subendothelium of SHR arteries, where there was infiltration by macrophages, the angiotensin-converting enzyme inhibitors could prevent the macrophage infiltration and attenuate the impairment of endothelium-dependent relaxation (Clozel *et al.*, 1991). These observations suggest that the abnormal function of macrophages *per se* may be associated with the hypertensive state in SHR. It is possible that hypertension may lead to changes in macrophage function or that the alteration of macrophage function and the elevated blood pressure may be coupled to a similar,

or possibly common, causative factor or genetic defect in SHR. The precise molecular mechanism of NO action that led to the inhibition of lymphocyte proliferation is not fully understood. One possibility is that it is identical to the mechanism responsible for the NO cytotoxic effect on tumor cells and micro-organisms, *i.e.* inactivation of iron-sulfur enzymes known to be crucial for ATP production and DNA replication (Hibbs *et al.*, 1991). NO could also inhibit the synthesis of some pro-stimulating cytokines. It has been reported that syndonimine, a NO supplying agent, decreased IL-1β synthesis in human monocytes and that this inhibition is correlated with an increase in intracellular cGMP level (Fulle *et al.*, 1991). The half-life of NO is very short (within seconds). This short half-life contributes to the oxidation of NO in the presence of oxygen and superoxide anions (Ignarro, 1991). This could explain the earlier result which showed that the culture supernatant of SHR spleen cells failed to inhibit lymphocyte proliferation because the NO had been inactivated.

It has been reported that endothelium-dependent relaxation was impaired in SHR suggesting that the NO released from endothelial cells is probably reduced (Diederich *et al.*, 1990; Koller and Huang, 1994). However, in this study it was observed that NO synthesis in SHR macrophages was increased. The explanation for this paradox is not known at this time and requires further investigation.

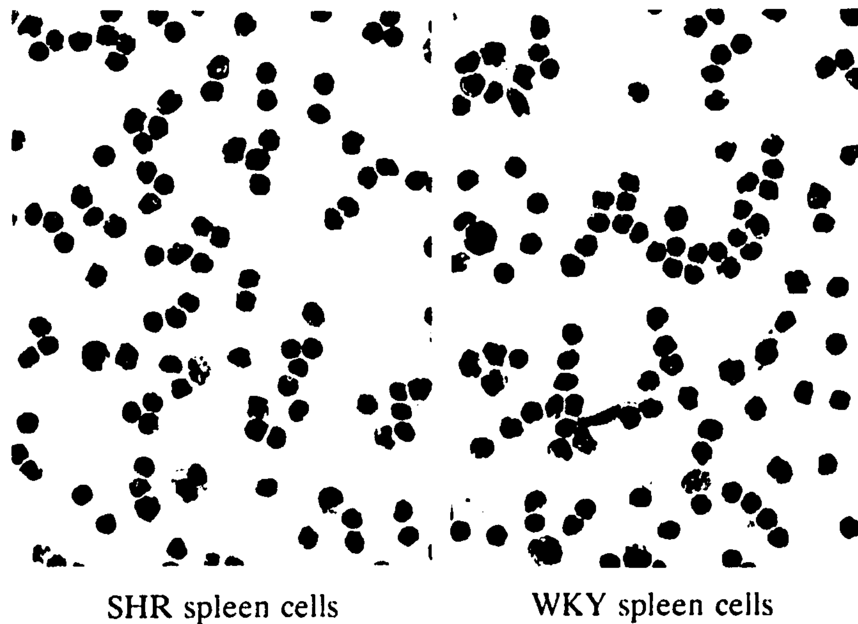
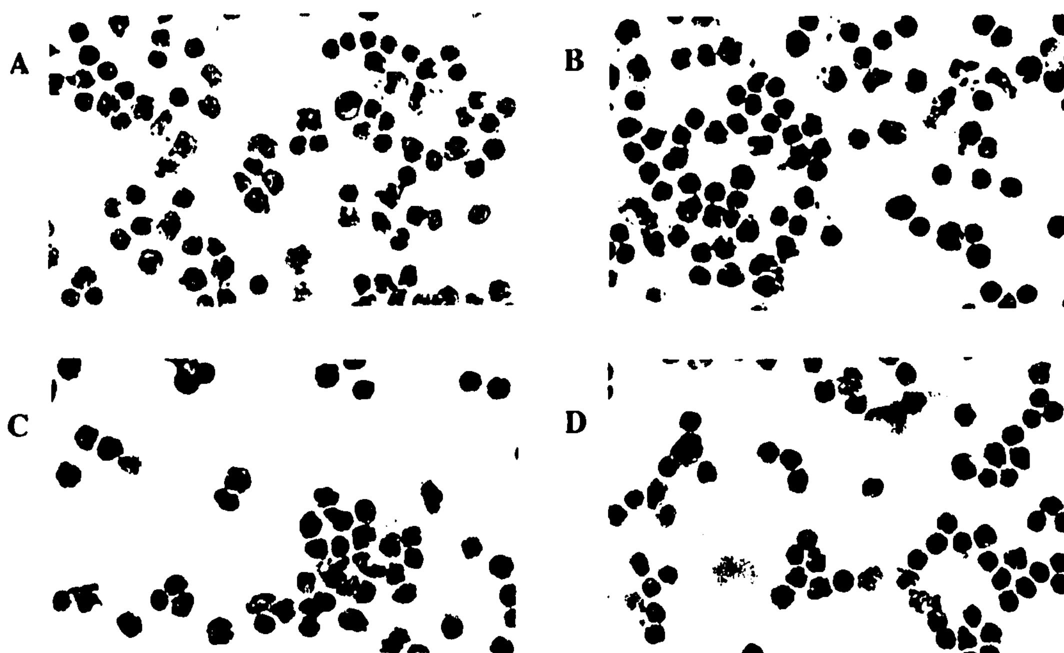


Figure IV-1. **Negative control of immunoperoxidase staining**. The fixed spleen cells were incubated on slides with mouse IgG_1 antibody (the same isotype as anti-rat ED1 and ED2 antibody). A goat anti-mouse IgG antibody was then incubated with the cells followed by a peroxidase staining. The cells were counter-stained by hematoxylin with a blue colour.

Figure IV-2

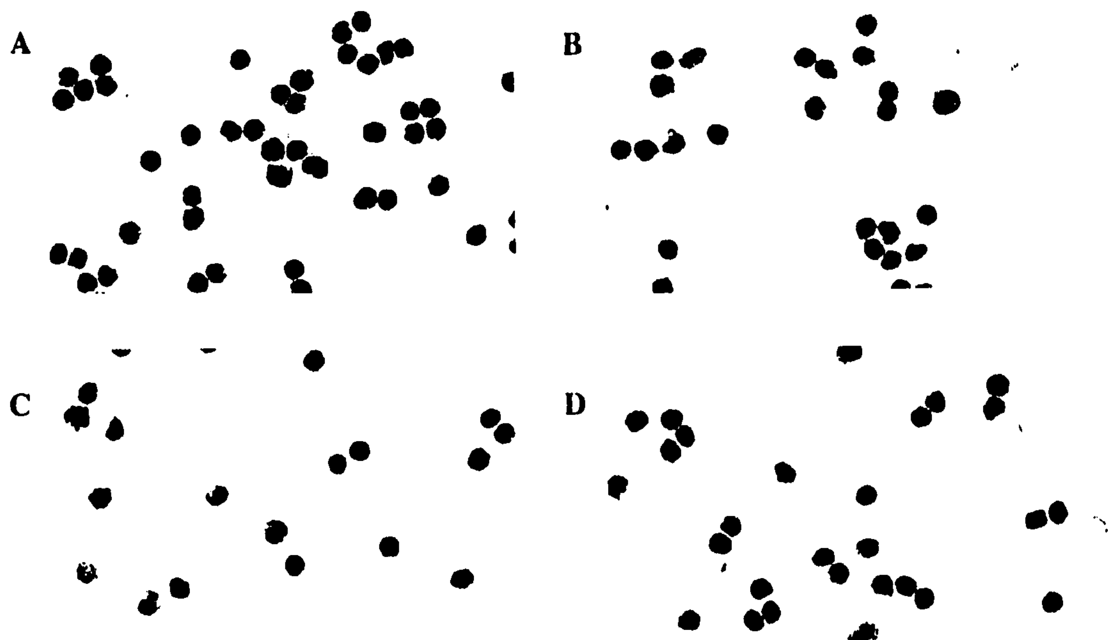


The expression of ED1 or ED2 antigen on spleen cells

	ED1 $^+$ cells (%)	ED2 $^+$ cells (%)
SHR spleen cells	23.1 $\pm$ 3.7	4.03 $\pm$ 2.1
WKY spleen cells	18.2 $\pm$ 1.1	6.7 $\pm$ 3.8

The fixed spleen cells were incubated on slides with mouse anti-rat ED1 (1:1000 dilution) or ED2 (1:800 dilution) monoclonal antibody. A goat anti-mouse IgG antibody was then incubated with the cells. The cells expressing ED1 and ED2 antigens were detected by an immunoperoxidase staining method. The values are Mean $\pm$ SE of positive cells counted from 3 areas in each slide, one slide for each 4 rats. **A: ED1 expressed on SHR spleen cells; B: ED1 expressed on WKY spleen cells; C: ED2 expressed on SHR spleen cells; D: ED2 expressed on WKY spleen cells.**

Figure IV-3



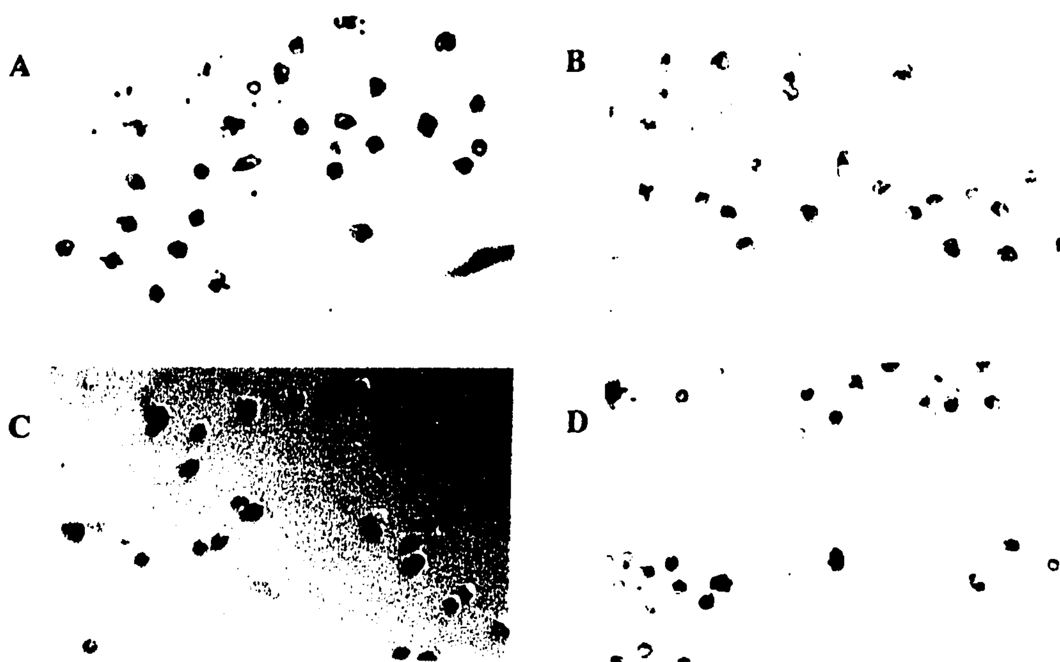
The expression of ED1 or ED2 antigen on non-adherent cells

	ED1 + cells (%)	ED2 + cells (%)
SHR non-adherent cells	3.2 ± 1.1	N/A *
WKY non-adherent cells	1.7 ± 0.9	N/A *

The fixed non-adherent cells were incubated on slides with mouse anti-rat ED1 (1:1000 dilution) or ED2 (1:800 dilution) monoclonal antibody. A goat anti-mouse IgG antibody was then incubated with the cells. The cells expressing ED1 and ED2 antigens were detected by an immunoperoxidase staining method. The values are Mean ± SE of positive cells counted from 3 areas in each of 2 slides. **A: ED1 expressed on SHR non-adherent cells; B: ED1 expressed on WKY non-adherent cell; C: ED2 expressed on SHR non-adherent cells; D ED2 expressed on WKY non-adherent cells.**

*: The number of cells in each area of the slide is too small to obtain an accurate percentage.

Figure IV-4



The expression of ED1 or ED2 antigen on adherent cells

	ED1 $^{+}$ cells (%)	ED2 $^{+}$ cells (%)
SHR adherent cells	93.6 ± 3.4	69.3 ± 0.6
WKY adherent cells	88.2 ± 4.2	67.3 ± 1.2

The fixed adherent cells in a 96 well tissue plate were incubated with mouse anti-rat ED1 (1:1000 dilution) or ED2 (1:800 dilution) monoclonal antibody. A goat anti-mouse IgG antibody was then incubated with the cells. The cells expressing ED1 and ED2 antigens were detected by an immunoperoxidase staining method. The values are Mean ± SE of positive cells counted from 3 areas in each well, 2-3 wells per tested antigen. **A: ED1 expressed on SHR adherent cells; B: ED1 expressed on WKY adherent cells; C: ED2 expressed on SHR adherent cells; D: ED2 expressed on WKY adherent cell.**

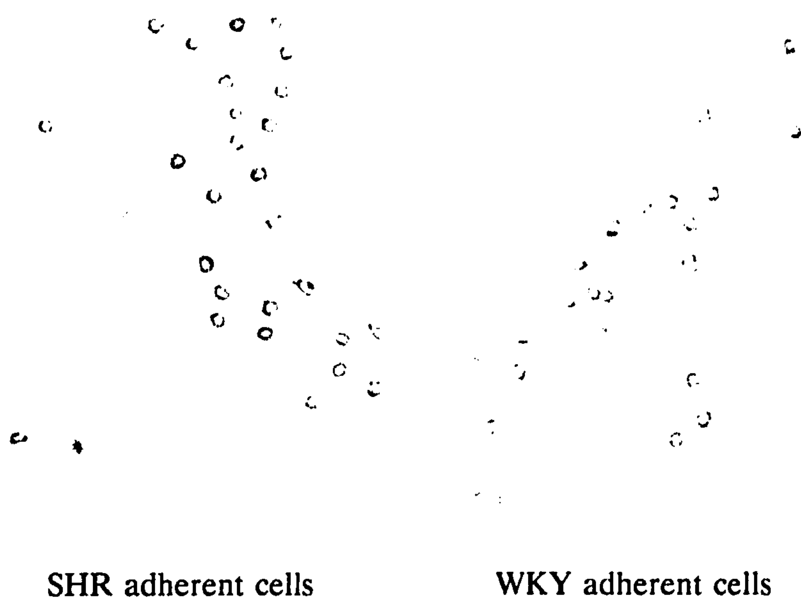


Figure IV-5. **Negative control of immunoperoxidase staining**. The fixed adherent cells in a 96 well plate were incubated with mouse IgG_1 antibody (the same isotype as anti-rat ED1 and ED2 antibody). A goat anti-mouse IgG antibody was then incubated with the cells followed by a peroxidase staining. There was no hematoxylin staining for nuclei of these cells.

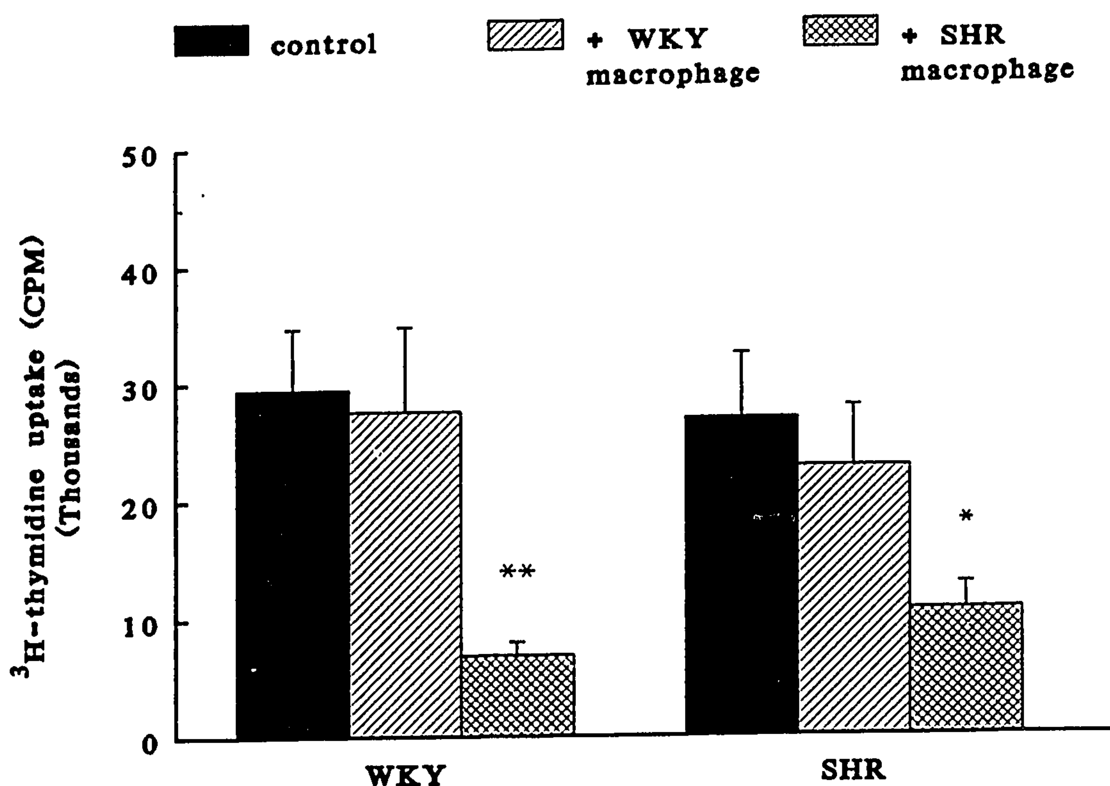


Figure IV-6. **Effect of macrophages on the proliferation response of macrophage-depleted lymphocytes from SHR and WKY.** Spleen cells (2×10^5 cells/well) were incubated in 96 well tissue culture plates for 90 minutes. The medium which contained nonadherent lymphocytes was decanted. The plates were washed twice with HBSS to remove any residual nonadherent cells. Macrophage-depleted lymphocytes from SHR or WKY were added onto plates, which contained adherent macrophages, in quadruplicate and incubated for 72 hours in the presence of 2.5 μg/ml Con A. Data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from six rats. Significant difference: * $P < 0.05$; ** $P < 0.01$, compared with respective control and + WKY macrophage group.

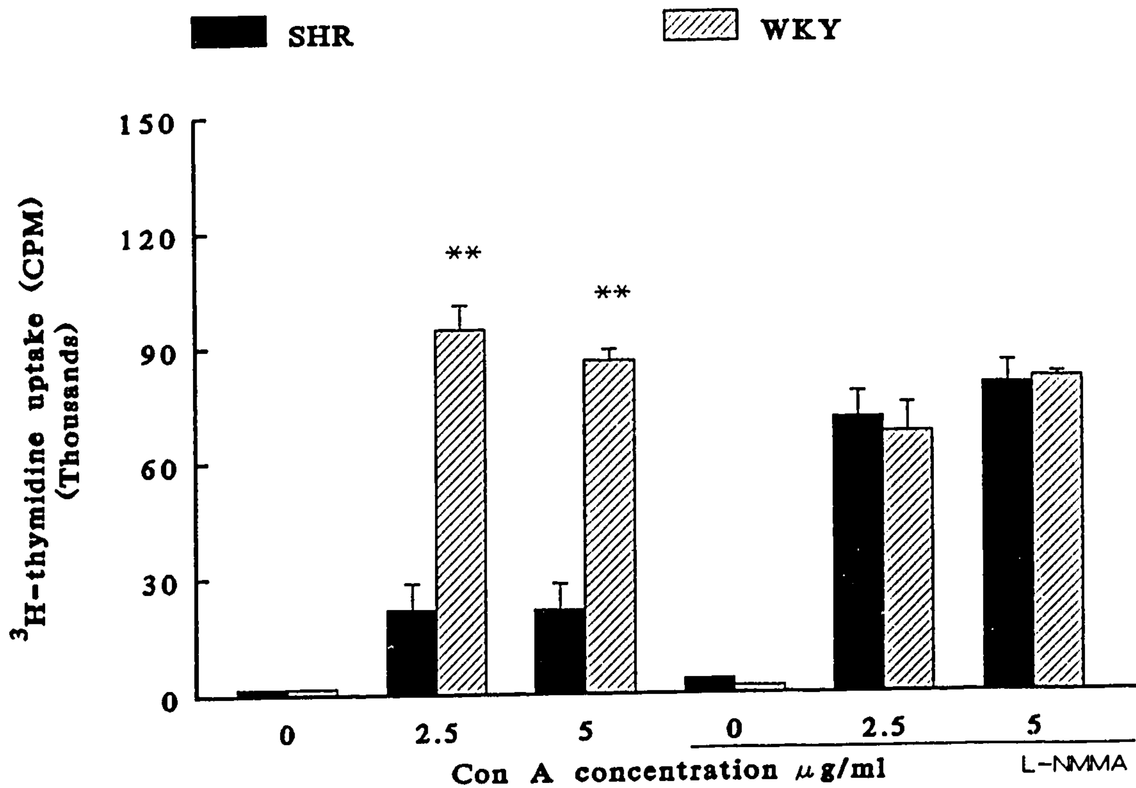


Figure IV-7. **Effects of L-NMMA on the proliferation response of SHR and WKY spleen cells.** L-NMMA (0.05 mM) was added to the culture simultaneously with Con A. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from six rats. Significant difference: * $P < 0.01$, compared with respective SHR spleen cells.

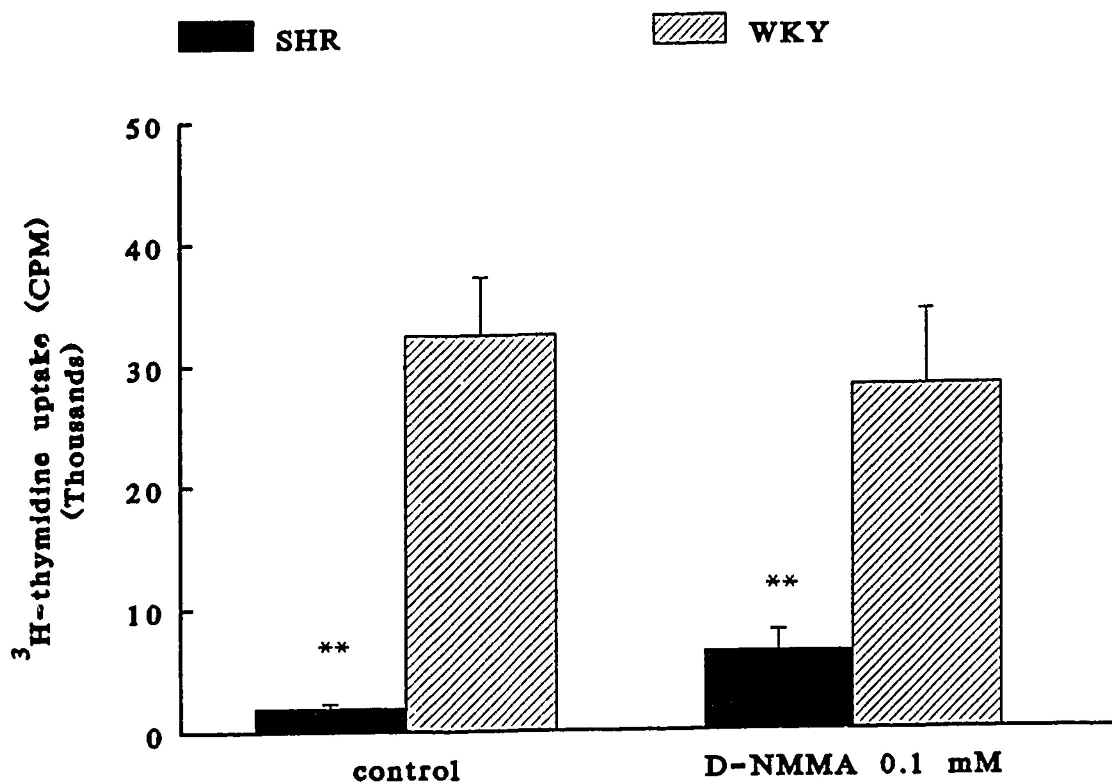


Figure IV-8. **Effect of D-NMMA on the proliferation response of SHR and WKY spleen cells.** D-NMMA (0.01 mM) was added to the culture simultaneously with 2.5 $\mu g/ml$ Con A. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from three rats. Significant difference: ** $P < 0.01$, compared with respective WKY spleen cells.

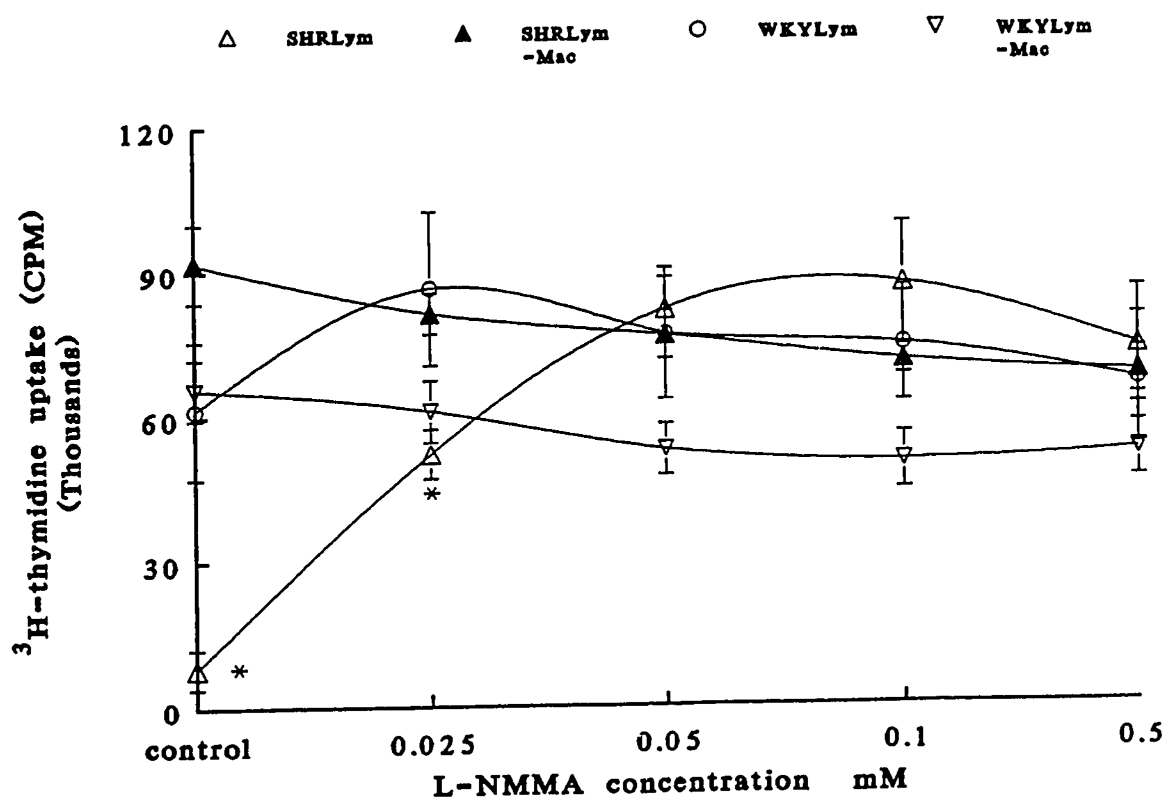


Figure IV-9. **Effects of L-NMMA on the proliferation response of spleen cells or macrophage-depleted lymphocytes obtained from SHR and WKY.** Various concentrations of L-NMMA were added to the culture simultaneously with 5 μg/ml Con A. SHRLym: SHR spleen cells; SHRLym-Mac: SHR macrophage-depleted lymphocytes; WKYLym: WKY spleen cells; WKYLym-Mac: WKY macrophage-depleted lymphocytes. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from six rats. Significant difference: * $P < 0.01$ when the responses of SHR spleen cells were compared with those of SHR macrophage-depleted lymphocytes.

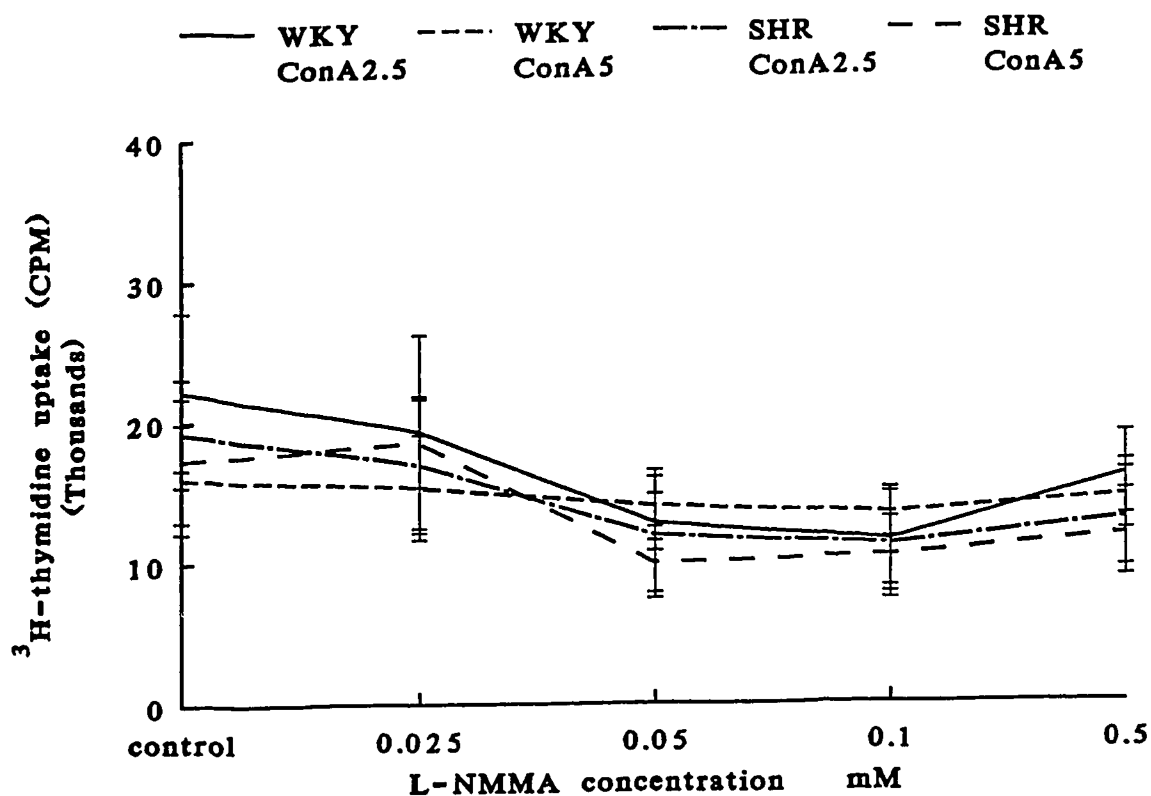


Figure IV-10. **Effects of L-NMMA on the proliferation response of thymocytes obtained from SHR and WKY.** Various concentrations of L-NMMA were added to the culture simultaneously with 2.5 or 5 μg/ml Con A. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from six rats.

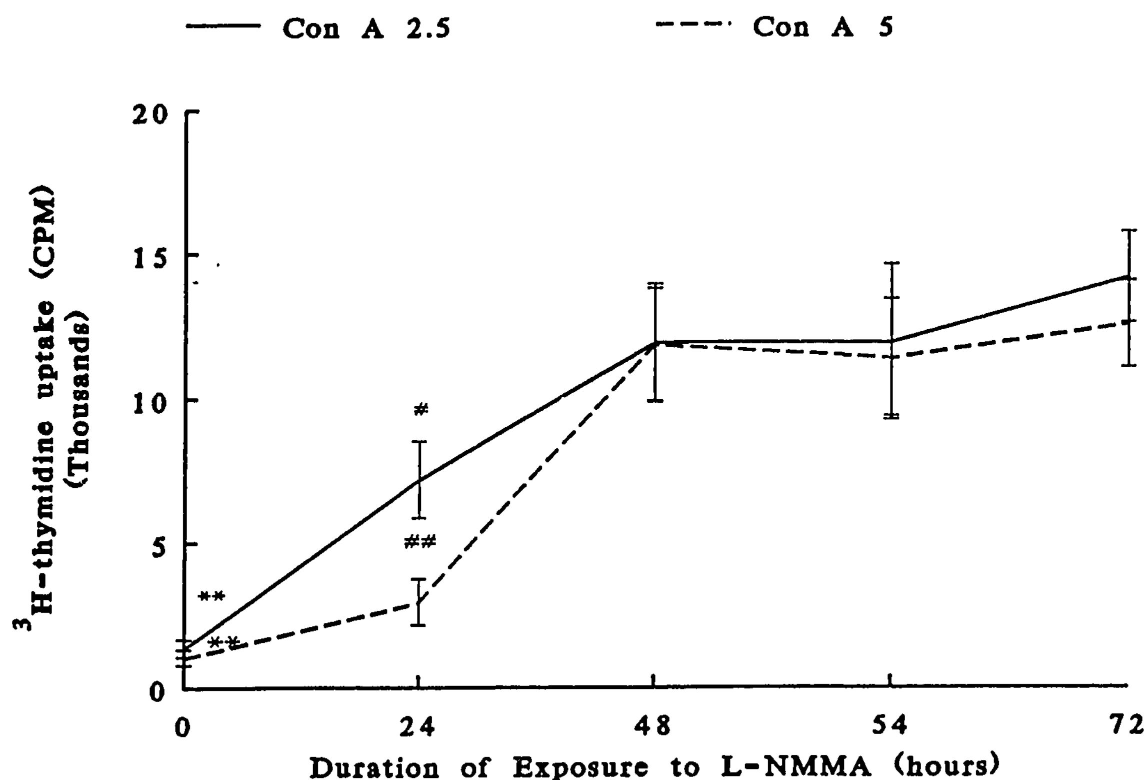


Figure IV-11. **The effect of L-NMMA exposure with time on the proliferation response of SHR spleen cells.** L-NMMA (0.05 mM) was added into the SHR spleen cell culture at various times during the 72 hours culture period. The time points represent the duration of exposure to L-NMMA at the termination of the culture, *i.e.* 24 hours represents the addition of L-NMMA at the final 24 hours of the culture period; 72 hours represents the addition of L-NMMA at the beginning of the culture. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from 3 rats. **: $P < 0.01$, compared with respective response at 24, 48, 58 and 72 hours (except the response to 5 μg/ml Con A at 24 hours). #: $P < 0.05$, compared with respective response at 72 hours. ## $P < 0.01$, compared with respective response at 48, 54 and 72 hours.

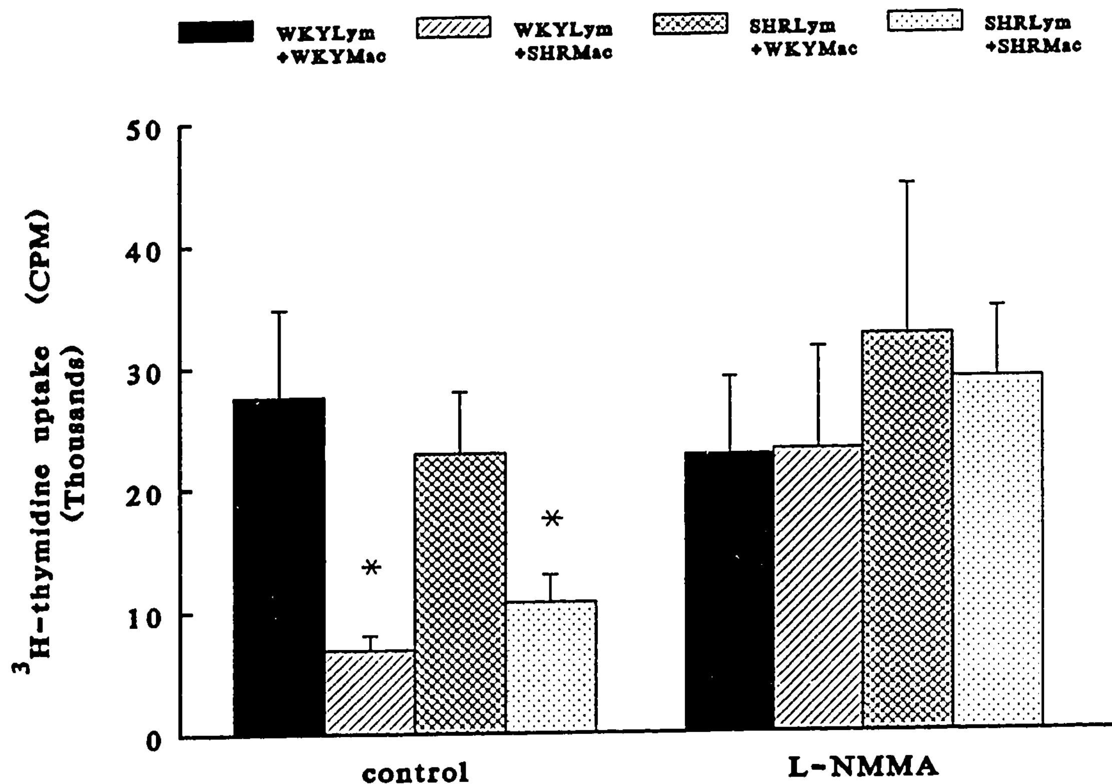


Figure IV-12. **Effect of L-NMMA on the inhibition of lymphocyte proliferation responses caused by SHR macrophages.** Macrophages in 96 well plates were prepared as described in Figure IV-1. Macrophage-depleted lymphocytes from SHR or WKY were added to the plates and incubated for 72 hours in the presence of 2.5 μg/ml Con A and 0.05 mM L-NMMA. WKYLym: WKY macrophage-depleted lymphocytes; WKYMac: WKY macrophages; SHRLym: SHR macrophage-depleted lymphocytes; SHRMac: SHR macrophages. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from six rats. Significant difference: * P < 0.01, compared with respective cells treated with L-NMMA.

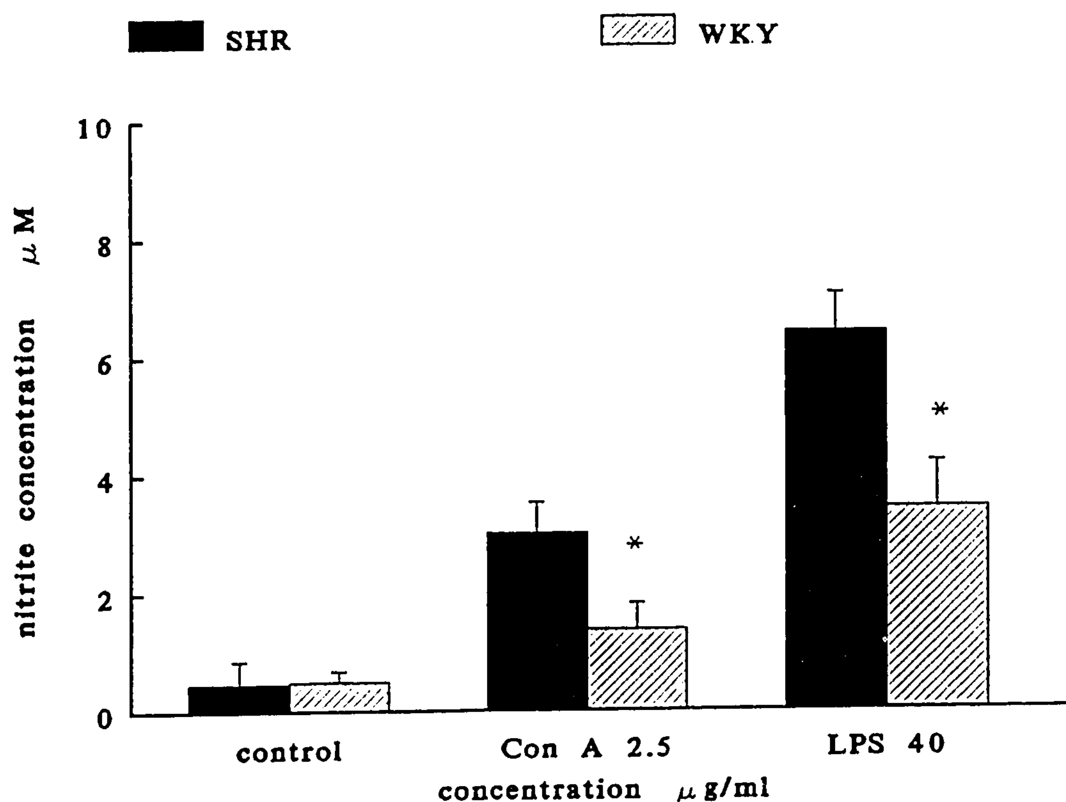


Figure IV-13. **Nitric oxide production by macrophages from SHR and WKY.** Five ml of 1×10^6 cells/ml spleen cells were incubated in 60x15 mm plastic dishes in complete medium for 90 minutes. The medium which contained nonadherent lymphocytes was decanted. The dishes were washed twice to remove any residual nonadherent cells and 5 ml of fresh medium was added. The adherent macrophages were then incubated for 72 hours in the presence of 2.5 μg/ml Con A or 40 μg/ml LPS. The supernatant was collected by centrifuging at 4°C, at 800 x g for 10 minutes. Nitrite concentration in the supernatant was determined by a colorimetric assay. Data represent mean ± SE from four experiments.
* $P < 0.05$, compared with SHR.

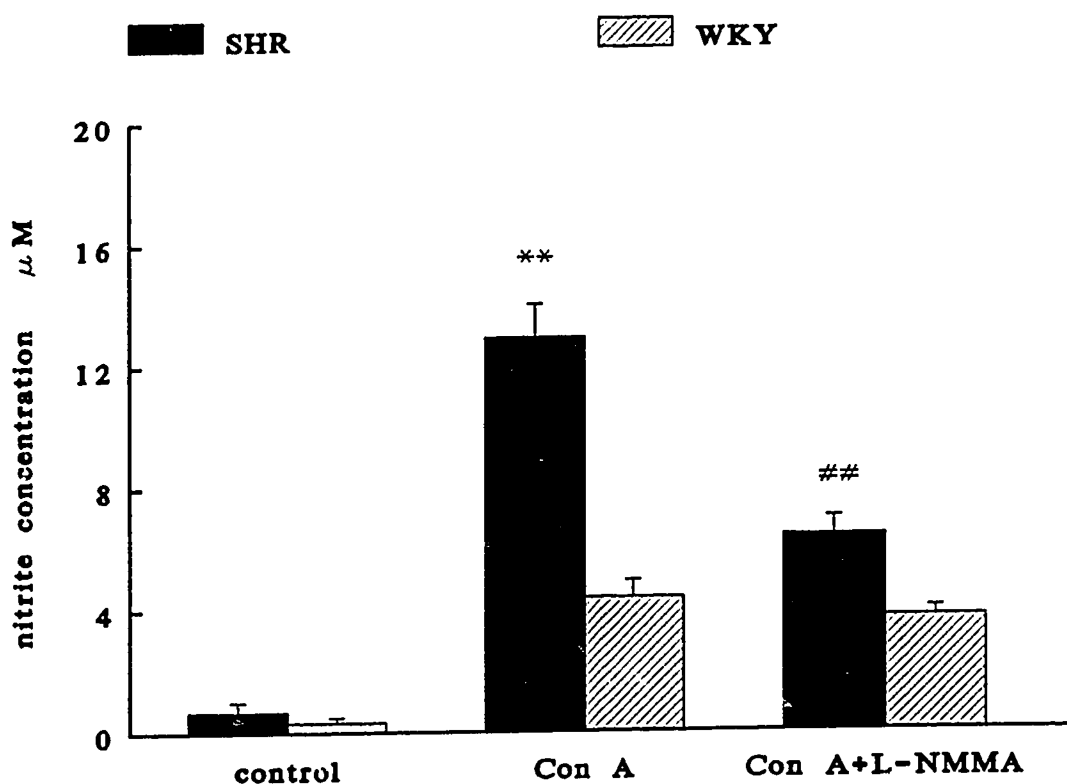


Figure IV-14. **Nitric oxide production by spleen cells from SHR and WKY.** Five ml of $1x10^6$ cells/ml spleen cells were incubated in 12 well tissue culture plates in complete medium for 72 hours in the presence of 2.5 μg/ml Con A or 2.5 μg/ml Con A plus 0.05 mM L-NMMA. The supernatant was collected by centrifugation at 4°C, 800 x g for 10 minutes. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. Data represent mean ± SE from four rats. ** $P < 0.01$, compared with WKY spleen cells. ## $P < 0.01$, compared with SHR spleen cells stimulated by Con A.

CHAPTER V. INTERACTION OF LYMPHOCYTES AND VSMC: THE ROLE OF THE NITRIC OXIDE PATHWAY

I. INTRODUCTION

The main objective of this thesis is to investigate the relationship between immune dysfunction and hypertension in SHR. Although it was shown in the last chapter that the overproduction of NO by macrophages is responsible for lymphocyte depression in SHR, it is possible that NO production by other cells may also contribute to this inhibition. Lymphocytes circulate throughout the body in the ongoing process of immune surveillance by travelling through the bloodstream, moving into tissues and then returning to the circulatory system via the lymphatics (Butcher, 1990; Shimizu *et al.*, 1992). It is possible that VSMC and endothelial cells of blood vessels can influence the properties of lymphocytes or *vice versa.*

The hallmark of hypertension is an increase in vascular resistance. VSMC are responsible for controlling the lumen diameter of resistance vessel and thus, controlling vascular resistance. In hypertension, VSMC exhibit abnormal growth (Lee, 1985; Mulvany, 1992) and responsiveness to vasoactive agents (Bohr *et al.*, 1991a; de Champlain *et al.*, 1991). It was of great interest to determine if any interaction exists between VSMC and lymphocytes. An understanding of this interaction in SHR may provide evidence for a relationship between immune dysfunction and hypertension. Therefore, the interaction between lymphocytes and

VSMC was investigated. The role of NO in this interaction and NO production by VSMC from SHR or WKY were also studied.

II. EXPERIMENTAL DESIGN

A. Mixed culture of lymphocytes with VSMC. As described in Chapter II, VSMC from SHR and WKY were plated in 96 well tissue plates at various cell concentrations for 36-40 hours to allow attachment of VSMC to the plates. To study the effect of VSMC on the proliferation response of lymphocytes, lymphocytes (0.2 x 10^6 cells/well) isolated from SHR or WKY were then co-cultured in quadruplicate with VSMC at a ratio of VSMC:lymphocytes of 1:40, 1:20 or 1:10 in the presence of Con A for 72 hours. ^{3}H-thymidine was added to the cell culture during the last 18 hours and the cells were harvested and counted. Because VSMC can take up a substantial amount of ^{3}H-thymidine thus making the interpretation of results difficult, the VSMC were irradiated using a ^{137}Cs r-irradiator at a dose of 4000 rads to prevent proliferation (Kruisbeek and Shevach, 1991). As shown later in this chapter, this irradiation dose sufficiently prevented VSMC proliferation. The mixed cell culture was carried out as described previously.

B. The effect of L-NMMA on the mixed cell culture. L-NMMA (0.05 mM) was simultaneously added to the mixed cell culture with 2.5 μg/ml of Con A and cultured for 72 hours. ^{3}H-thymidine was added to the culture during the last 18 hours. The cells were harvested and counted as described previously.

C. NO production by VSMC from SHR and WKY.

1. Comparison of NO production in VSMC of SHR and WKY. VSMC were incubated in 24 well tissue culture plates in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent (stopping at G_0 phase in the cell cycle). To induce NO production, VSMC were cultured with either LPS, IL-1β, TNFα, INFγ or a cytokine mixture containing 5 units/ml IL-1β, 5 nM TNFα, 200 units/ml IFNγ and 10 μg/ml LPS in 10% FCS DMEM medium for 48 hours. The amount of NO in the culture supernatant was determined by a colorimetric assay.

2. Time-dependent and dose-dependent NO production by VSMC. After being rendered quiescent, VSMC from SHR or WKY were cultured with LPS or a cytokine mixture as described previously. At different times during the culture period, the supernatant was collected and kept in 4°C until tested. VSMC were cultured in the presence of various concentrations of LPS for 48 hours as described previously. The amount of NO in the supernatant was tested by a colorimetric assay.

D. Growth of SHR and WKY VSMC.

1. Proliferation study. VSMC (1 x 10^4 cells/well) were cultured in 96 well tissue culture plates in 10% FCS DMEM medium for 24 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 4 days to render the VSMC quiescent. The quiescent VSMC were, then, cultured in 10% FCS DMEM medium in the presence of either LPS or L-NMMA or both for

72 hours. ^{3}H-thymidine was added to the culture for the final 24 hours. The VSMC were harvested by trypsin treatment and counted.

2. Protein synthesis in VSMC. Protein synthesis in cells is correlated with growth rate. In order to examine the growth of SHR and WKY VSMC with time, 1.5 ml of 1 x 10^5 cells/ml VSMC were cultured in 24 well tissue culture plates in 10% FCS DMEM medium for 36-40 hours and were then rendered quiescent. The medium was then replaced with 10% FCS DMEM medium. At various times, the VSMC were washed twice with HBSS and solubilized. The protein content in VSMC was determined as described in Chapter II.

E. Effect of cytokines on VSMC proliferation. VSMC (1 x 10^4 cells/well) were cultured in 96 well tissue culture plates in 10% FCS DMEM medium for 24 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 4 days to render the VSMC quiescent. The medium was, then, replaced with 5% FCS DMEM medium in the presence of IL-2, IL-1β, INFγ or IL-6. After 24 hours of incubation, the VSMC were pulsed with ^{3}H-thymidine and incubated for another 40 hours. The VSMC were harvested by trypsin treatment and counted.

III. RESULTS

A. Two non-irradiated SHR VSMC preparations significantly inhibited the proliferation response of WKY lymphocytes. SHR lymphocytes had reduced

proliferation responses as seen before. When these cells were co-cultured with SHR VSMC, the proliferation response was further reduced. WKY VSMC had no significant effect on the lymphocyte proliferation response in either SHR or WKY. (Figure V-1).

B. Figure V-2 shows that after VSMC were irradiated the amount of ^{3}H-thymidine incorporation was minimal, suggesting that the proliferation of VSMC ceased. The effect of irradiation on cell viability was tested by trypan blue exclusion. The results showed that more than 90% of VSMC were viable.

C. Irradiated SHR VSMC significantly inhibited the proliferation of WKY (Figure V-3) and SHR (Figure V-4) lymphocytes and macrophage-depleted lymphocytes in response to 2.5 μg/ml of Con A. The lymphocytes co-cultured with SHR VSMC exhibited only one-seventh to one-ninth of the control response. When macrophages were removed from SHR spleen cells, the proliferation response increased dramatically. However, the increased response was again suppressed by co-culturing with SHR VSMC (Figure V-4). WKY VSMC had no significant effect on lymphocyte proliferation responses.

D. Figure V-5 shows that SHR VSMC inhibited lymphocyte proliferation in a dose-dependent manner. As the proportion of VSMC increased, the inhibition of lymphocyte proliferation increased.

E. At a concentration of 0.05 mM, L-NMMA fully corrected the inhibited proliferation response of WKY lymphocytes (Figure V-6) and WKY macrophage-depleted lymphocytes (Figure V-7) caused by SHR VSMC . L-NMMA not only

reversed the inhibitory effect of SHR VSMC on SHR lymphocyte proliferation but also further increased the SHR lymphocyte proliferation to a much higher level even though SHR VSMC were present (Figure V-8). L-NMMA also fully corrected the inhibition of SHR VSMC on the proliferation response of SHR macrophage-depleted lymphocytes (Figure V-9).

F. The results of NO production by irradiated VSMC from SHR and WKY are shown in Figure V-10. The irradiated SHR VSMC produced a significantly larger amount of NO than did irradiated WKY VSMC after stimulation by LPS. Without stimulation a small amount of NO was produced by VSMC. Similar results were obtained when non-irradiated VSMC were used in this study. After stimulation by 40 μg/ml LPS, 25 units/ml IL-1β or a cytokine mixture, the non-irradiated SHR VSMC produced a significantly greater amount of NO than did those from WKY (Figure V-11). However, no significant amount of NO was produced when the VSMC were stimulated by either TNFα or IFNγ alone (Figure V-12). When 0.05 mM L-NMMA was added to the VSMC culture stimulated by LPS, the increased NO production in SHR VSMC was inhibited (Figure V-13).

G. Figure V-14 shows time-dependent changes in NO production by VSMC from SHR and WKY, stimulated by a the cytokine mixture. At each interval, SHR VSMC produced a significantly greater amount of NO than did WKY VSMC. A similar time-dependent relationship was observed when the VSMC were stimulated with LPS (Figure V-15). Again, SHR VSMC produced larger amounts of NO than did WKY VSMC.

H. LPS stimulated NO production by VSMC from SHR or WKY in a dose-dependent manner (Figure V-16). At each concentration tested, SHR VSMC produced a significantly greater amount of NO than did WKY VSMC.

I. Time-dependent protein synthesis in VSMC is shown in Figure V-17. There was no significant difference in the protein content of VSMC of SHR and WKY during the first 48 hours of the culture period which started after quiescence. However, the protein content of SHR VSMC increased significantly after that period of culture time.

J. Figure V-18 shows the effect of LPS or LPS plus L-NMMA on VSMC proliferation. When cultured in 10% FCS DMEM medium for 72 hours, SHR VSMC exhibited a greater proliferation rate than did WKY VSMC. When SHR VSMC were cultured in the presence of LPS, the increased proliferation rate was inhibited. L-NMMA reversed the inhibited SHR VSMC proliferation caused by LPS.

K. The effect of cytokines on VSMC proliferation is shown in Figure V-19. IL-2, IL-1β, INFγ and IL-6 did not show a significant stimulatory or inhibitory effect on the proliferation of VSMC.

IV. DISCUSSION

No direct evidence is available concerning the interaction between lymphocytes and VSMC in hypertension. Although SHR macrophages were found to be responsible for the lymphocyte inhibition defect in SHR, the involvement of

other SHR cell types could not be excluded (Fabry *et al.*, 1990a). To test this possibility, the interaction between lymphocytes and VSMC was investigated. The results showed that SHR VSMC significantly inhibited the proliferation response of lymphocytes from SHR or WKY. The fast growth of SHR VSMC could cause changes in culture conditions and ^{3}H-thymidine uptake and makes the interpretation of the results difficult. To avoid this, the VSMC were irradiated at 4000 rad to stop the proliferation. The results from lymphocytes co-cultured with irradiated VSMC would then more closely reflect lymphocyte proliferation. When lymphocytes were co-cultured with irradiated SHR VSMC, the proliferation of lymphocytes was inhibited to the same degree as by non-irradiated SHR VSMC. Because SHR macrophages were involved in the suppressed lymphocyte proliferation in SHR, macrophages were removed from lymphocytes before co-culturing to exclude this effect. The same inhibition was seen in the proliferation response of macrophage-depleted lymphocytes when co-cultured with SHR VSMC, suggesting that SHR VSMC were the cells involved in this inhibition. This is further supported by the inhibitory effect of SHR VSMC on WKY spleen cells with or without normal macrophages.

To elucidate the mechanism involved in the inhibition of lymphocyte proliferation caused by SHR VSMC, the NO synthesis pathway was studied. When L-NMMA was added to the mixed culture, the inhibition of the proliferation responses of lymphocytes and macrophage-depleted lymphocytes caused by SHR VSMC was fully corrected. In the case of SHR lymphocytes, L-NMMA not only

reversed the inhibition but also increased the response dramatically, suggesting that L-NMMA inhibited NO synthesis in both SHR macrophages and SHR VSMC. In addition, both irradiated and non-irradiated SHR VSMC produced a significantly greater amount of NO than did WKY VSMC. This provides direct evidence for increased NO synthesis in SHR VSMC. This overproduction of NO by SHR VSMC mediated the inhibition of lymphocyte proliferation in the mixed culture system. The overactive NO synthesis in SHR VSMC may also have mediated lymphocyte depression *in vivo* because lymphocytes are constantly in contact with cells within the blood vessel wall and the lymphocytes are continuously recirculated between the blood stream and lymphoid tissue. It has been shown that vascular smooth muscle and endothelium can influence lymphocyte function by antigen presentation (Fabry *et al.*, 1990a) and cytokine production (Warner and Libby, 1989). The interaction between VSMC and lymphocytes has been suggested to be important in conditions such as vasculitis, atherosclerosis and multiple sclerosis (Hart *et al.*, 1985; Fabry *et al.*, 1990b). The present study demonstrates for the first time that a significant interaction between VSMC and lymphocytes exists in hypertension, and that this interaction is mediated through the NO synthesis pathway (Xiao and Pang, 1994a).

Regarding NO synthesis in VSMC, it is possible that the increased NO production in cultured SHR VSMC may be due to the greater number of VSMC present in the culture because SHR VSMC grow more rapidly than do WKY VSMC. This possibility was examined in the experiment which demonstrated that the difference in growth rate between SHR VSMC and WKY VSMC was only observed

after 48 hours in culture. In this experiment, VSMC from SHR or WKY had been rendered quiescent (stop at G_0 phase of the cell cycle) by a 4-day culture in 0.4% FCS DMEM medium. That the difference in growth rate was only evident after 48 hours agrees with an earlier report that the difference in growth rates between SHR and WKY became significant only after 4 days in culture (Hadrava *et al.*, 1989). Therefore, it is unlikely that the increase in the number of VSMC contributed to the greater NO production in SHR VSMC since the VSMC were only cultured for 48 hours after quiescence. This fact is also supported by the finding that even at 24 hours in culture, SHR VSMC produced a larger amount of NO than did WKY VSMC (Xiao and Pang, 1994b). The results of the present study agree with a recent report that the production of NO evoked by IL-1β in SHR VSMC was greater than that from WKY VSMC (Junquero *et al.*, 1993).

Since the increased NO synthesis in SHR VSMC was only observed after stimulation by LPS or cytokines, this suggests that the enhanced NO production may result from an alteration in inducible NOS. This agrees with previous finding that like NOS in macrophages, NOS in VSMC can be induced by cytokines or LPS (Busse and Mulsch, 1990; Beasley *et al.*, 1991; Xie *et al.*, 1992). It is well established that the constitutive NO synthesis pathway in endothelial cells is important in the regulation of blood pressure. The involvement of NO synthesis, however, especially inducible NO synthesis in VSMC in the hypertensive state is not clear. Shear stress, arginine vasopressin, norepinephrine, histamine and thrombin have all been reported to stimulate endothelial cells in blood vessels to produce NO (Shepherd and Katusic,

1991). The production and release of these factors could be altered in hypertensive state. Recently, it was reported that the cGMP content of blood vessels was increased in SHR (Mourlon-Le Grand *et al.*, 1992). L-arginine has been demonstrated to induce a greater fall in blood pressure in SHR (Schleiffer *et al.*, 1991). These observations suggested that the NO synthesis system was probably increased in SHR. These studies, however, did not distinguish the inducible NO synthesis from the constitutive NO synthesis. The present study provides evidence that the inducible NO synthesis was elevated in SHR VSMC. Upon stimulation by vasoconstrictors, SHR arteries release endothelium-derived contracting factors (EDCF) (Luscher and Vanhoutte, 1986; Dai *et al.*, 1992). Among EDCF, superoxide anion is a potent chemical inactivator of NO (Rubanyi, 1991; Moroi *et al.*, 1994) Cosentino *et al.*, 1994). It has been speculated that an imbalance between NO and EDCF may exist in SHR (Rubanyi, 1992; Ito and Carretero, 1992; Junquero *et al.*, 1992). The overproduction of NO in SHR macrophages and VSMC suggests that there may be a general alteration in inducible NO synthesis. The activated NO synthesis system in macrophages and VSMC reflect a general compensatory protective mechanism against the vasoconstricting effect of EDCF and other vasoconstricting substances. This general activation of inducible NO synthesis, especially in macrophages, results in the lymphocyte depression in SHR.

It has been well characterized that the responsiveness of resistance arteries to endothelium-dependent vasodilating substance such as acetylcholine was reduced in SHR and patients with essential hypertension (Tesfamariam and Halpern, 1988;

Deng *et al.*, 1993; Malinski *et al.*, 1993; Li *et al.*, 1994). It has been suggested that a defect in the endothelium-derived NO synthesis system may at least partly account for both the increased vascular resistance and the impaired response to endothelium-dependent vasodilators (Tesfamariam and Halpern, 1988; Panza *et al.*, 1993). However, it was also reported that SHR coronary artery exhibited a higher sensitivity to the endothelium-dependent vasodilator, bradykinin, paralleled by a higher release of NO into the coronary circulation (Kelm *et al.*, 1992). Recently it was shown that UV light-activated NO release from aorta was greater in SHR than that in WKY (Kubaszewski *et al.*, 1994). Furthermore, the release rate of endothelium-derived NO into kidney did not differ between SHR and WKY (Hayakawa *et al.*, 1993). It has been reported that despite a reduced vascular response to acetylcholine, endothelium-derived NO synthesis was normal in isolated microperfused afferent arterioles of SHR (Ito and Carretero, 1992). These results suggest that the reduced endothelium-dependent relaxation in hypertension may not be due to an insufficient NO synthesis by constitutive NO synthase in endothelial cells. The reduced endothelium-dependent relaxation may be due to factors other than endothelium-derived NO, such as increased EDCF or decreased endothelium-derived hyperpolarization factor (EDHF).

The NO synthesis pathway has been recognized to not only play a significant role in the regulation of blood pressure and also in the regulation of VSMC proliferation (Nakaki *et al.*, 1990). It was reported that IFNr inhibited VSMC proliferation by NO generation in normal rats (Nunokawa and Tanaka, 1992). The

present study shows that SHR VSMC proliferate to a significantly greater extent than do those of WKY after 72 hours in culture. However, when SHR VSMC were cultured with LPS, the proliferation was significantly inhibited and this inhibition could be completely reversed by the addition of L-NMMA to the culture. This study provides the first evidence suggesting that the enhanced NO synthesis pathway in SHR VSMC may have an important role in the prevention of VSMC proliferation in the hypertensive state. This inhibitory effect may serve as a negative feedback mechanism. However, this mechanism is not strong enough to counterbalance the increased VSMC proliferation and, hence, in hypertension the vascular system still exhibits hypertrophy and hyperplasia.

The present study also examined the possibility that the immune system affects VSMC. This was accomplished by studying the effect of cytokines on VSMC proliferation. IL-2 and INF γ, the two T cell-derived cytokines, and also IL-1 and IL-6, the cytokines mainly produced by macrophages, were tested. The results showed that these cytokines had no significant effect on VSMC proliferation in either SHR or WKY. It has been reported that IL-1 promoted proliferation of VSMC in coordination with platelet derived growth factor (PDGF) (Ikeda *et al.*, 1990). In contrast to a report that IL-6 stimulated c-myc (an oncogene involved in DNA replication) expression and proliferation of VSMC (Nabata *et al.*, 1990), this study showed that IL-6 did not have a stimulatory effect on VSMC proliferation. The reason for this discrepancy is not clear at this time.

In summary, the present study demonstrated that 1) SHR VSMC significantly

inhibited the proliferation response of lymphocytes from SHR and WKY, 2) L-NMMA corrected the inhibited lymphocyte proliferation caused by SHR VSMC, 3) the overproduction of NO by SHR VSMC was responsible for this inhibition, 4) upon stimulation, NO synthesis in SHR VSMC was greater compared with that in WKY VSMC. These results suggest that a general activation of the inducible NO synthesis system may exist in SHR to serve as a compensatory protective mechanism against the elevated blood pressure. The alteration of NO synthesis in VSMC may be an important factor contributing to lymphocyte depression in hypertension. However, the precise relationship, *i.e.* causative or parallel, between lymphocyte depression and hypertension is still not clear at this time.

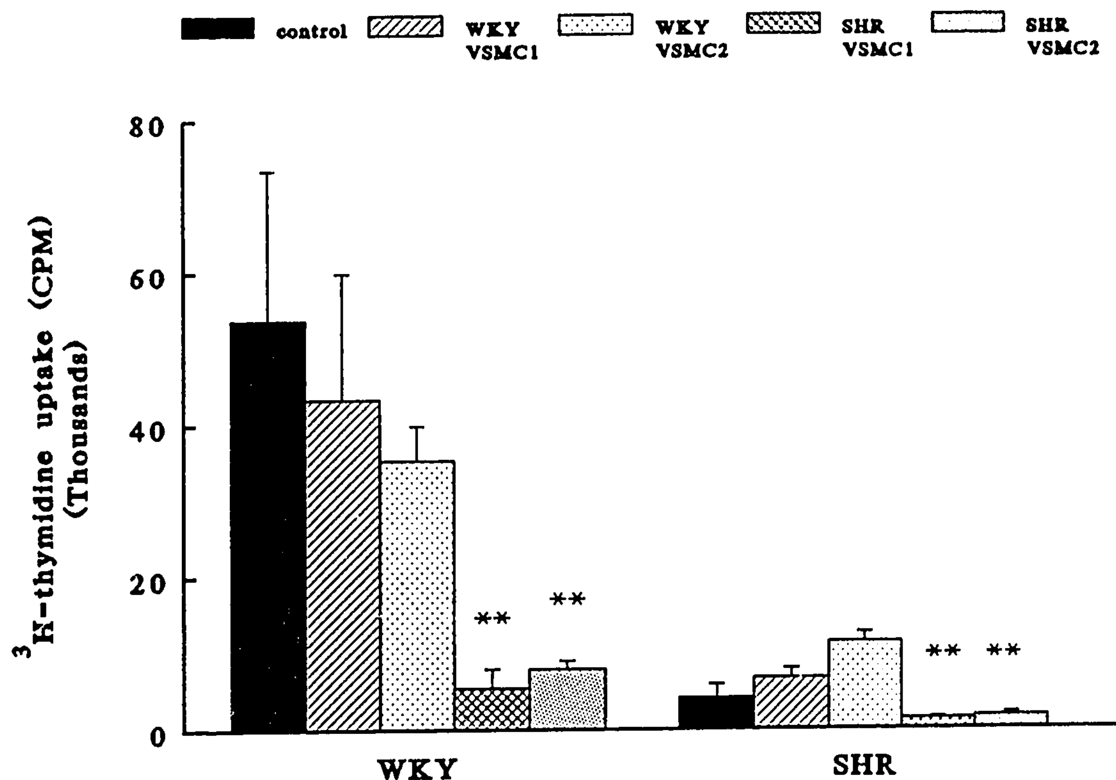


Figure V-1. **Effect of non-irradiated VSMC on the proliferation response of lymphocytes.** Two SHR and two WKY subcultured VSMC preparations (2 x 10^4 cells/well) were planted in 96 well tissue culture plates and incubated in 10% FCS DMEM medium. After incubated for 36-40 hours, the medium was decanted. The lymphocytes from SHR and WKY were then added in quadruplicate onto the plates at a lymphocyte : VSMC ratio of 10:1. The cells were incubated in 10% FCS RPMI-1640 in the presence of 2.5 μg/ml Con A for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from three experiments. **: $P < 0.01$, compared with respective control group (lymphocytes only) and the groups which contained lymphocytes plus WKY VSMC.

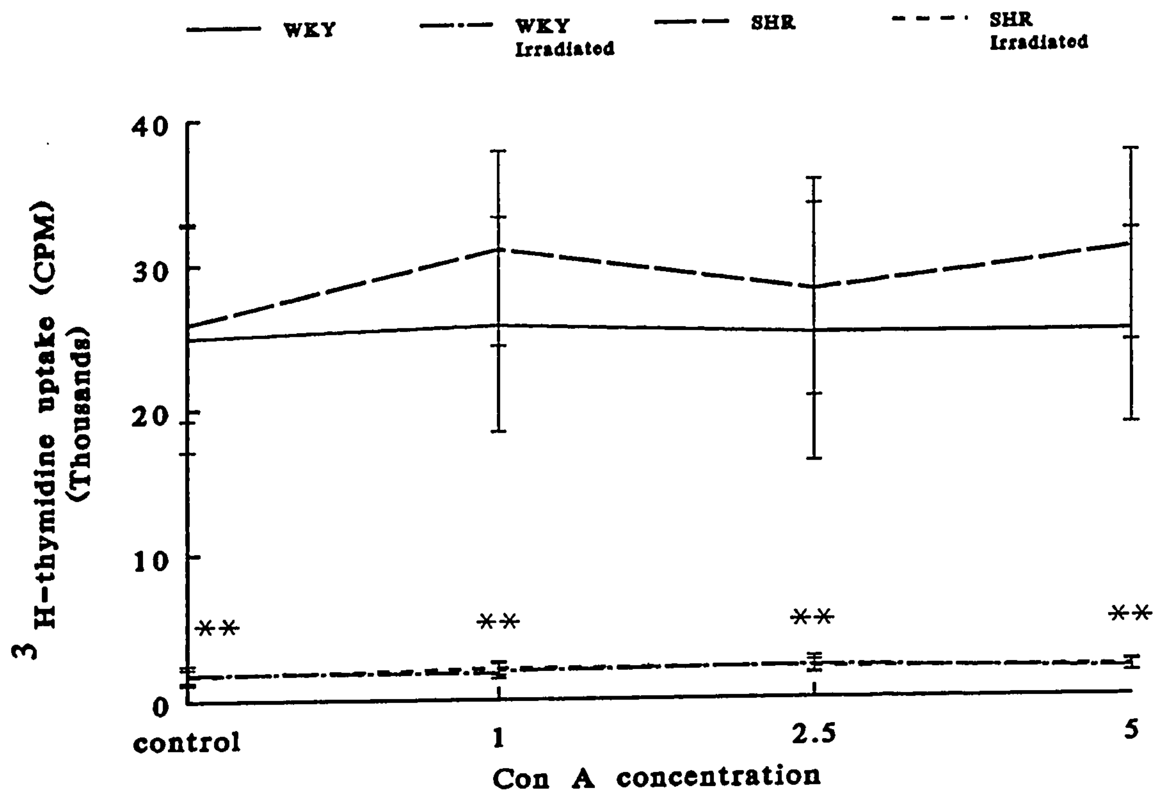


Figure V-2. **Effect of irradiation on VSMC proliferation.** VSMC (2 x 10^4 cells/well) were planted in 96 well plates and incubated for 36-40 hours. One plate was then irradiated using a ^{137}Cs r-irradiator at a dose of 4000 rads. Another plate was not irradiated. VSMC in the plates were washed twice with HBSS and cultured in 10% FCS DMEM medium for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N=9) from 3 cell preparations, 3 experiments for each preparation. **: $P < 0.01$, compared with respective non-irradiated VSMC.

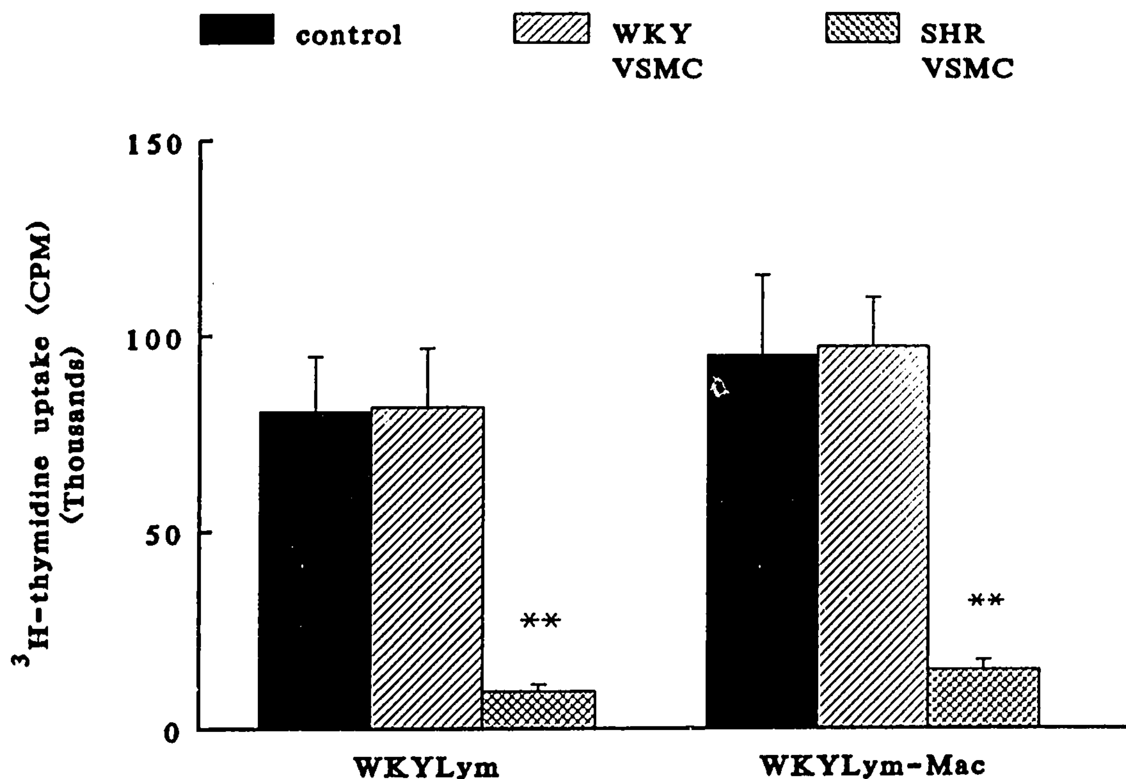


Figure V-3. **Effect of irradiated VSMC on the proliferation response of WKY lymphocytes.** Irradiated VSMC were prepared as described in Figure V-2. WKY lymphocytes (WKYLym) or WKY macrophage-depleted lymphocytes (WKYLym-Mac) were subsequently added in quadruplicate onto the plates at a lymphocyte : VSMC ratio of 10:1. The cells were incubated for 72 hours in the presence of 5 μg/ml Con A in 10% FCS RPMI-1640 medium. ^{3}H-thymidine was added to the culture for the final 18 hours. Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with respective control group (lymphocytes only) and the group which contained lymphocytes plus WKY VSMC.

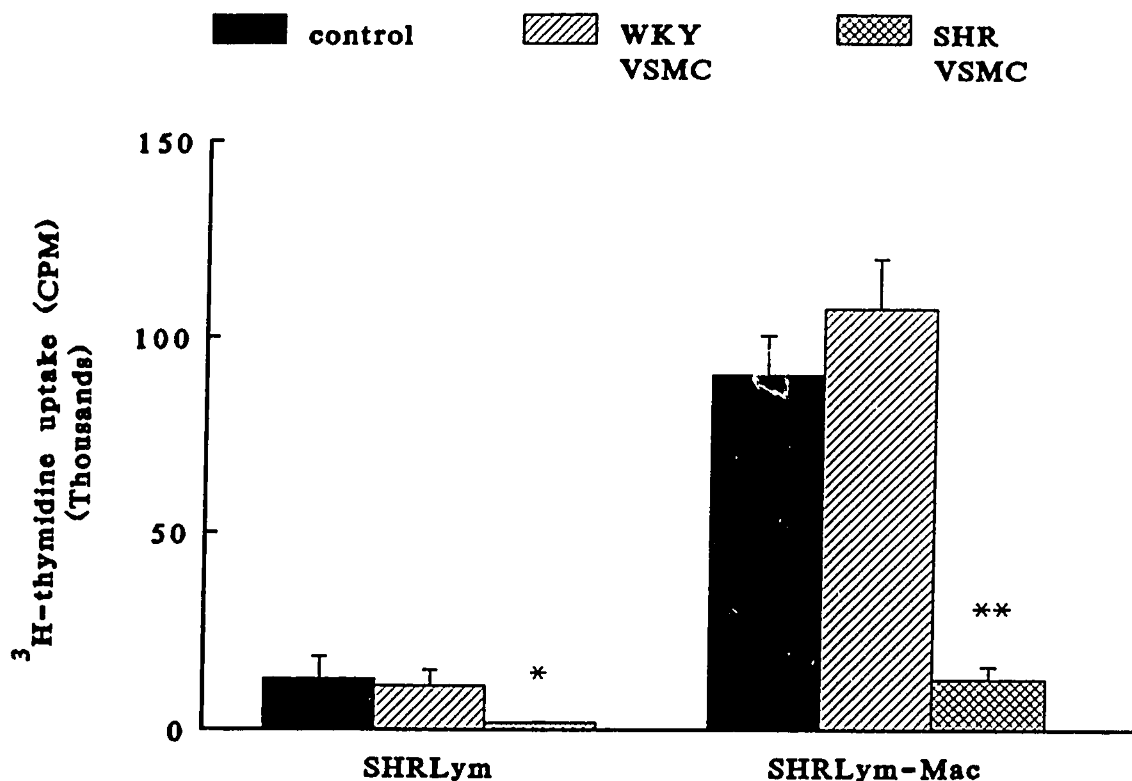


Figure V-4. **Effect of irradiated VSMC on the proliferation response of SHR lymphocytes.** Irradiated VSMC were prepared as described in Figure V-2. SHR lymphocytes (SHRLym) or SHR macrophage-depleted lymphocytes (SHRLym-Mac) were subsequently added in quadruplicate onto plates at a lymphocyte : VSMC ratio of 10:1. The cells were incubated for 72 hours in the presence of 5 μg/ml Con A in 10% FCS RPMI-1640 medium. ^{3}H-thymidine was added to the culture for the final 18 hours. Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well $\pm$ SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation.
*: $P < 0.05$, **: $P < 0.01$, compared with respective control group (lymphocytes only) and the group which contained lymphocytes plus WKY VSMC.

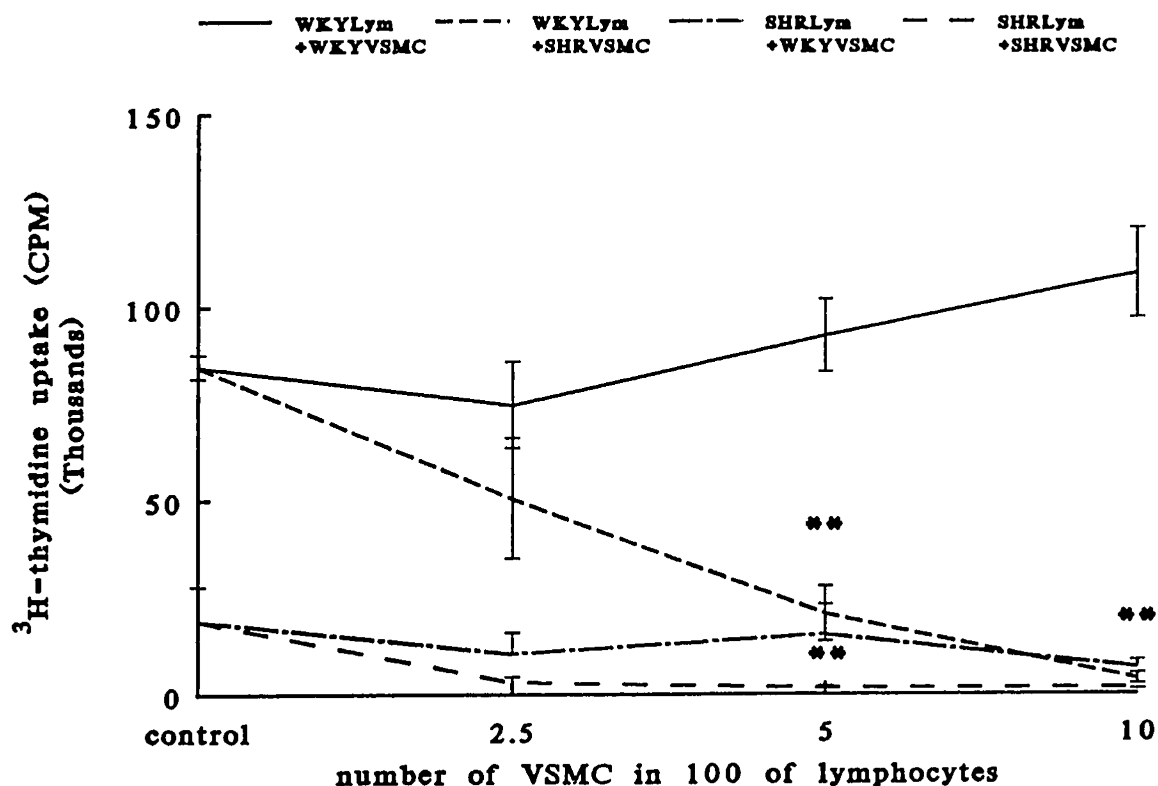


Figure V-5. **Dose dependent effect of VSMC on the proliferation response of lymphocytes from SHR and WKY.** Various concentrations of VSMC were planted in 96 well plates and incubated for 36-40 hours. The plates were then irradiated at 4000 rads. Lymphocytes (2 x 10^5cells/well) from SHR or WKY were subsequently added in quadruplicate onto plates at a lymphocyte : VSMC ratio of 40:1, 20:1 and 10:1. The cells were incubated for 72 hours in the presence of 5 μg/ml Con A. Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N = 12) from 3 VSMC preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with respective group which contained lymphocytes plus WKY VSMC.

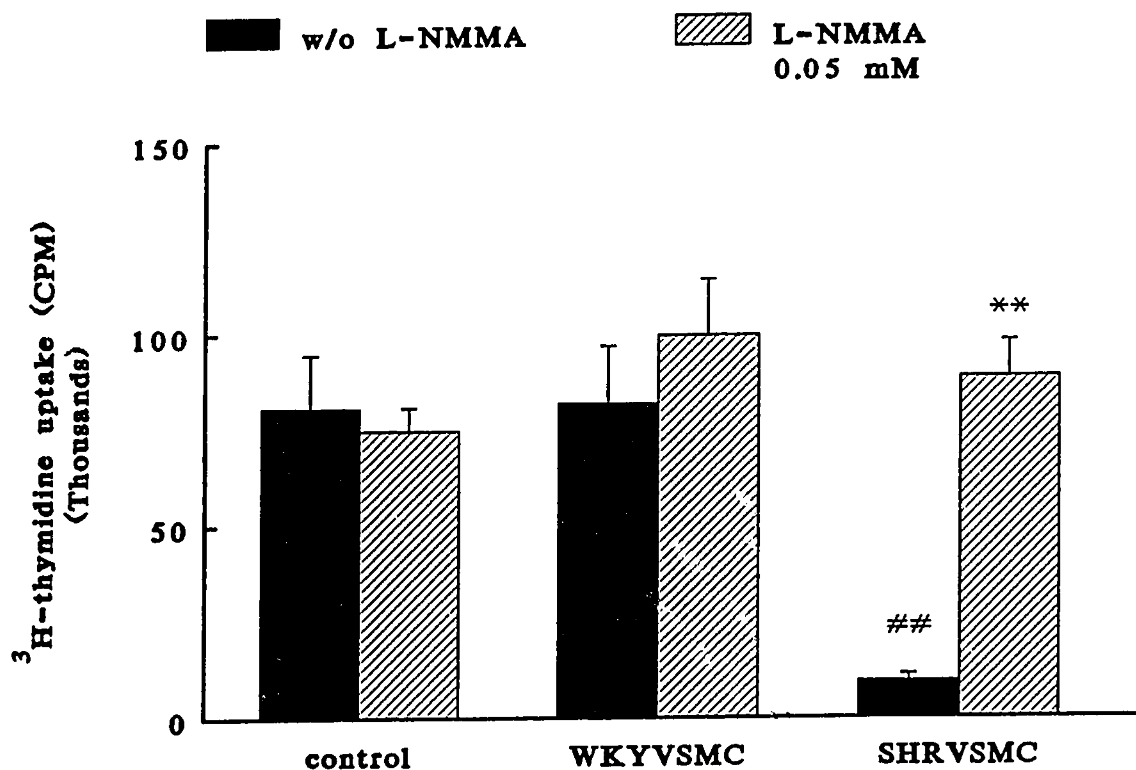


Figure V-6. **Effect of L-NMMA on the interaction of VSMC and WKY lymphocytes.** L-NMMA (0.05 mM) was added to the co-cultures of WKY lymphocytes with VSMC from SHR or WKY as described in Figure V-3 in the presence of 2.5 μg/ml Con A. (**control**: WKY lymphocytes; **WKYVSMC**: WKY lymphocytes plus WKY VSMC; **SHRVSMC**: WKY lymphocytes plus SHR VSMC). Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation. **: P < 0.01, compared with SHRVSMC group that without L-NMMA treatment. ##: P < 0.01, compared with both control and WKYVSMC groups that without L-NMMA treatment.

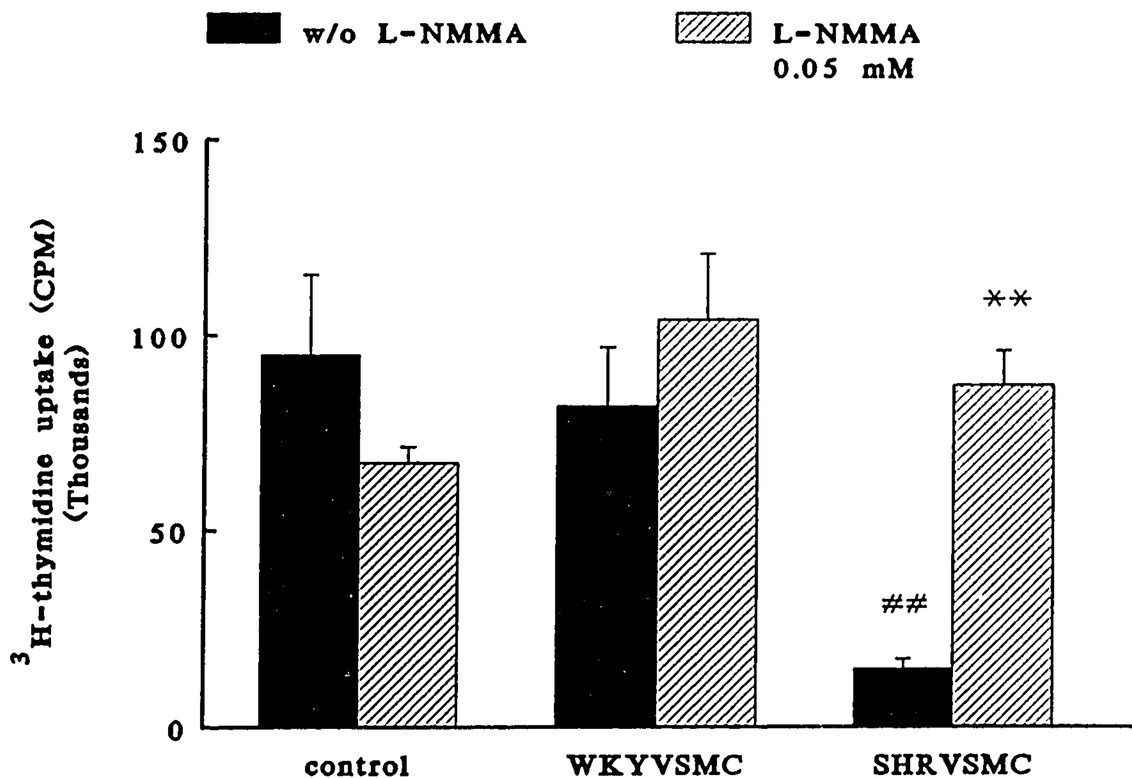


Figure V-7. **Effect of L-NMMA on the interaction of VSMC and WKY macrophage-depleted lymphocytes.** L-NMMA (0.05 mM) was added to the co-cultures of WKY macrophage-depleted lymphocytes with VSMC from SHR or WKY as described in Figure V-3 in the presence of 2.5 μg/ml Con A. (**control**: WKY macrophage-depleted lymphocytes; **WKYVSMC**: WKY macrophage-depleted lymphocytes plus WKY VSMC; **SHRVSMC**: WKY macrophage-depleted lymphocytes plus SHR VSMC). Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation.
**: $P < 0.01$, compared with SHRVSMC group that without L-NMMA treatment.
##: $P < 0.01$, compared with both control and WKYVSMC groups that without L-NMMA treatment.

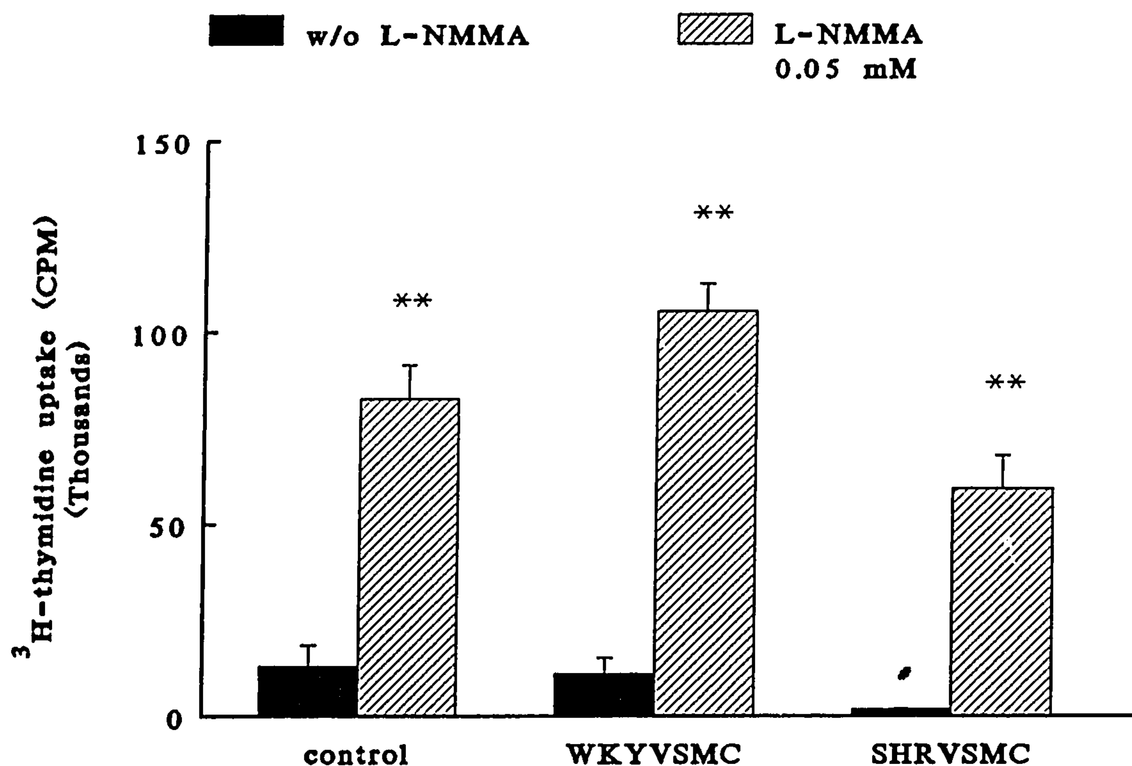


Figure V-8. **Effect of L-NMMA on the interaction of VSMC and SHR lymphocytes.** L-NMMA (0.05 mM) was added to the co-cultures of SHR lymphocytes with VSMC from SHR or WKY as described in Figure V-3 in the presence of 2.5 μg/ml Con A. (**control**: SHR lymphocytes; **WKYVSMC**: SHR lymphocytes plus WKY VSMC; **SHRVSMC**: SHR lymphocytes plus SHR VSMC). Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with respective group that without L-NMMA treatment. #: $P < 0.05$, compared with both control and WKYVSMC groups that without L-NMMA treatment.

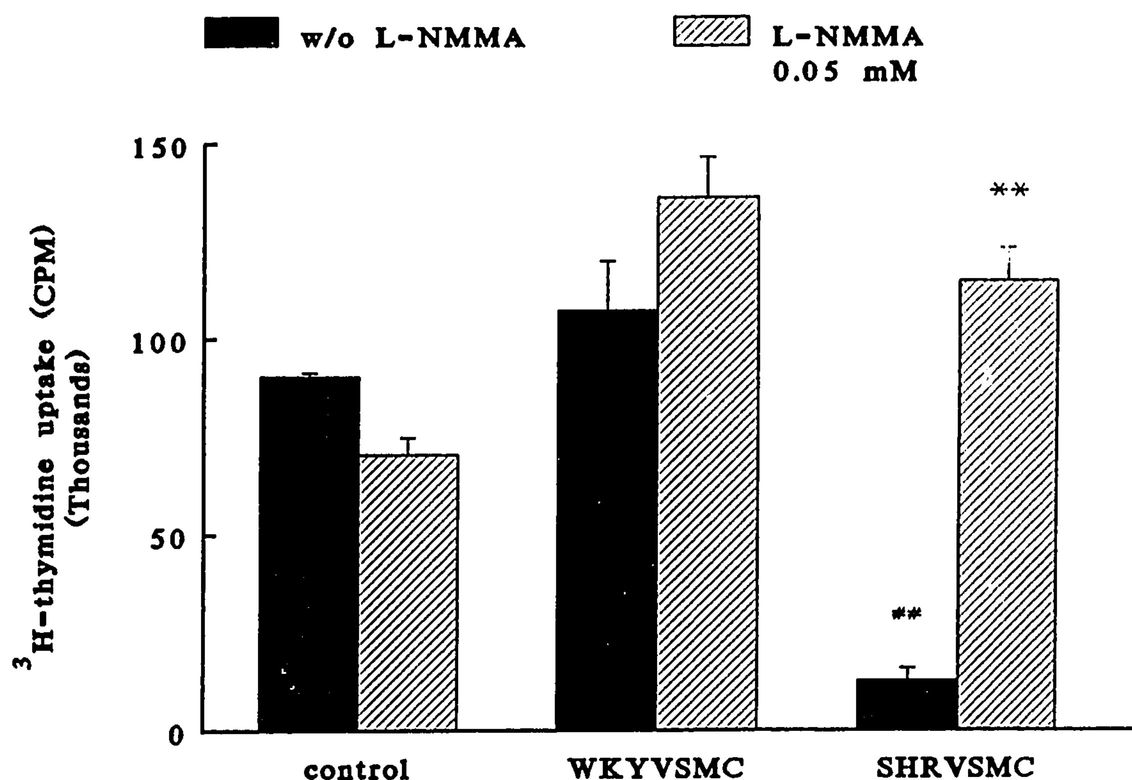


Figure V-9. **Effect of L-NMMA on the interaction of VSMC and SHR macrophage-depleted lymphocytes.** L-NMMA (0.05 mM) was to the co-cultures of SHR macrophage-depleted lymphocytes with VSMC from SHR or WKY as described in Figure V-3 in the presence of 2.5 μg/ml Con A. (**control**: SHR macrophage-depleted lymphocytes; **WKYVSMC**: SHR macrophage-depleted lymphocytes plus WKY VSMC; **SHRVSMC**: SHR macrophage-depleted lymphocytes plus SHR VSMC). Data represent means of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with SHRVSMC group that without L-NMMA treatment. ##: $P < 0.01$, compared with both control and WKYVSMC groups that without L-NMMA treatment.

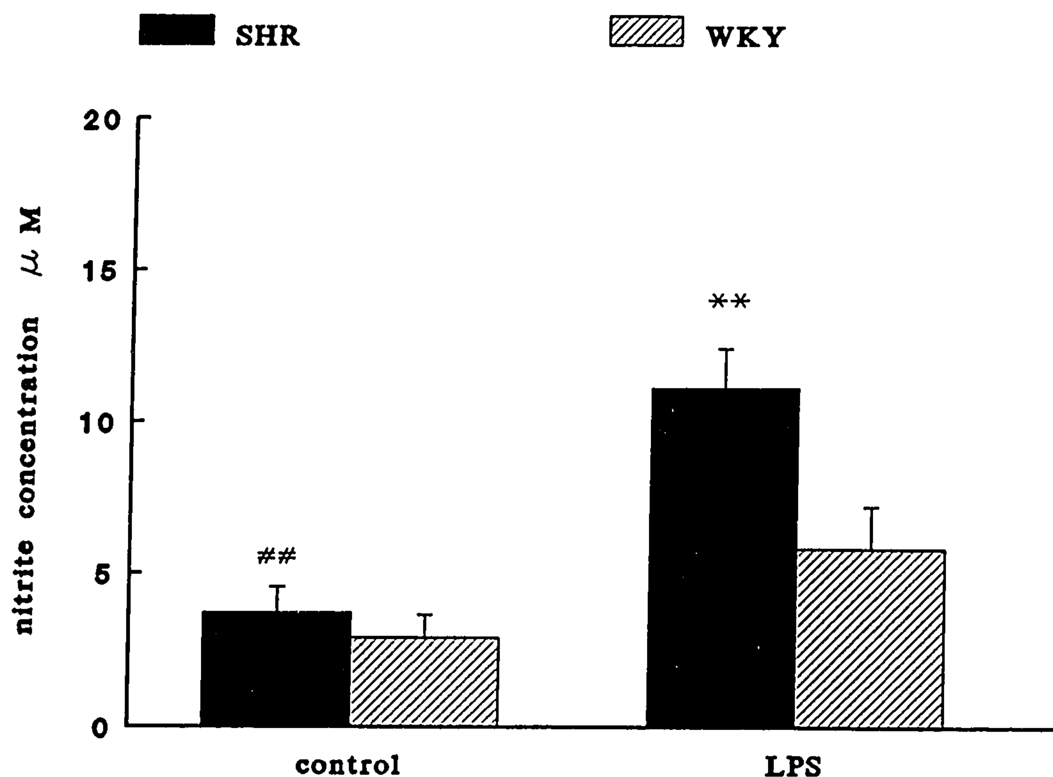


Figure V-10. **Nitric oxide production by irradiated VSMC from SHR and WKY induced by LPS.** VSMC were incubated in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. The VSMC were then irradiated using a ^{137}Cs-irradiator at dose of 4000 rads. To induce NO production, the irradiated VSMC were cultured with 40 μg/ml LPS in 10% FCS DMEM medium for 48 hours. The amount of NO in the culture supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N=6) from 3 VSMC preparations, 2 experiments for each preparation.
**: $P < 0.01$, compared with WKY VSMC treated with LPS. ##: $P < 0.01$, compared with SHR VSMC treated with LPS.

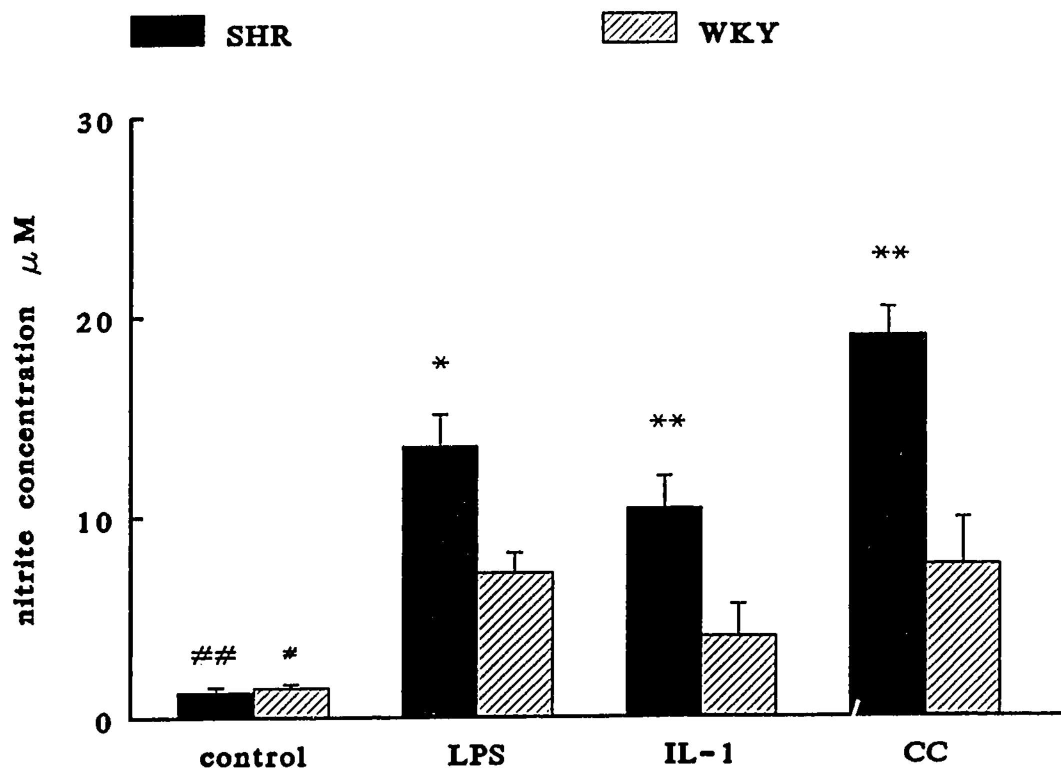


Figure V-11. **Nitric oxide production by VSMC from SHR and WKY induced by LPS, IL-1β and a cytokine mixture.** VSMC were incubated in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. To induce NO production, VSMC were cultured with either 40 μg/ml LPS, 25 units/ml IL-1β or a cytokine mixture (CC) containing 5 units/ml IL-1β, 5 nM TNFα, 200 units/ml IFNγ and 10 μg/ml LPS in 10% FCS DMEM medium for 48 hours. The amount of NO in the culture supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation. *: $P < 0.05$, **: $P < 0.01$ compared with respective WKY VSMC. ##: $P < 0.01$, compared with SHR VSMC treated with LPS, IL-1β or the cytokine mixture. #: $P < 0.05$, compared with WKY VSMC treated with LPS, IL-1β or the cytokine mixture.

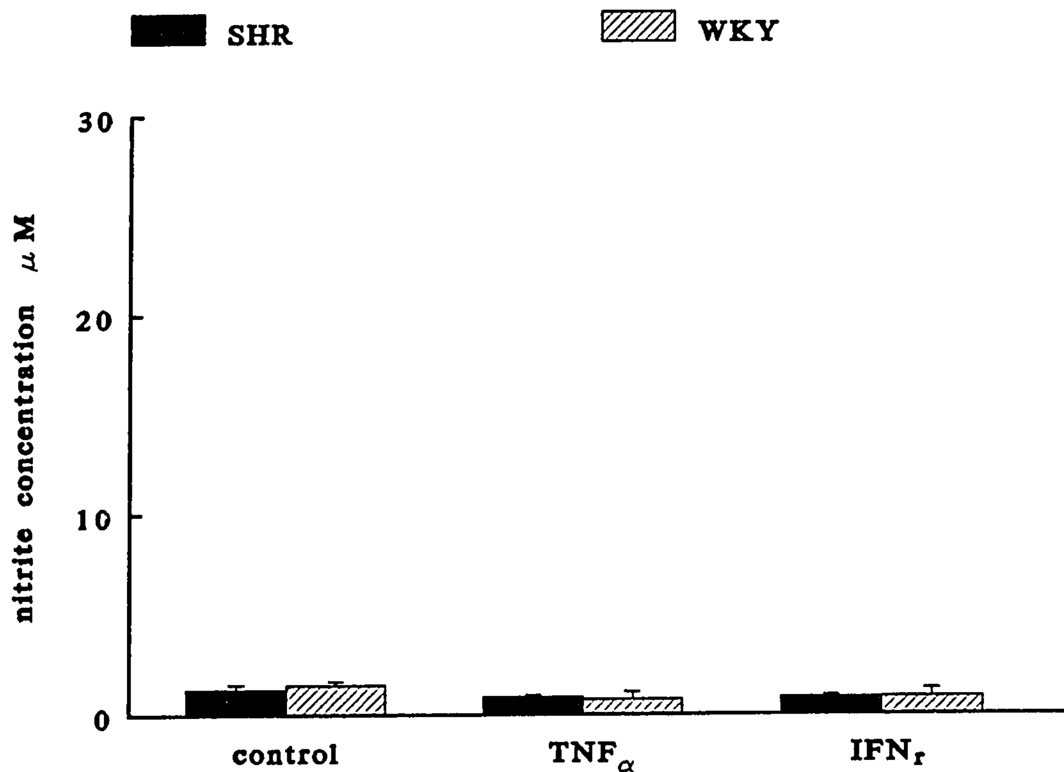


Figure V-12. **Nitric oxide production by VSMC from SHR and WKY induced by TNFα and IFNγ.** VSMC were incubated in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. To induce NO production, VSMC were cultured with either 5 nM TNFα or 200 units/ml IFNγ in 10% FCS DMEM medium for 48 hours. The amount of NO in the culture supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N=12) from 3 preparations, 4 experiments for each preparation.

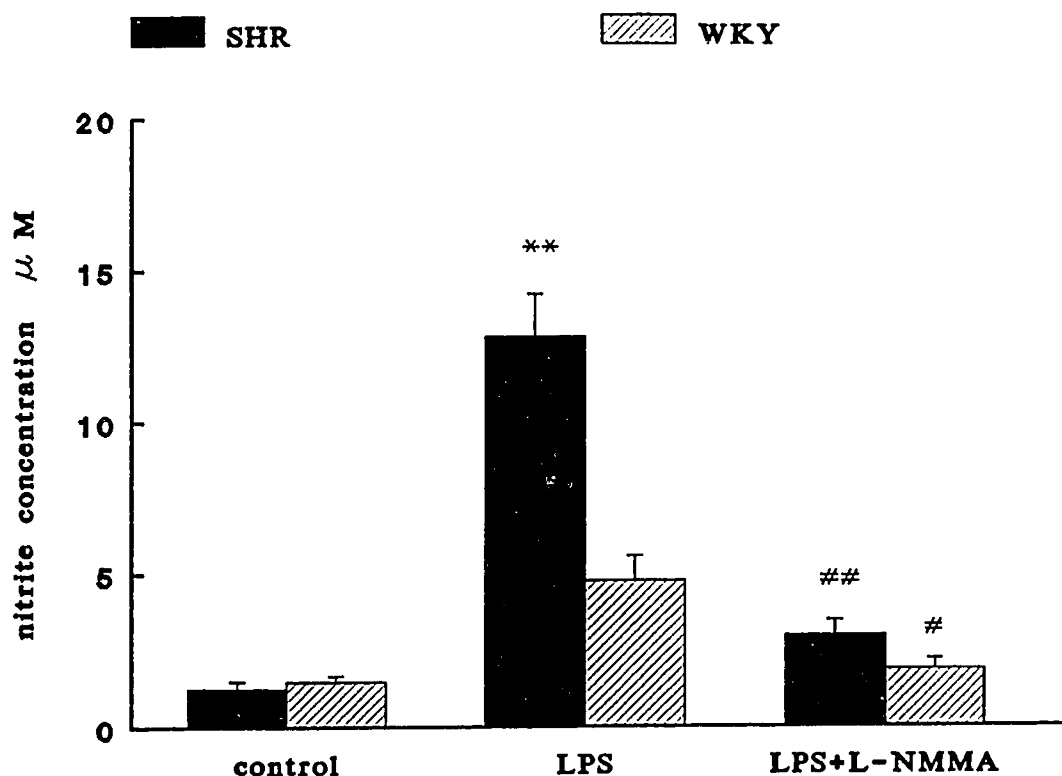


Figure V-13. **Effect of L-NMMA on VSMC NO production induced by LPS.** L-NMMA (0.05 mM) was added to VSMC culture as described in Figure V-10. In these experiments, the concentration of LPS was 40 μg/ml. The amount of NO in the culture supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N=12) from 3 VSMC preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with WKY VSMC treated with LPS only. ##: $P < 0.01$, compared with SHR VSMC treated with LPS only. #: $P < 0.05$, compared with WKY VSMC treated with LPS only.

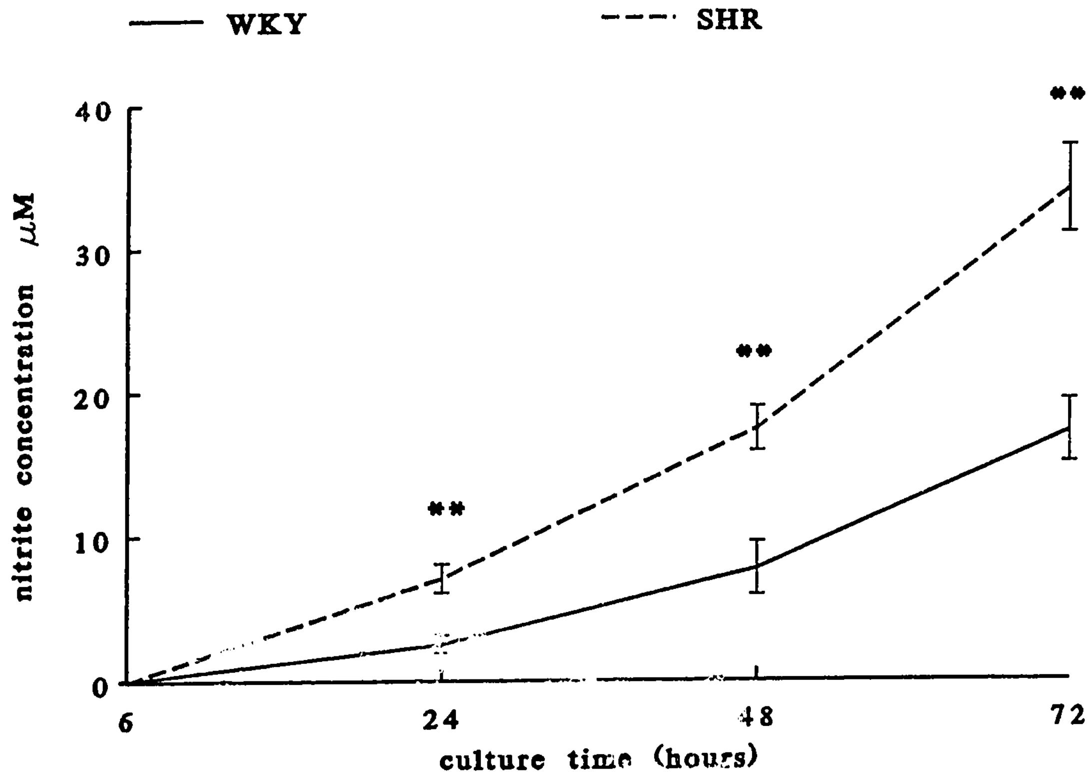


Figure V-14. **Time course of NO production in VSMC induced by a cytokine mixture.** VSMC were incubated in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. VSMC were then cultured with a cytokine mixture in 10% FCS DMEM medium as described in Figure V-11. At various times, the culture supernatant was collected. The amount of NO in the supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N=12) from 3 preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with respective WKY VSMC.

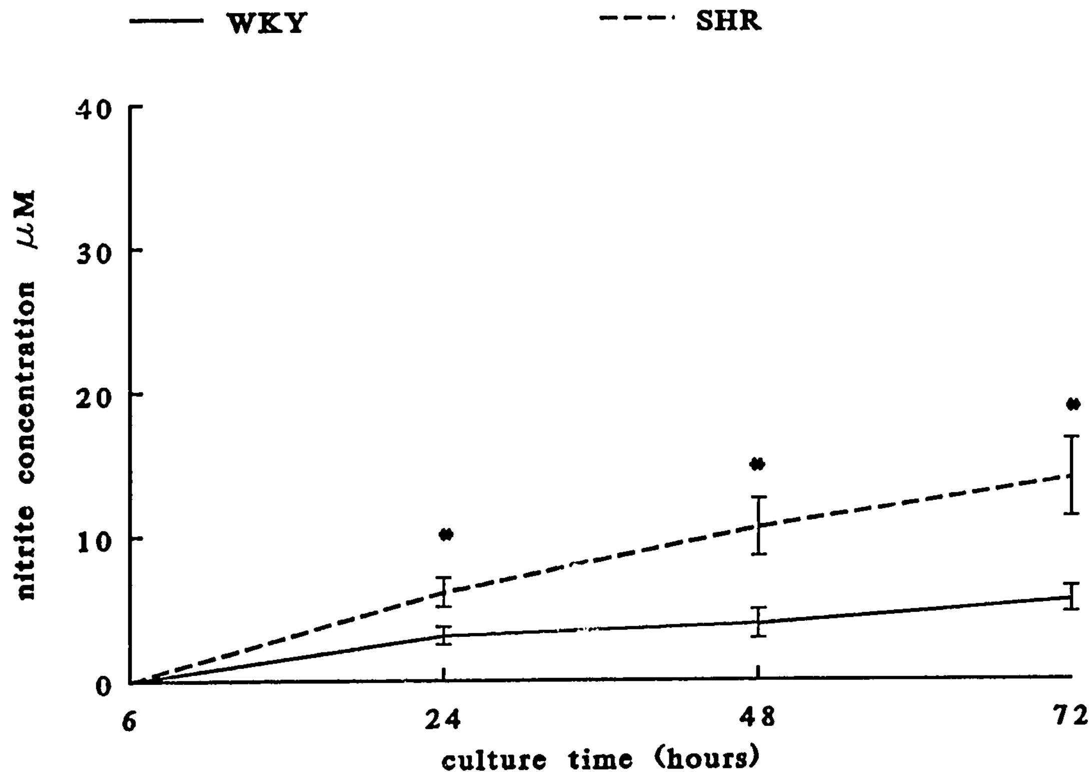


Figure V-15. **Time course of NO production in VSMC induced by LPS.** VSMC were incubated in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. VSMC were then cultured in 10% FCS DMEM medium in the presence of 40 μg/ml LPS. At various times, the culture supernatant was collected. The amount of NO in the supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N = 12) from 3 preparations, 4 experiments for each preparation. *: $P < 0.05$, compared with respective WKY VSMC.

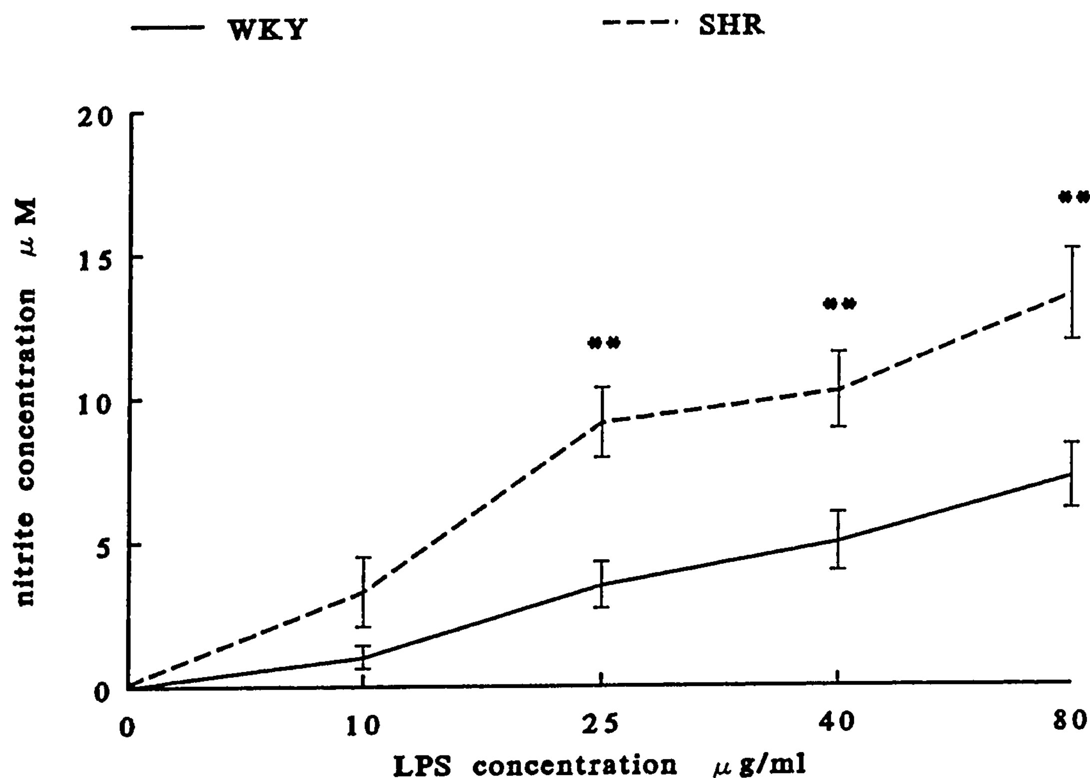


Figure V-16. **Dose dependent NO production in VSMC induced by LPS.** VSMC were incubated in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. VSMC were cultured with various concentrations of LPS in 10% FCS DMEM medium for 48 hours. The amount of NO in the culture supernatant was determined by a colorimetric assay. Data represent means of nitrite concentrations ± SE (N=12) from 3 preparations, 4 experiments for each preparation. **: $P < 0.01$, compared with respective WKY VSMC.

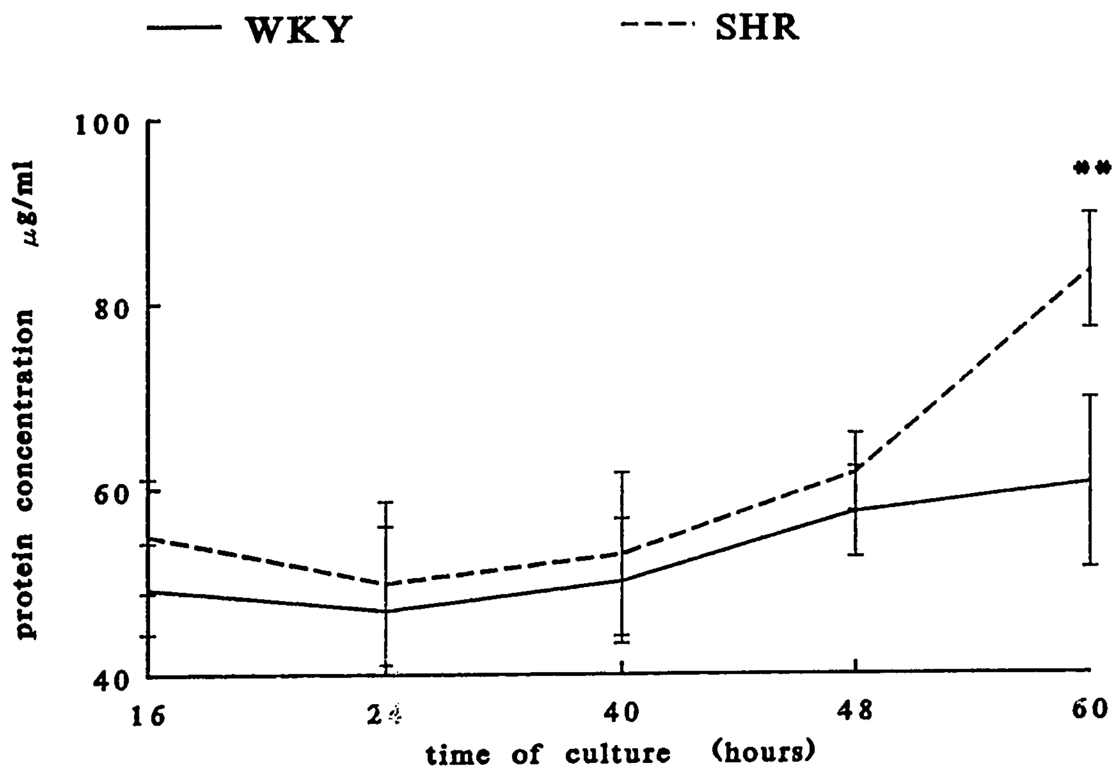


Figure V-17. **Time dependent protein synthesis in VSMC from SHR and WKY.** VSMC were incubated in 24 well tissue culture plates in 10% FCS DMEM medium for 36-40 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 48 hours to render the VSMC quiescent. VSMC were then cultured in 10% FCS DMEM medium. At various times, VSMC in the wells were washed twice with HBSS and solubilized with 1M NaOH (0.2 ml/well). The protein content of the VSMC was determined by the Pierce BCA protein assay. Data represent means of protein concentrations per well (μg/ml) ± SD (N=9) from 3 preparations, 3 experiments for each preparation. **: $P < 0.01$, compared with that of WKY VSMC cultured for 60 hours.

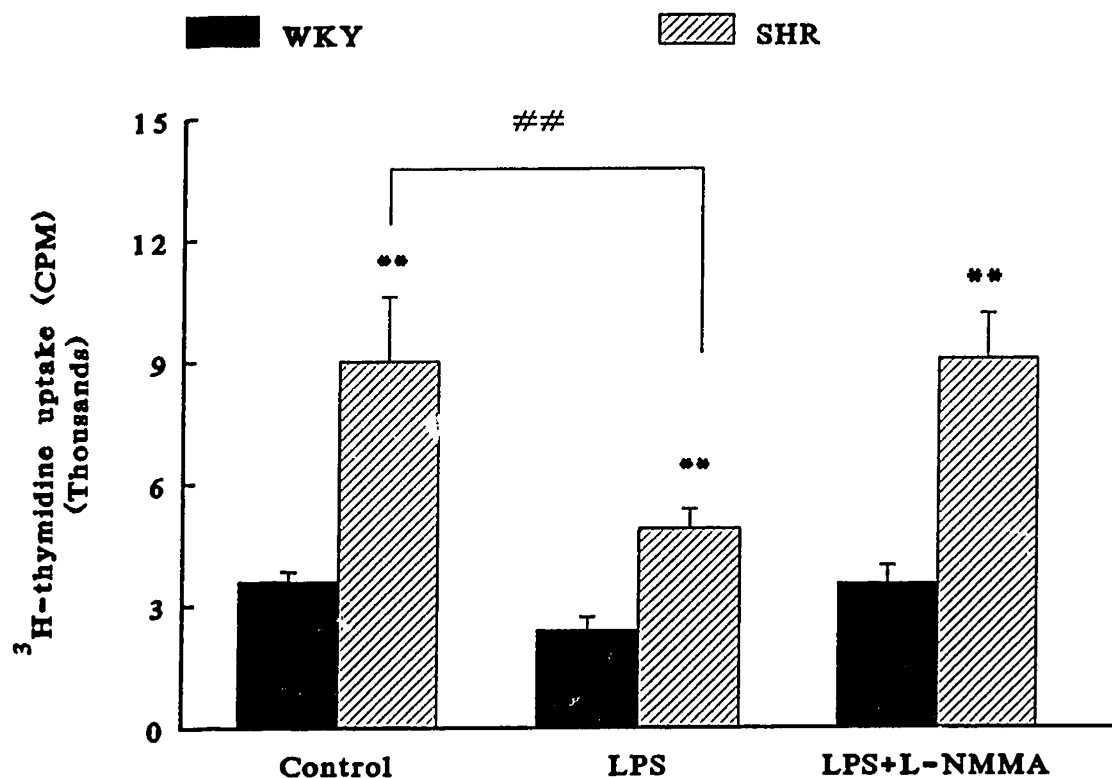


Figure V-18. **Effect of LPS and L-NMMA on VSMC proliferation.** VSMC ($1x10^4$ cells/well) were cultured in 96 well plates in 10% FCS DMEM medium for 24 hours to allow attachment of VSMC to the plates. The medium was changed to 0.4% FCS DMEM medium for 4 days. The quiescent VSMC were then cultured in 10% FCS DMEM medium for 72 hours in the presence or absence of 40 μg/ml LPS or LPS plus 0.05 mM L-NMMA. ^{3}H-thymidine was added for the final 24 hours The VSMC were harvested by trypsin treatment. Data represent means of ^{3}H-thymidine uptake by VSMC (CPM) per well ± SE (N=9) from 3 VSMC preparations, 3 experiments for each preparation. **: $P < 0.01$ compared with respective WKY VSMC. ##: $P < 0.01$, when control SHR VSMC was compared with SHR VSMC treated with LPS.

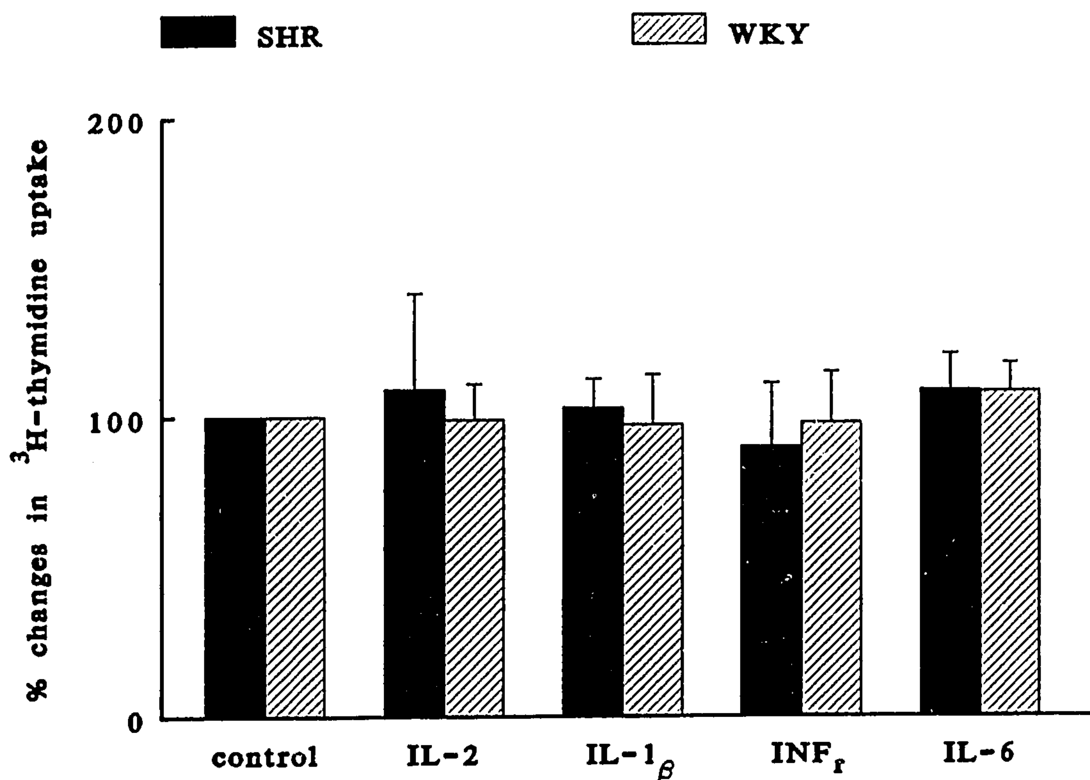


Figure V-19. **Effect of cytokines on VSMC proliferation.** VSMC (1 x 10^4cells/well) were cultured in 96 well plates as described in Figure V-18. The VSMC were then cultured in 5% FCS DMEM medium in the presence of 20 units/ml IL-2, 5 units/ml IL-1β, 50 units/ml INFγ or 100 units/ml IL-6. After 24 hours of incubation, the VSMC were pulsed with ^{3}H-thymidine and incubated for another 40 hours. The VSMC were harvested by trypsin treatment. Data represent the percentage changes in ^{3}H-thymidine uptake by VSMC ± SE (N=6) from two VSMC preparations, three experiments for each preparation.

CHAPTER VI. RELATIONSHIP OF IMMUNE DYSFUNCTION, NO SYNTHESIS ALTERATION AND HYPERTENSION

I. INTRODUCTION

In the previous chapters the enhancement of NO synthesis in SHR macrophages was described. This increased NO production resulted in lymphocyte suppression. It was also shown that SHR VSMC produced a significantly higher amount of NO upon stimulation. Furthermore, SHR VSMC inhibited the proliferation response of lymphocytes from either SHR or WKY. This inhibition was mediated by overproduction of NO in SHR VSMC. Whether the activation of NO synthesis is related to age and the development of hypertension is not clear. In order to elucidate the role of NO in hypertension and to understand the relationship between immune dysfunction and hypertension in SHR, the time course of the development of hypertension, NO synthesis alteration in macrophages and VSMC and lymphocyte depression were investigated.

Although the age-related study could provide information on the time sequence of these events which occur in SHR, it may not provide direct evidence on the effect of high blood pressure *per se* on NO synthesis alteration. Thus, it is necessary to examine NO synthesis alteration in a hypertensive state and a normotensive state. One possible approach is to use pharmacological means to lower blood pressure in SHR and then examine the changes in NO synthesis and

lymphocyte depression. However, results from the pharmacological study may be difficult to interpret since the drug used to lower blood pressure may have other effects in addition to those on the NO synthesis pathway. The borderline hypertensive rat (BHR) is a genetic model for environmentally induced hypertension. BHR is the first generation offspring of a mating between a female SHR and a male WKY. BHR possesses genetic information from both a normotensive WKY and a hypertensive SHR parent. The BHR becomes permanently hypertensive when subjected to a time-limited period of exposure to environmental stress or to increased dietary sodium intake (Lawler *et al.*, 1981; Lawler *et al.*, 1987; DiBona and Jones, 1993). The study of the NO synthesis pathway and the proliferation response of lymphocytes in both normotensive BHR and hypertensive BHR may be another way to provide supportive evidence for the effect of high blood pressure on these changes.

II. EXPERIMENTAL DESIGN

A. Investigation of age related blood pressure elevation, lymphocyte proliferation and NO synthesis alteration in SHR compared with those of WKY. To study the relationship between hypertension and immune dysfunction, the time courses of development of hypertension, lymphocyte inhibition and NO synthesis activation in macrophages and VSMC were investigated. As described in the Materials and Methods chapter, 3, 6 and 10 weeks old male SHR and WKY were purchased and housed for one or two weeks to allow adjustment to a new

environment and then used at 4, 8 and 12 weeks of age. Some rats were housed for up to one year. After blood pressure was measured under anaesthetized condition, the rat was sacrificed and the spleen and aorta were collected. The proliferation responses of spleen cells and macrophage-depleted lymphocytes were determined. NO production by spleen cells containing macrophages was also tested as described previously. VSMC were isolated and subcultured under the same conditions as described in Chapter II. VSMC NO production induced by LPS or a cytokine mixture was examined in cells between passage 3 and 10.

B. Examination of blood pressure, lymphocyte proliferation and NO synthesis in hypertensive BHR, normotensive BHR and WKY. To study the effect of high blood pressure on lymphocytes and the activation of NO synthesis, hypertension was induced in BHR. As described in Chapter II, 5 weeks old BHR and WKY were assigned to two groups. One group received a normal diet containing 1% NaCl, another group received an 8% sodium diet for 8 weeks. At 13 weeks of age, blood pressures were measured and the tissues were then collected. The proliferation response of lymphocytes, NO production by spleen macrophages and NO production by VSMC were examined as described previously.

III. RESULTS

A. Age Study:

1. SHR had significantly higher mean arterial blood pressure than did WKY

at all ages. The blood pressure increased continuously from 4 weeks of age and reached a plateau value of about 150 mmHg by 12 weeks of age (Figure VI-1).

2. At 4 weeks of age, SHR spleen cells showed the lowest lymphocyte proliferation response of any age groups, exhibiting only 5-10% of the response of WKY cells. As age advanced, the response of SHR spleen cells increased but remained significantly lower than that of WKY spleen cells. At 4 weeks of age, the proliferation response of SHR spleen cells was significantly lower compared with that of SHR spleen cells at 8 or 12 weeks of age. The depressed SHR lymphocyte proliferation response persisted for up to one year (Figure VI-2).

3. Figure VI-3 shows that in all age groups the depressed proliferation responses of SHR spleen cells could be fully reversed by L-NMMA. After removing macrophages from SHR spleen cells, the proliferation response of these cells was significantly increased, except in the one year old group. Neither L-NMMA nor the removal of macrophages had any effect on the proliferation response of WKY spleen cells (Figure VI-4).

4. The results of NO synthesis by spleen cells, which contained macrophages at different ages are shown in Figure VI-5. After stimulation by Con A or LPS, SHR spleen macrophages produced a significantly greater amount of NO than did WKY spleen cells. Compared to 8 or 12 weeks of age groups, SHR spleen macrophages produced larger amounts of NO at 4 weeks of age, but the difference was not significant. This increased NO synthesis by SHR spleen macrophages was inhibited by L-NMMA (Figure VI-6). L-NMMA also inhibited NO production in WKY spleen

macrophages (Figure VI-7).

5. After being induced by the cytokine mixture, VSMC isolated from SHR produced a significantly greater amount of NO than did WKY VSMC in all age groups (Figure VI-8). At 4 weeks of age, the difference in NO production was already profound. There was an increase in NO synthesis in VSMC from 4 weeks, which reached a plateau at 12 weeks of age. A similar result in NO production was obtained in VSMC stimulated with LPS (Figure VI-9).

6. Figure VI-10 shows that without stimulation, only a trace amount of NO was produced in SHR VSMC. It was also shown that L-NMMA inhibited NO production in SHR VSMC in all age groups. The same result was observed in WKY VSMC (Figure VI-11).

7. When lymphocyte proliferation response was plotted against the rise in blood pressure, no correlation was observed between the lymphocyte proliferation and the blood pressure in either WKY or SHR. The correlation coefficients (r) were 0.18 and 0.03, respectively (Figure VI-12).

8. There was no correlation between spleen macrophage NO production and the increase in blood pressure in SHR (r= -0.15) (Figure VI-13, Panel B). In addition, there was no correlation between spleen macrophage NO production and blood pressure in WKY (r = -0.24) (Figure VI-13, Panel A).

9. Figure VI-14 shows the relationship between blood pressure and VSMC NO synthesis induced by a cytokine mixture. When NO synthesis in SHR VSMC was plotted against the blood pressure in SHR, there was a significant positive correlation

($r = 0.71, P = 0.0003$) (Panel B). However, this correlation did not exist in WKY ($r = 0.13$) (Panel A). Figure VI-15 also shows that a similar relationship exists when VSMC NO synthesis was induced by LPS. There was a significantly positive correlation in SHR ($r = 0.69, P = 0.0006$) (Panel B). No significant correlation was found in WKY ($r = -0.07$) (Panel A).

B. <u>BHR Study</u>:

1. The blood pressure of BHR was slightly, but not significantly higher than that of WKY under normal diet. After an 8 week high sodium diet, the blood pressure was significantly increased in BHR. There was an increment of more than 30 mmHg compared with the blood pressure of BHR fed a normal diet. High sodium intake did not, however, significantly affect blood pressure in WKY (Figure VI-16).

2. The proliferation response of spleen cells was tested (Figure VI-17). The BHR which had high blood pressure exhibited a normal lymphocyte proliferation response similar to that of normotensive BHR and WKY. The proliferation response of WKY spleen cells was also not affected by the high sodium intake.

3. Figure VI-18 shows spleen cell (containing macrophages) NO production induced by Con A in BHR and WKY. In hypertensive BHR, the NO synthesis was not significantly different from that of BHR with normal blood pressure or WKY. In WKY fed a high sodium diet, NO synthesis in spleen macrophages was very similar to that of spleen macrophages of WKY fed a normal diet. Similar results

were observed when spleen macrophages were stimulated by LPS (Figure VI-19). There was no difference in NO production between hypertensive BHR and normotensive BHR. 4. However, BHR with high blood pressure exhibited an elevated NO synthesis in VSMC. There was a more than 2 fold increase in NO production in VSMC of hypertensive BHR compared with that of normotensive BHR after stimulation by a cytokine mixture (Figure VI-20). When stimulated by LPS, the VSMC of hypertensive BHR also produced a greater amount of NO than did VSMC of normotensive BHR (Figure VI-21). High sodium intake did not produce any significant effect on VSMC NO synthesis in WKY.

5. Figure VI-22 shows there was no correlation between blood pressure and spleen macrophage NO production induced by the cytokine mixture in hypertensive BHR fed a high sodium diet ($r = -0.01$, Panel A). No correlation was observed in normotensive BHR fed a control diet ($r = -0.37$, Panel B), WKY fed a high sodium diet ($r = -0.03$, Panel C) and WKY fed a control diet ($r = -0.1$, Panel D).

6. When VSMC NO production induced by the cytokine mixture was plotted against the blood pressure, there was a significant positive correlation in hypertensive BHR, which were fed a high sodium diet, ($r = 0.87$, $P = 0.02$) (Figure VI-23, Panel A). No such correlation was found in normotensive BHR, which fed a control diet ($r = -0.28$, Panel B), WKY fed a high sodium diet ($r = 0.14$, Panel C) and WKY fed a control diet ($r = -0.16$, Panel D).

IV. DISCUSSION

Previous studies have demonstrated that the proliferation response of spleen cells was severely depressed in 12 weeks old SHR. This depression was mediated by overproduction of NO from SHR macrophages. In addition, it has been shown that SHR VSMC produced a greater amount of NO than WKY VSMC. Furthermore, SHR VSMC were capable of inhibiting lymphocyte proliferation responses in either SHR or WKY. These observations indicate that there may be a general activation of the inducible NO synthesis system in SHR, contributing to the lymphocyte proliferation defect. The present study was designed to determine if the development of hypertension, lymphocyte depression and changes in the inducible NO synthesis system are related.

As soon as it was technically possible to cannulate the carotid artery (4 weeks of age), the blood pressure of SHR was found to be significantly higher than that of WKY. Similar results have been reported by other investigators, *i.e.*, at 4 to 5 weeks of age the blood pressure was elevated in SHR (Head and Adams, 1992; Pascual *et al.*, 1993). In this study, the proliferation response of SHR spleen cells was already significantly reduced at 4 weeks of age. As blood pressure continued to increase with advancing age, the reduced lymphocyte proliferation response in SHR persisted but to a lesser extent than that observed at 4 weeks of age. An age dependent lymphocyte depression has been reported by Pascual and co-workers (1992). They showed that at 4 weeks of age the proliferation response of SHR spleen cells was

similar to that of WKY spleen cells. The reason for this disparity in these results is not clear. It is possible that different experimental systems were used. In the present study, the data were generated from 10 rats, 4 wells for each individual rat, while their data were presented from only 5 wells, in one experiment. Because lymphocyte proliferation depression was observed at 4 weeks of age when the blood pressure was already significantly higher, it is difficult to determine whether a causative relationship exists between these two changes or whether these changes happen concurrently. However, taking into consideration the observation that the greatest lymphocyte depression occurred at 4 weeks when the blood pressure was the lowest, it is likely that there is a dissociation between blood pressure and lymphocyte depression in SHR. This dissociation is supported by the observation that showed no correlation exists between lymphocyte depression and blood pressure in SHR.

The fact that L-NMMA dramatically increased the proliferation response of SHR lymphocytes in all age groups indicates the involvement of NO in the lymphocyte proliferation defect throughout the development of hypertension. Removal of macrophages from SHR spleen cells also corrected the lymphocyte depression in the 4, 8 and 12 weeks of age groups indicating that the activation of NO synthesis in SHR macrophages is responsible for the lymphocyte proliferation defect during development. It is not clear why removal of macrophages did not show any significant effect on the lymphocyte proliferation response in the one year old group. It is possible that the adhering ability of macrophages onto plastic surfaces was decreased in aged SHR, resulting in incomplete depletion of macrophages from

spleen cells. It is also possible that one year of hypertension causes activation of the NO synthesis in SHR spleen cells other than macrophages, *i.e.*, granulocytes and dendritic cells and that these cells were not removed by the adhering process. Direct evidence of NO synthesis activation was provided by measuring NO production in SHR spleen cells containing macrophages, in all age groups. After stimulation by Con A or LPS, SHR spleen macrophages produced more than two fold the amount of NO compared with those of WKY in all ages. Compared with the 8 or 12 weeks of age groups, NO production by spleen macrophages at 4 weeks of age was higher. This greater NO production correlated with the lowest lymphocyte proliferation response in SHR (4 weeks of age), suggesting that the onset of NO synthesis activation in SHR and the resulting suppression of lymphocyte proliferation may either occur at a very early stage of life as soon as blood pressure begins to rise or may be genetic in origin. That the higher NO production in spleen macrophages occurred at the time when the blood pressure was lowest also suggests that the activation of NO synthesis in spleen macrophages may not be related to the increase in blood pressure in SHR. However, the correlation analysis showed that there was no correlation between activation of NO synthesis in spleen macrophages and hypertension, and no correlation between lymphocyte depression and hypertension. These results implicated a dissociation between lymphocyte depression and hypertension in SHR.

The present study demonstrates that after induction by LPS or the cytokine mixture, SHR VSMC produced significantly greater amounts of NO than did WKY

VSMC in all age groups. Unlike NO synthesis in macrophages, when NO production in SHR VSMC is plotted against blood pressure there is a significant positive correlation, indicating that the increase in NO synthesis in VSMC is significantly associated with the rise in blood pressure in SHR. Because the increase in NO synthesis in VSMC correlated with blood pressure with advancing age, age itself may affect NO synthesis in SHR VSMC. However, when age was analyzed as a covariant, it did not show any significant effect on NO synthesis. It is not yet understood what is the mechanism responsible for activation of NO synthesis in SHR VSMC. It is possible that this mechanism and the mechanism responsible for the activation of NO synthesis in SHR macrophages may or may not be the same.

This study is the first to demonstrate that NO synthesis in SHR macrophages and VSMC is activated throughout all stages of the development of hypertension, supporting the hypothesis that a general alteration of inducible NO synthesis system may exist in SHR (Xiao and Pang, 1994c). However, this age related study did not elucidate whether a causative or parallel relationship exists between the development of hypertension and the lymphocyte depression as a result of activation of NO synthesis in SHR macrophages.

The BHR study was designed to further define the relationship between lymphocyte depression, NO synthesis alteration and hypertension. The results of this study demonstrated that blood pressure was significantly increased in BHR fed a high sodium diet. The lymphocyte proliferation response in hypertensive BHR was very similar to the response of normotensive BHR and WKY. NO synthesis in spleen

macrophages of hypertensive BHR was not significantly different from that of normotensive BHR. Correlation analysis showed that there was no correlation between blood pressure and spleen macrophage NO synthesis in hypertensive BHR. These observations suggest that lymphocyte depression and NO synthesis activation in macrophages are not related to high blood pressure, at least in salt-induced hypertension. This confirms the results of the age study which showed that NO synthesis in SHR spleen macrophages are not related to blood pressure. The normal response of lymphocyte proliferation and normal production of NO by spleen macrophages in hypertensive BHR suggest that lymphocyte depression and hypertension are two different, and perhaps parallel, phenomena which occur in hypertension.

However, unlike NO synthesis in macrophages of hypertensive BHR, this study showed that NO synthesis was significantly increased in VSMC of the hypertensive BHR but not in those of normotensive BHR. In addition, there was a significant correlation between blood pressure and VSMC NO synthesis in hypertensive BHR, but not in normotensive BHR and WKY. These observations support the results of the age study which showed that the blood pressure is positively correlated with the increase in VSMC NO synthesis in SHR. These findings suggest that increased blood pressure may influence the activation of the NO synthesis in VSMC. In SHR and hypertensive BHR, both a dissociation between macrophage NO synthesis and blood pressure, and an association between VSMC NO synthesis and blood pressure suggest that the activation of NO synthesis in VSMC and macrophages in SHR may be two

separate parallel events. This also suggests that the mechanisms for activation of NO synthesis in SHR VSMC and macrophages may be different.

In BHR, dietary sodium intake induced a transition from the normotensive state (that of the WKY parent) to the hypertensive state (that of the SHR parent). The hypertensive BHR exhibit many characteristics of the SHR phenotype such as sustained increase in arterial pressure, exaggerated natriuresis and increased efferent renal sympathetic nerve activity. These characteristics persist even after dietary sodium has return to normal levels (DiBona and Jones, 1991). The mechanism involved in the induction of hypertension in BHR fed a high sodium diet is not fully understood. Several lines of evidence suggest that neural mechanisms may contribute to the development of salt-induced hypertension. Increased dietary sodium intake can act on the central nervous system resulting in alterations in the regulation of the peripheral sympathetic nervous system activity, especially the control of renal function (DiBona and Jones, 1991). Increased renal sympathetic nerve activity has been suggested to be involved in genetically predisposed individuals (Tucker and Hunt, 1993). The hypothesis of the involvement of neural mechanisms was also supported by the observation that salt-induced hypertension in BHR could be prevented by disrupting central nervous system control of sympathetic activity (Sanders and Johnson, 1989). However, it has also been suggested that the increase in sympathetic activity may have occurred during the initial exposure to high sodium at an early phase in the rise in blood pressure. Long-term elevation of blood pressure was maintained by other factors (Tucker and Hunt, 1993). It has also been

demonstrated that salt-induced hypertension is produced via a mechanism related to extracellular fluid volume and increased cardiovascular and renal responses (DiBona and Jones, 1992; 1993). Various abnormalities in microvascular structure and function have been documented in animals with salt-induced hypertension (Vial *et al.*, 1989; Takenaka *et al.*, 1992). The ability to excrete electrolytes and water was reportedly impaired in BHR on a high sodium diet compared to similarly treated WKY (Lawler *et al.*, 1987). Thus, it is possible that a higher blood pressure is needed in BHR to excrete a given load of sodium. Whatever the underlying mechanism, the blood pressure was significantly and persistently increased in BHR fed a high sodium diet.

It has become clear that NO plays an important role in extracellular volume regulation as a natriuretic factor. Several recent studies showed that NO production increased in response to increased dietary salt intake in rats. It has been reported that both plasma concentrations of nitrite and nitrate and urine excretion of nitrite and nitrate were increased in salt-induced hypertension (Shultz and Tolins, 1993). Circulating and excreted nitrite and nitrate reflect *in vivo* production of NO and are also correlated with urinary cGMP excretion (Shultz and Raij, 1991). Chen and Sanders (1991) reported that an increased dietary salt load resulted in increased activity of the NO system in Sprague-Dawley rats and in salt-resistant, but not salt-sensitive, Dahl rats. Later, the same authors reported that plasma concentrations of citrulline, a by-product of NO synthase, did not differ between salt-sensitive and salt-resistant Dahl rats. Administration of L-arginine lowered blood pressure to

normotensive levels and simultaneously increased urinary nitrate excretion in rats fed a high sodium diet (Chen and Sander, 1993). Contradictory to their earlier findings, their later observations suggested that NO production was enhanced in salt-induced hypertension. In these studies, the source of the increased NO detected in response to increased salt intake was not clear and was only hypothesized to be vascular tissue and the kidney. It has been shown that NO synthase inhibition results in renal vasoconstriction and decreased GFR (Shultz and Toline, 1993). NO synthase inhibition also attenuated the natriuresis and diuresis induced by extracellular volume expansion (Atucha *et al.*, 1994). These results suggested that in addition to the effect of regulating vascular tone, the endogenous NO system may play an important role in the modulation of renal sodium handling, extracellular and blood volume regulation, and, therefore, in blood pressure regulation.

The current study showed that the blood pressure of WKY fed a high salt diet did not differ from that of WKY fed a normal diet. In addition, despite the high salt intake, the NO synthesis in WKY VSMC was very similar to that of WKY fed a normal sodium diet. These results are different from the results reported by others (Chen and Sanders, 1991; Shultz and Tolins, 1993). Explanations for these differences are probably related to experimental design. Previous investigators measured NO production in plasma and urine whereas in this study induced NO production in VSMC was measured. Since high salt intake affects NO synthesis in neither BHR macrophages nor WKY macrophages or VSMC, the increased NO synthesis in VSMC of BHR fed a high sodium diet maybe related to the elevated

blood pressure.

The mechanism for increase in NO synthesis in VSMC of salt-induced hypertensive rats is not clear at this time. It has been reported that persistent hypertension caused damage to resistance vessels and the renal vasculature (Ager, 1993; Ruilope *et al.*, 1994). There is evidence suggesting that tissue and organ damage might initiate an immunological response (Norman *et al.*, 1988; Hancock *et al.*, 1993). The cytokines, especially inflammatory cytokines such as IL-1 and TNF, produced and released in immunological reactions, are potent inducers of NO synthase expression. The increase in cytokine production in local arterial walls may result in increased expression of inducible NO synthase. Dexamethasone has been shown to prevent expression of inducible, but does not alter constitutive NO synthase in the arterial system (Knowles *et al.*, 1990). When dexamethasone was added with the high salt diet, despite supplementation with L-arginine, hypertension developed in a fashion typical of salt-induced hypertension (Chen and Sander, 1993). This observation suggested that inducible NO synthase was involved in the increase in NO production in response to high sodium intake. The present study provides direct evidence that inducible NO production was increased in VSMC in salt-induced hypertension.

The functional consequences of NO formation by inducible NO synthase in VSMC have not been clearly determined. Because massive infiltration of inflammatory cells was observed in vascular lesions in hypertensive humans and animals (Olsen, 1972; Ishimitsu *et al.*, 1992), it seems possible that inducible NO

synthesized in VSMC may modulate the formation of hypertensive vascular lesion. NO may decrease the severity of such lesions via inhibitory effects on adhesion and aggregation of platelets, superoxide anion production, leukocyte adhesion, endothelin generation, and VSMC proliferation. Furthermore, the enhanced NO synthesis in the vasculature may play a compensatory role in states of elevated blood pressure. NO synthesized in the vascular wall may counter-balance the effect of vasoconstrictive factors whose production is usually elevated in the hypertensive state.

In summary, the current study showed that lymphocyte depression and NO synthesis activation in SHR macrophages persisted throughout the development of hypertension. The alteration of NO synthesis in macrophages and the resulting lymphocyte depression were not associated with the increase in blood pressure in SHR. Inducible NO synthesis in VSMC was significantly elevated in SHR throughout the development of hypertension and there was a positive correlation between the increase in NO synthesis in SHR VSMC and the rise in blood pressure. Hypertensive BHR induced by high salt intake exhibited normal lymphocyte proliferation responses and normal NO synthesis in macrophages. However, inducible NO synthesis in VSMC was significantly enhanced in hypertensive BHR. These results suggest that hypertension and the lymphocyte depression as a result of NO synthesis activation in macrophages are two parallel, but perhaps unrelated phenomena that occur in SHR. In VSMC, the NO synthesis was closely associated with high blood pressure in both SHR and BHR, suggesting that inducible NO synthase in VSMC may be related to elevated blood pressure.

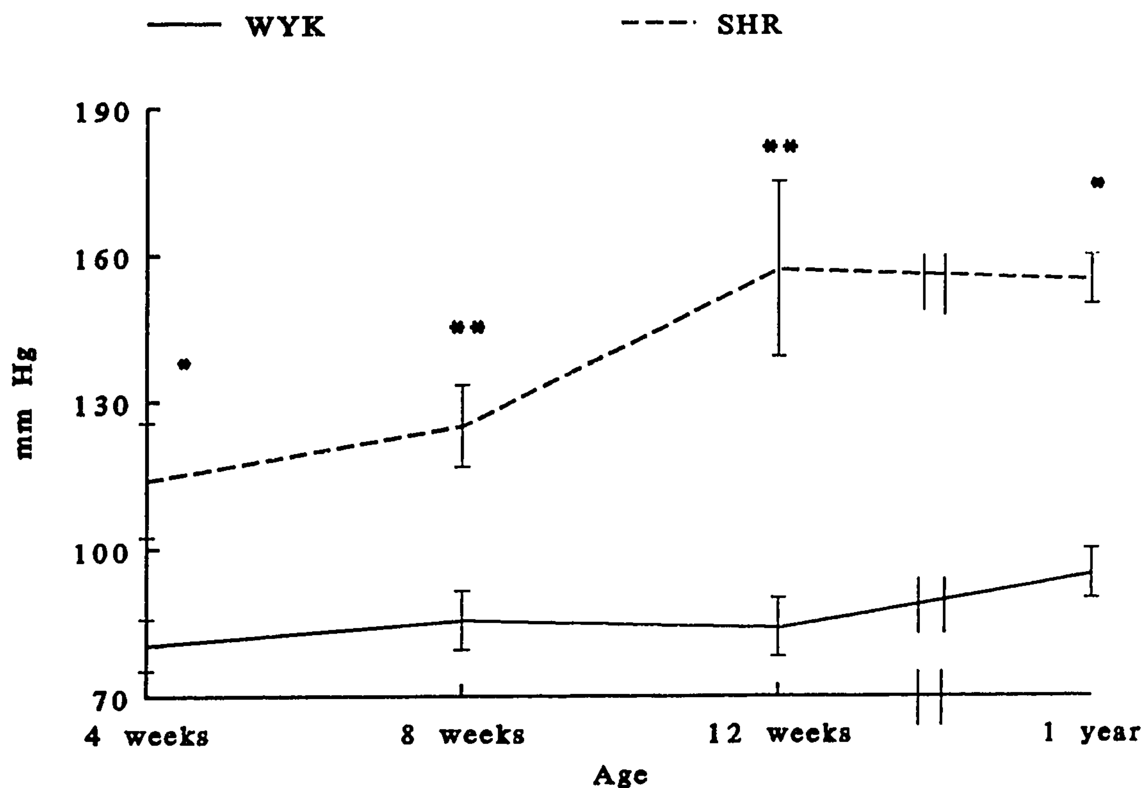


Figure VI-1. **Mean arterial blood pressure of SHR and WKY from age 4 weeks to one year.** The blood pressure was measured via carotid artery cannulation under anaesthetized condition. The values represent means of arterial blood pressure ± SD for age 4 weeks (n=6), 8 weeks (n=10), 12 weeks (n=5) and one year (n=3).
*: $P < 0.05$, **: $P < 0.01$, compared with respective WKY

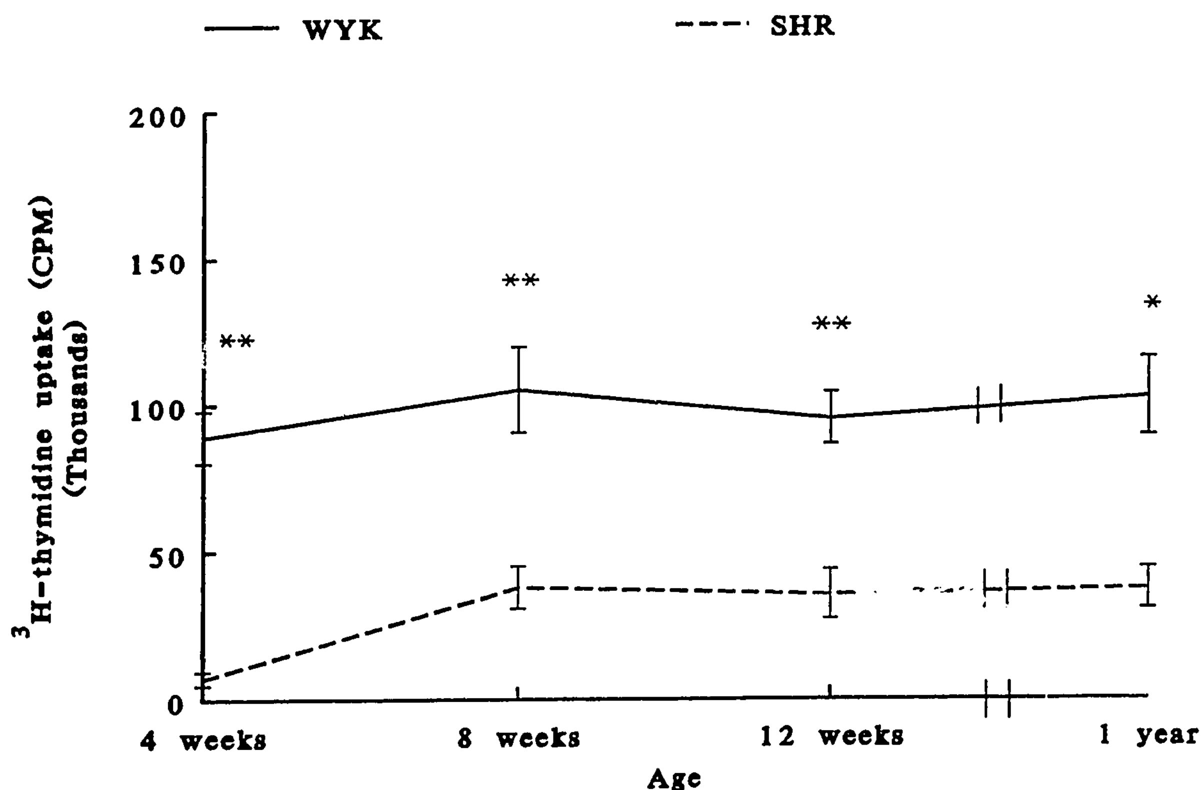


Figure VI-2. **The proliferation responses of spleen cells of SHR and WKY from age 4 weeks to one year.** The spleen cells (2 x 10^5cells/well) were dispensed in 96 well tissue culture plates in quadruplicate in the presence of 2.5 or 5 μg/ml Con A which produced similar effects. The cells were cultured for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The data represent the means of ^{3}H-thymidine uptake by cells (CPM) per well ± SE from age of 4 weeks (n=10), 8 weeks (n=10), 12 weeks (n=5) and one year (n=3). *: $P < 0.05$, **: $P < 0.01$, compared with respective SHR.

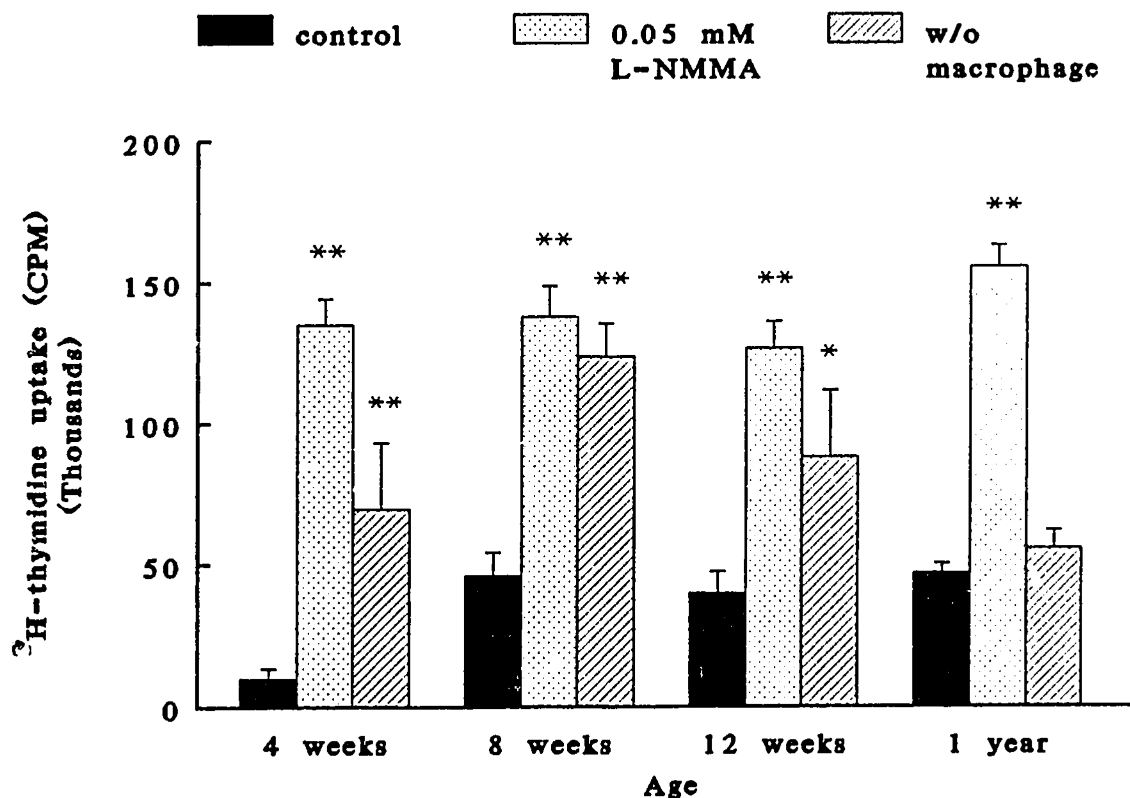


Figure VI-3. **Effect of L-NMMA or macrophage depletion on the proliferation responses of SHR spleen cells from age 4 weeks to one year.** L-NMMA (0.05 mM) was added to the spleen cell culture in quadruplicate in the presence of 2.5 μg/ml Con A. Macrophages were removed from spleen cells by adherence onto plastic. The cells were cultured under the conditions described in Figure VI-2. The data represent the means of ^{3}H-thymidine uptake by cells (CPM) per well ± SE from age 4 weeks (n=10), 8 weeks (n=10), 12 weeks (n=5) and one year (n=3). * :$P < 0.05$, **: $P < 0.01$, compared with respective control group.

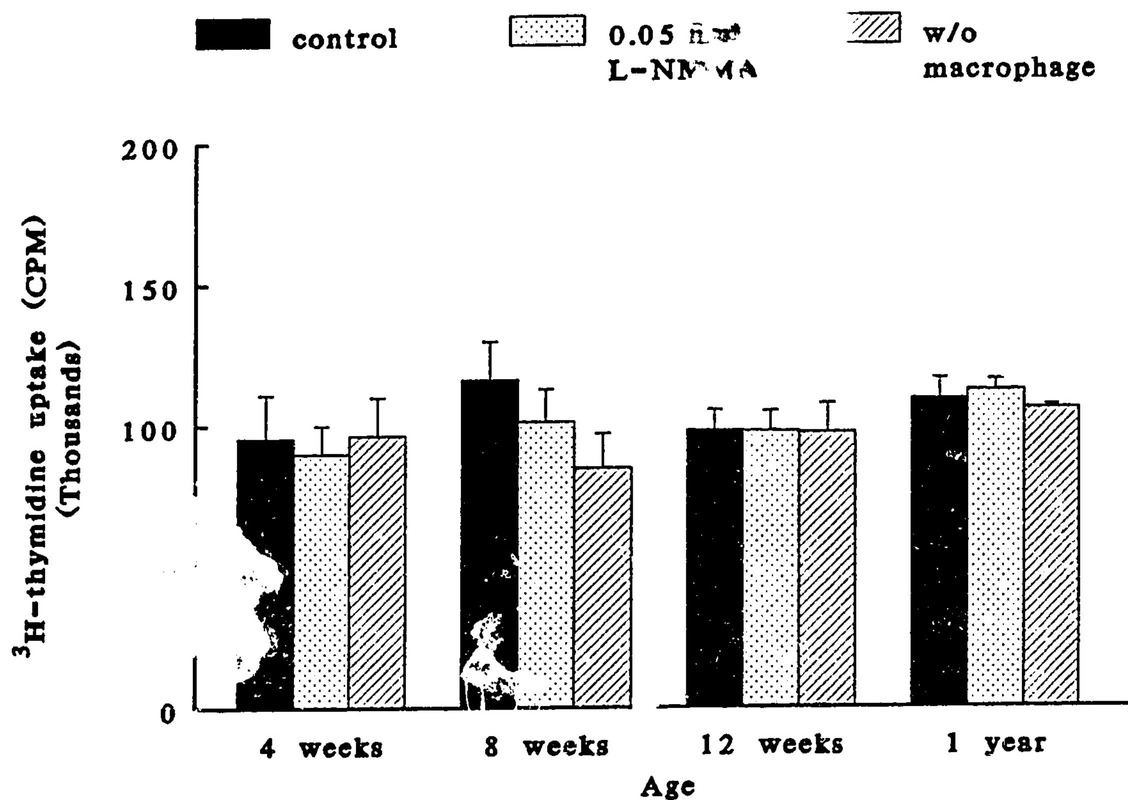


Figure VI-4. **Effect of L-NMMA or macrophage depletion on the proliferation responses of WKY spleen cells from age 4 weeks to one year.** L-NMMA (0.05 mM) was added to the spleen cell culture in quadruplicate in the presence of 2.5 μg/ml Con A. Macrophages were removed from spleen cells by adherence onto plastic. The cells were cultured under the conditions described in Figure VI-2. The data represent the means of ^{3}H-thymidine uptake by cells (CPM) per well ± SE from age of 4 weeks (n=10), 8 weeks (n=10), 12 weeks (n=[illegible]) and one year (n=3).

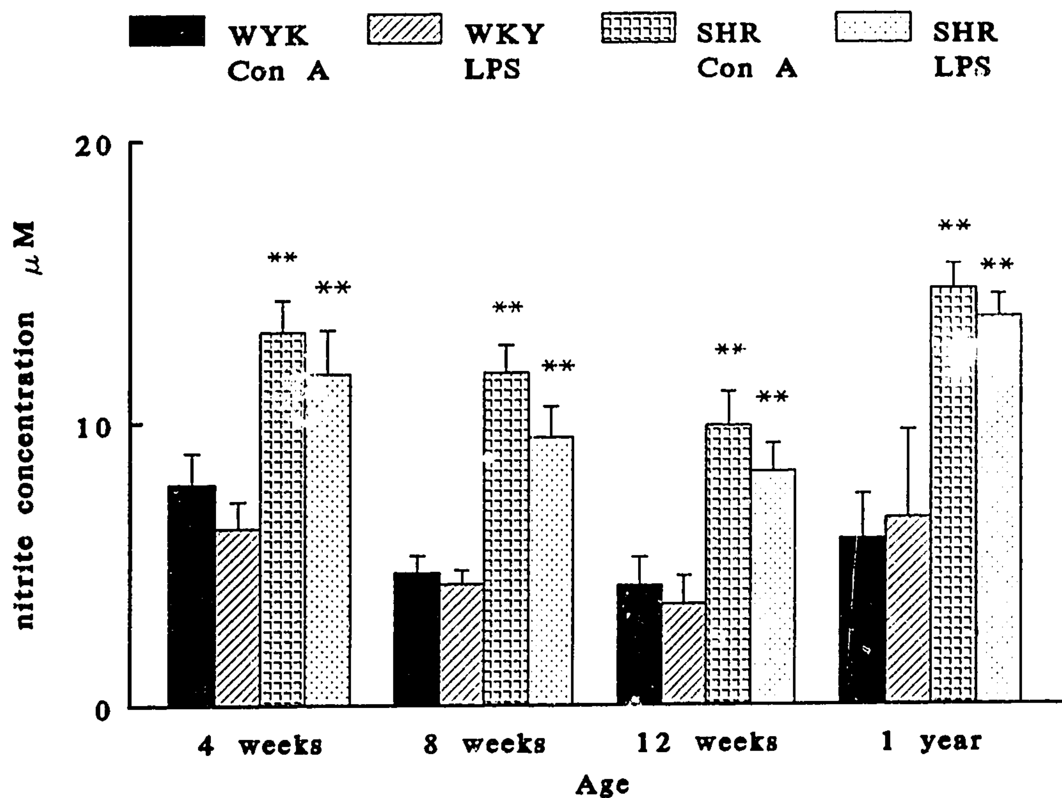


Figure VI-5. **Nitric oxide production by spleen macrophages of SHR and WKY from age 4 weeks to one year.** Spleen cells were cultured with 2.5 μg/ml Con A or 40 μg/ml LPS for 72 hours. The nitrite concentration of the culture supernatant was determined by a colorimetric assay. The data represent the means of nitrite concentration ± SE from age of 4 weeks (n=10), 8 weeks (n=10), 12 weeks (n=5) and one year (n=3). **: $P < 0.01$, compared with respective WKY.

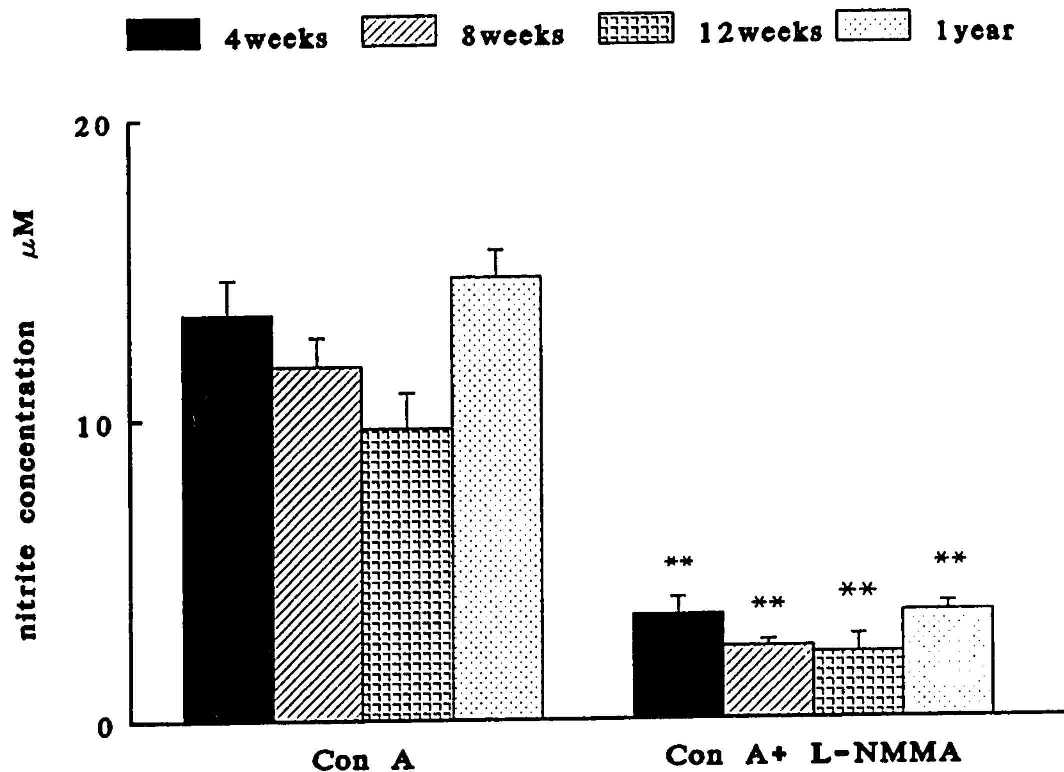


Figure VI-6. **Effect of L-NMMA on nitric oxide production by SHR spleen cells containing macrophages from age 4 weeks to one year.** L-NMMA (0.1 mM) was added to SHR spleen cell culture in the presence of 2.5 μg/ml Con A. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE from age of 4 weeks (n=10), 8 weeks (n=10), 12 weeks (n=5) and one year (n=3). **: $P < 0.01$, compared with the same age group treated with Con A.

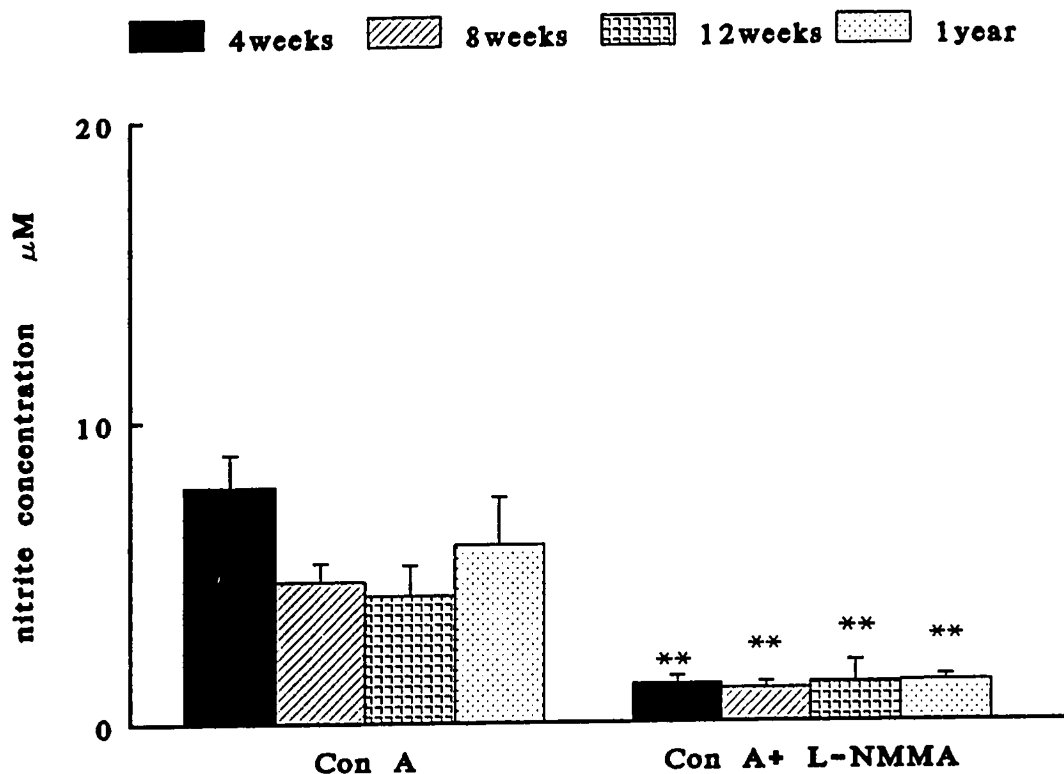


Figure VI-7. **Effect of L-NMMA on nitric oxide production by WKY spleen cells containing macrophages from age 4 weeks to one year.** L-NMMA (0.1 mM) was added to WKY spleen cell culture in the presence of 2.5 μg/ml Con A. The nitrite concentration of the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE from age of 4 weeks (n=10), 8 weeks (n=10), 12 weeks (n=5) and one year (n=3). **: $P < 0.01$, compared with the same age group treated with Con A.

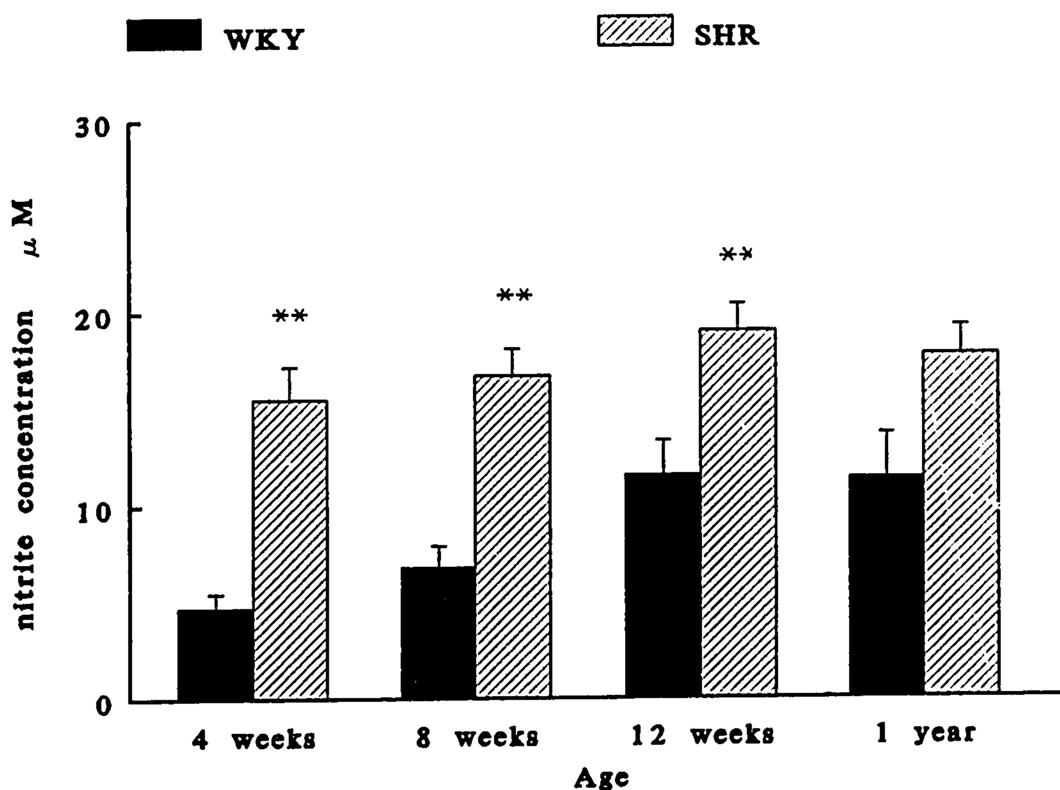


Figure VI-8. **Nitric oxide production by VSMC of SHR and WKY from age 4 weeks to one year induced by a cytokine mixture.** At least 5 SHR and 5 WKY VSMC preparations (for one year group, SHR n=3, WKY n=2) were used between passages 4 to 9. Quiescent VSMC were incubated with a cytokine mixture containing 5 units/ml IL-1β, 5 nM TNFα, 200 units/ml INFγ and 10 μg/ml LPS for 48 hours. The nitrite concentration in the supernatant was determined by a colorimetric assay. The data represent the means ± SE (N=15) from 5 VSMC preparations, 3 experiments for each preparation (N=9 for one year SHR, N=6 for one year WKY). **: $P < 0.01$, compared with respective WKY.

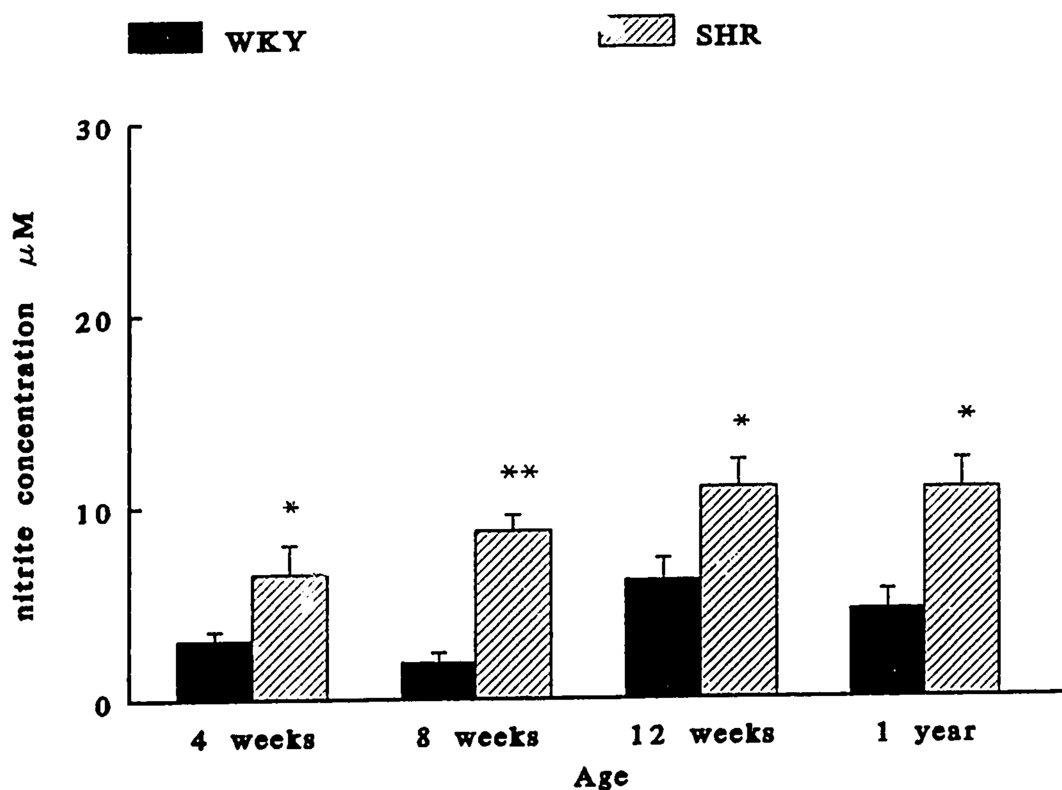


Figure VI-9. **Nitric oxide production by VSMC of SHR and WKY from age 4 weeks to one year induced by LPS.** At least 5 SHR and 5 WKY VSMC preparations (for one year group, SHR n=3, WKY n=2) were used between passages 4 to 9. Quiescent VSMC were incubated with 40 μg/ml LPS for 48 hours. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE (N=15) from 5 VSMC preparations, 3 experiments for each preparation (N=9 for one year SHR, N=6 for one year WKY). *: $P < 0.05$, **: $P < 0.01$, compared with respective WKY.

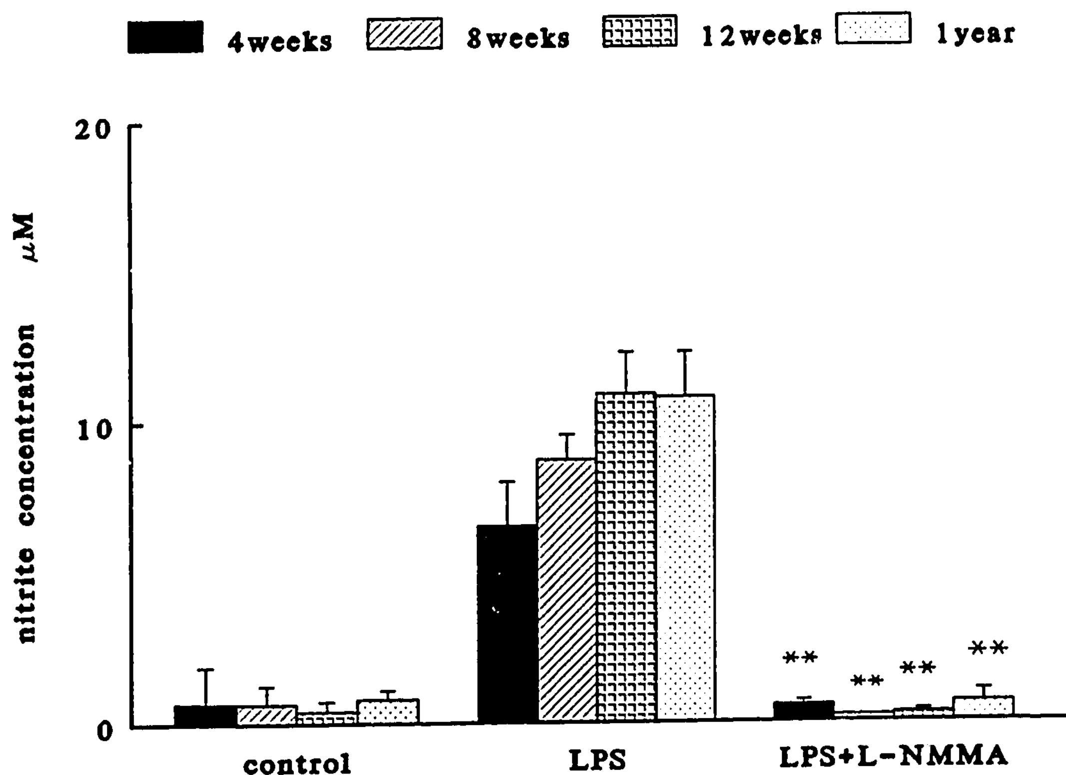


Figure VI-10. **Effect of L-NMMA on nitric oxide production by SHR VSMC from age 4 weeks to one year.** L-NMMA (0.1 mM) was added to SHR VSMC culture as described in Figure VI-9. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE (N=15) from 5 VSMC preparations, 3 experiments for each preparation (N=9 for one year SHR, N=6 for one year WKY). **: $P < 0.01$, compared with the same age group treated with LPS.

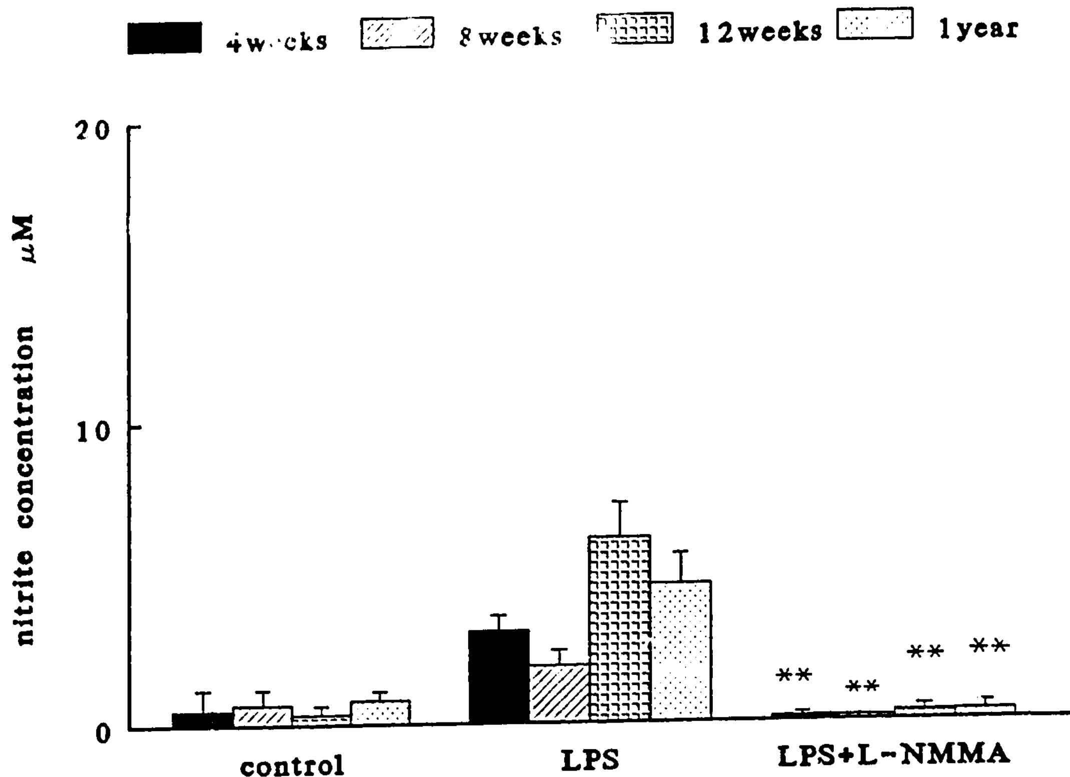


Figure VI-11. **Effect of L-NMMA on nitric oxide production by WKY VSMC from age 4 weeks to one year.** L-NMMA (0.1 mM) was added to SHR VSMC culture as described in Figure VI-9. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE from at least 5 VSMC preparations, 3 experiments for each preparation (N=9 for one year SHR, N=6 for one year WKY). **: $P < 0.01$, compared with the same age group treated with LPS.

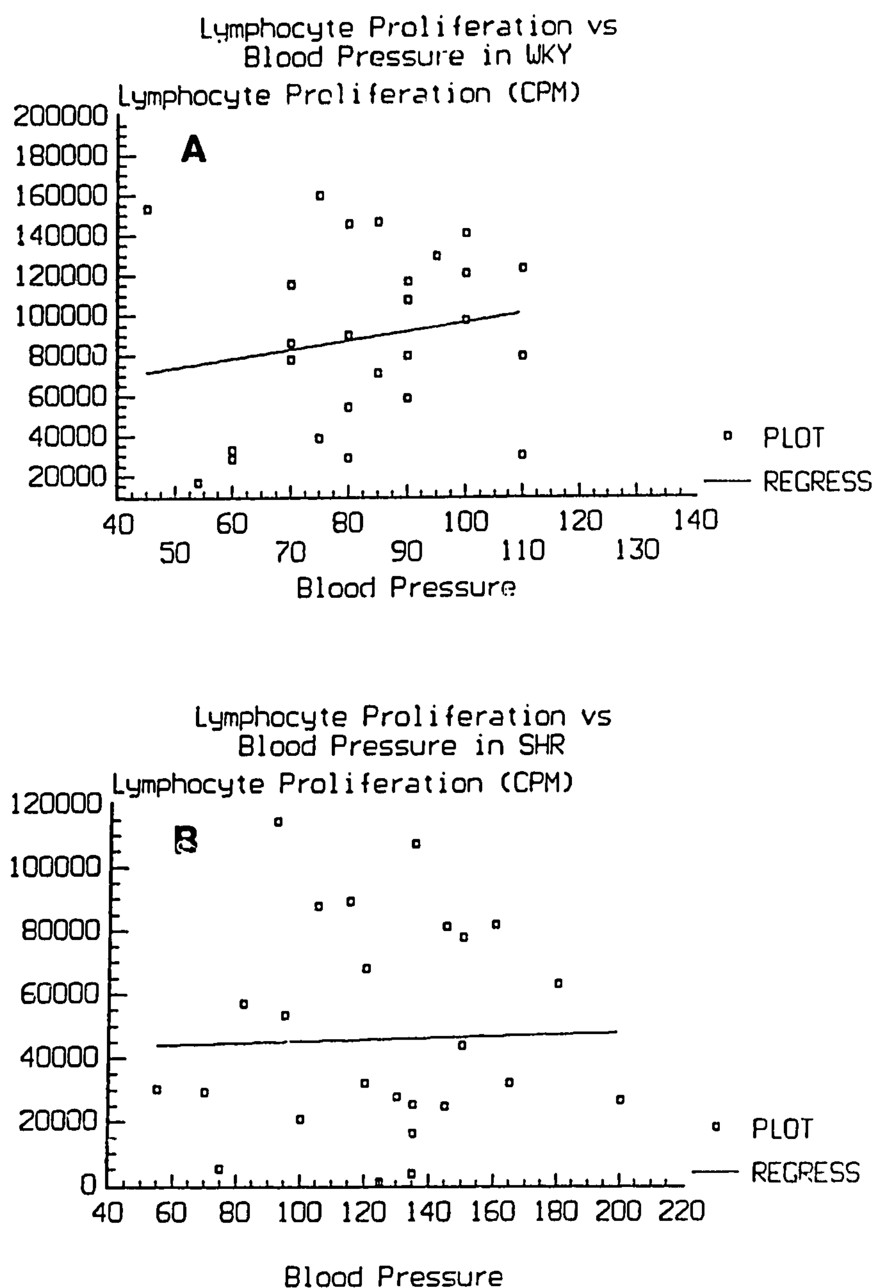
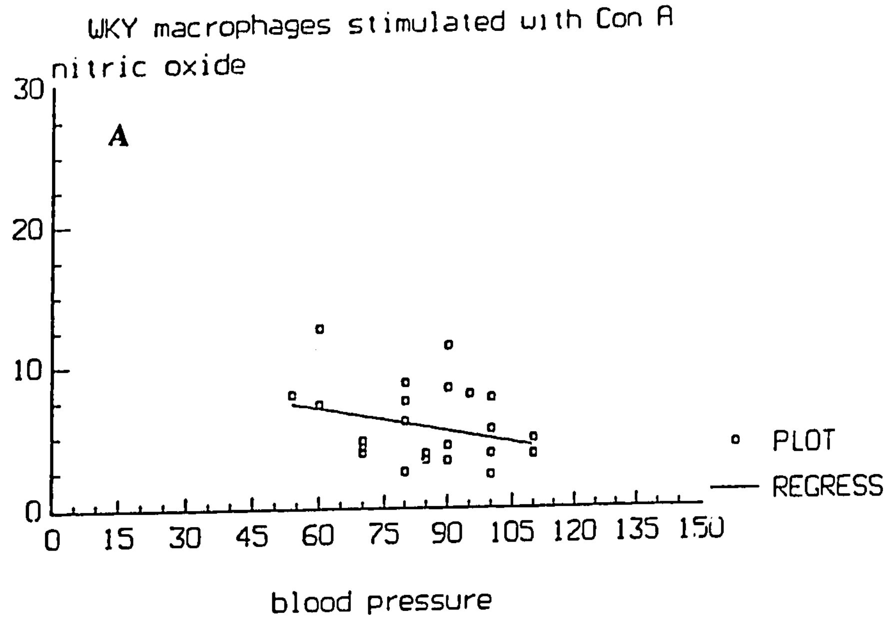


Figure VI-12. **Relationship between blood pressure and lymphocyte proliferation response in WKY and SHR.** Panel A: lymphocyte proliferation response to 2.5 μg/ml Con A (CPM) *versus* mean blood pressure (mm Hg) in WKY (**r= 0.18**). Panel B: lymphocyte proliferation response to Con A *versus* mean blood pressure in SHR (**r= 0.03**).



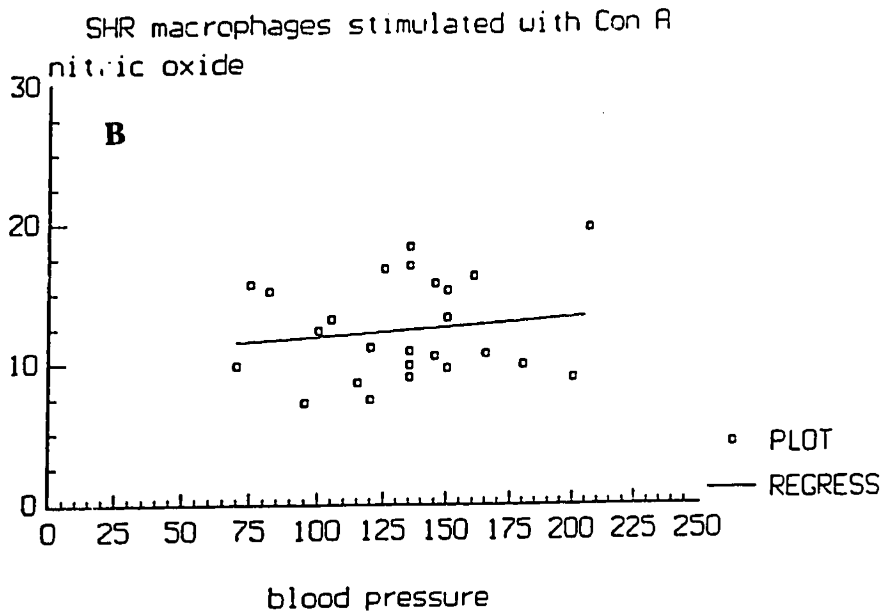
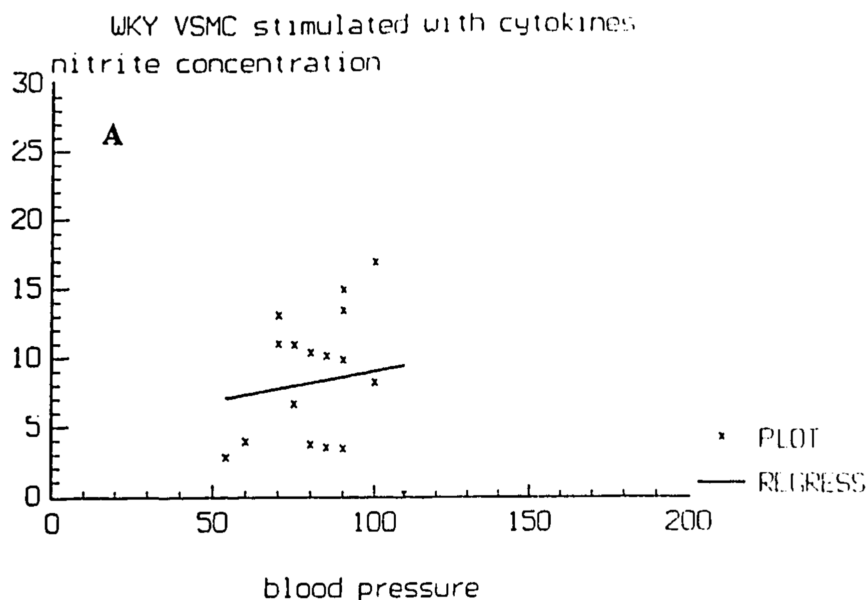


Figure VI-13. **Relationship between blood pressure and nitric oxide production in spleen cells containing macrophages.** Panel A: nitric oxide synthesis in WKY spleen macrophages induced by 2.5 μg/ml Con A (nitrite concentration μM) *versus* mean blood pressure (mm Hg) in WKY (**r = -0.24**). Panel B: nitric oxide synthesis in SHR spleen macrophages induced by Con A *versus* SHR mean blood pressure (**r = -0.15**).



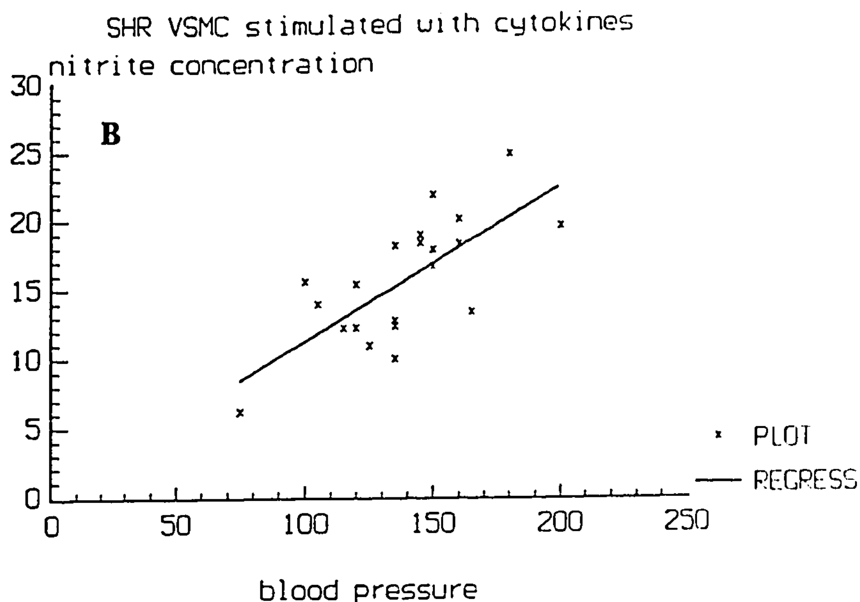
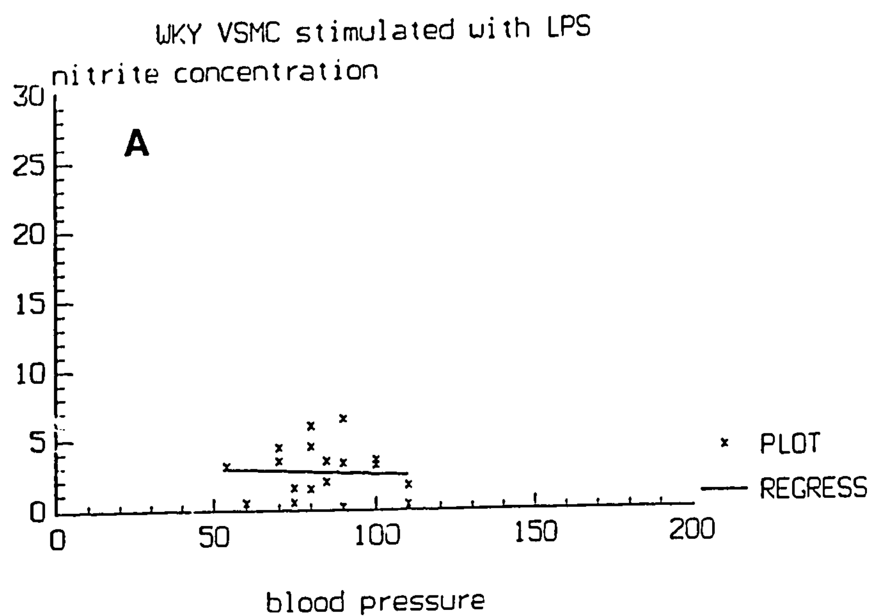


Figure VI-14. **Relationship between blood pressure and nitric oxide production in VSMC induced by a cytokine mixture.** Panel A: nitric oxide synthesis in WKY VSMC induced by a cytokine mixture (nitrite concentration μM) *versus* mean blood pressure (mm Hg) in WKY (**r = 0.13**). Panel B: nitric oxide synthesis in SHR VSMC induced by the cytokine mixture *versus* SHR mean blood pressure (**r = 0.71**, P = 0.0003).



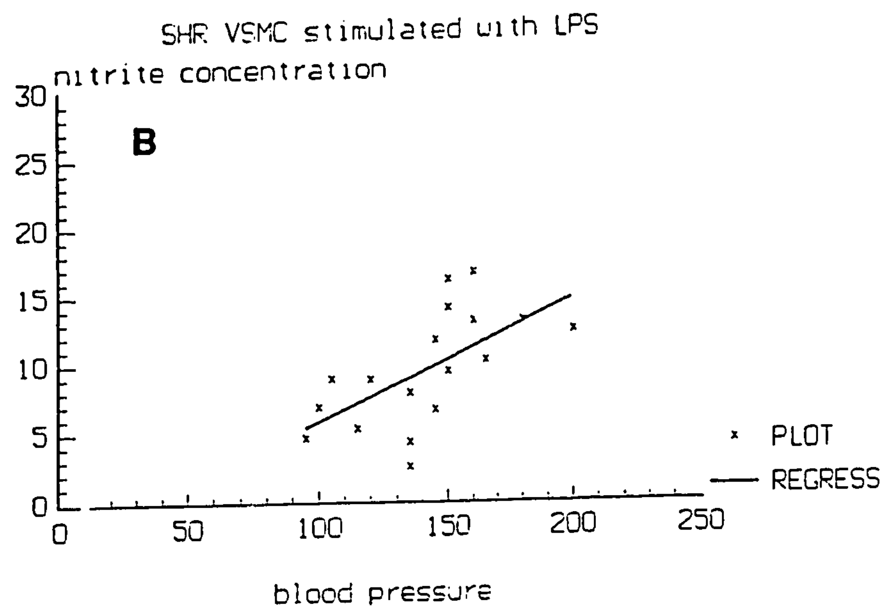


Figure VI-15. **Relationship between blood pressure and nitric oxide production in VSMC induced by LPS.** Panel A: nitric oxide synthesis in WKY VSMC induced by 40 μg/ml LPS (nitrite concentration μM) *versus* mean blood pressure (mm Hg) in WKY (**r = -0.07**). Panel B: nitric oxide synthesis in SHR VSMC induced by LPS *versus* SHR mean blood pressure (**r = 0.69, P** = 0.0006).

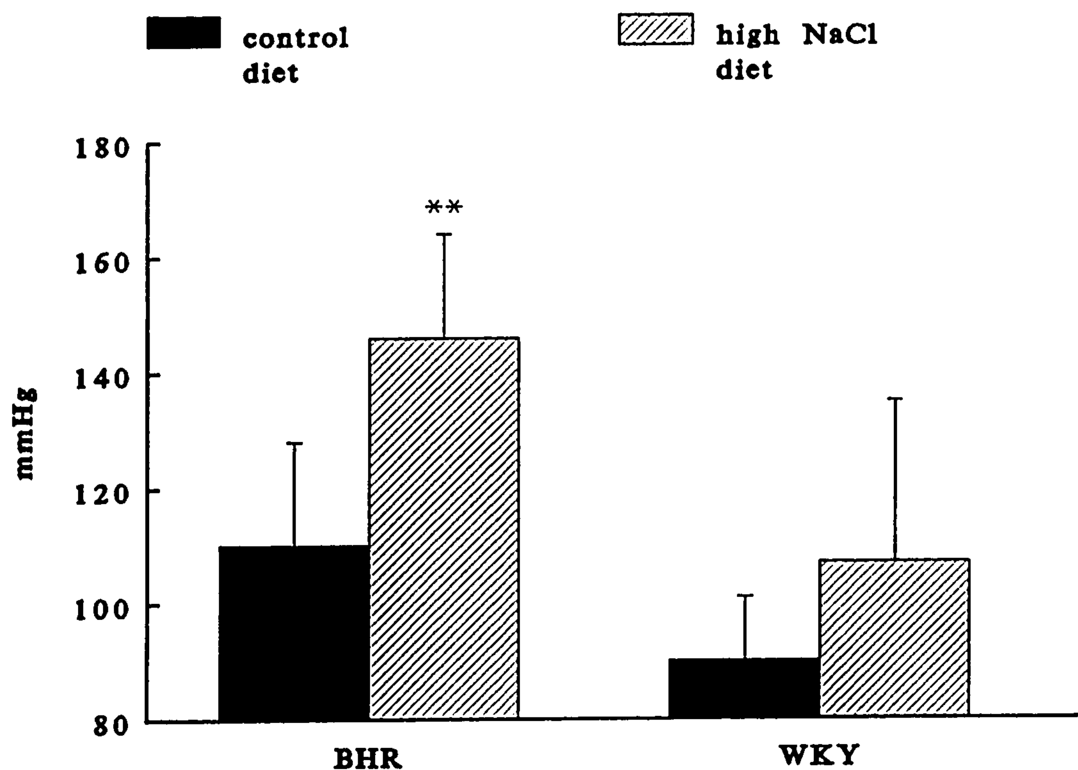


Figure VI-16. **Mean arterial blood pressures of BHR and WKY.** Five weeks old BHR and WKY were assigned to two groups. One group received a normal diet (1% NaCl), another group received a 8% NaCl diet for 8 weeks. At 13 weeks of age, blood pressures were measured in either anaesthetized BHR or WKY. The values represent means of arterial blood pressure (mm Hg) ± SD from 7 rats. **: $P < 0.01$, compared with BHR or WKY fed a normal diet and WKY fed a 8% NaCl diet.

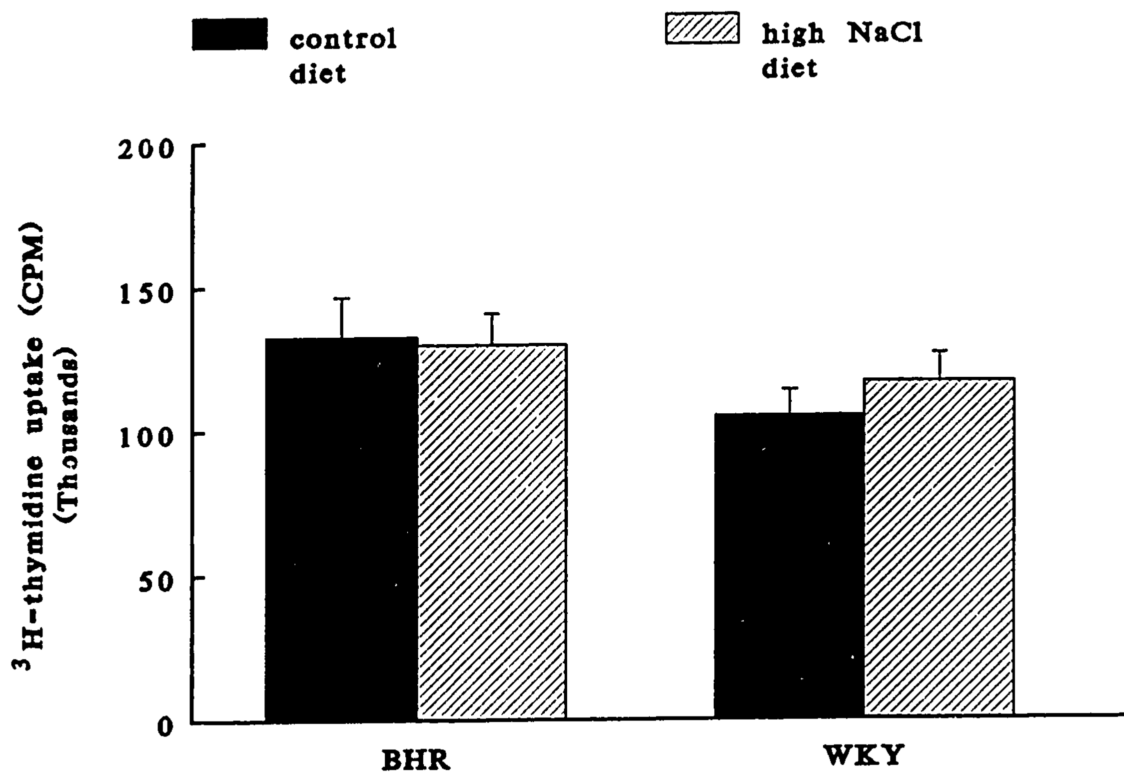


Figure VI-17. **The proliferation responses of spleen cells from BHR and WKY fed a high sodium diet.** The spleen cells from BHR or WKY (2 x 10^5cells/well) were dispensed in 96 well tissue culture plates in quadruplicate in the presence of 2.5 μg/ml Con A. The cells were cultured for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The data represent the means of ^{3}H-thymidine uptake by cells (CPM) per well ± SE from 7 rats.

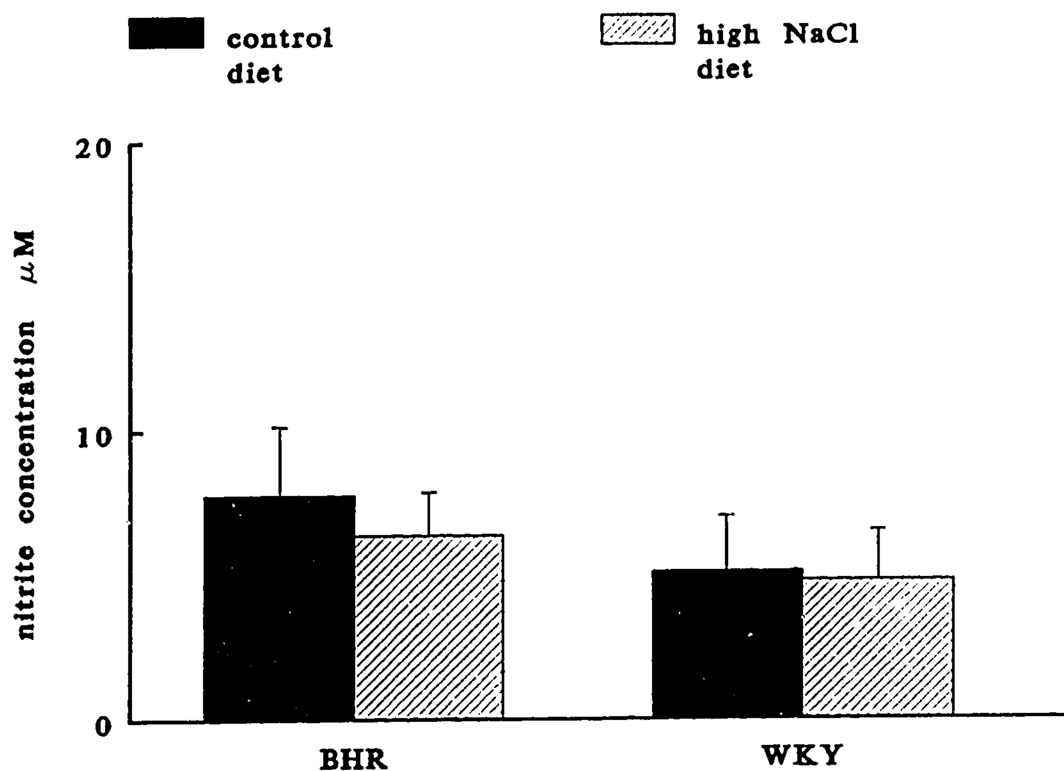


Figure VI-18. **Con A-induced nitric oxide production by spleen cells containing macrophages of BHR and WKY fed a high sodium diet.** Spleen cells were cultured with 2.5 μg/ml Con A for 72 hours. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE from 7 rats.

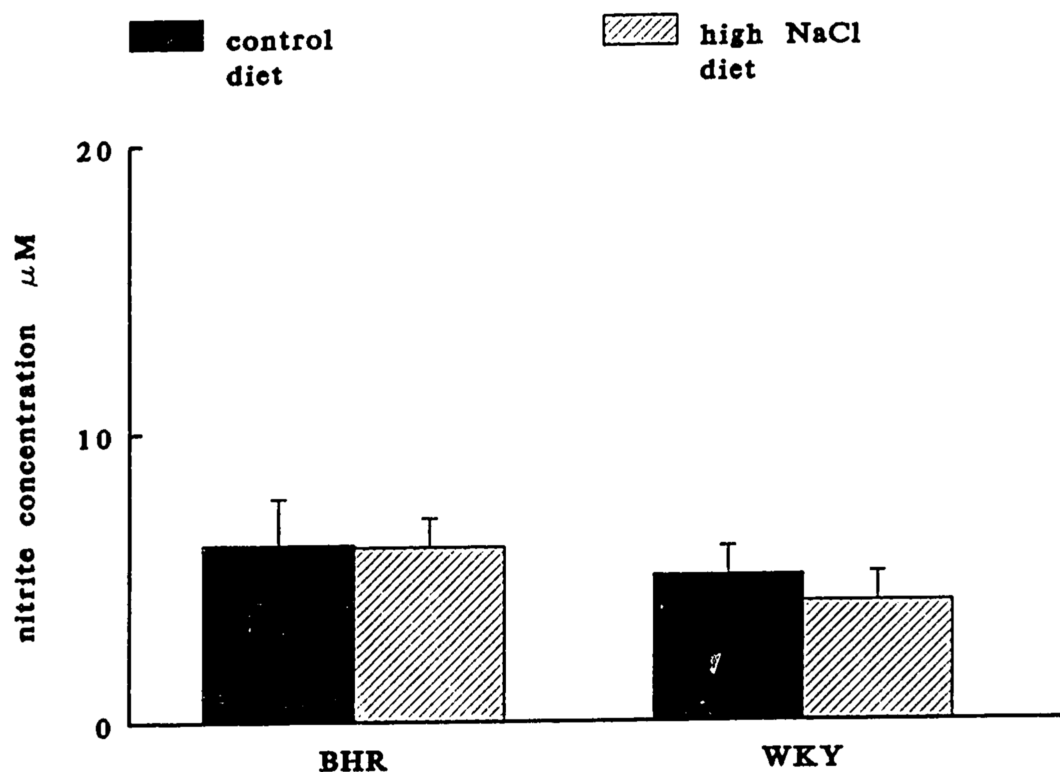


Figure VI-19. **LPS-induced nitric oxide production by spleen cells containing macrophages of BHR and WKY fed a high sodium diet.** Spleen cells were cultured with 40 μg/ml LPS for 72 hours. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE from 7 rats.

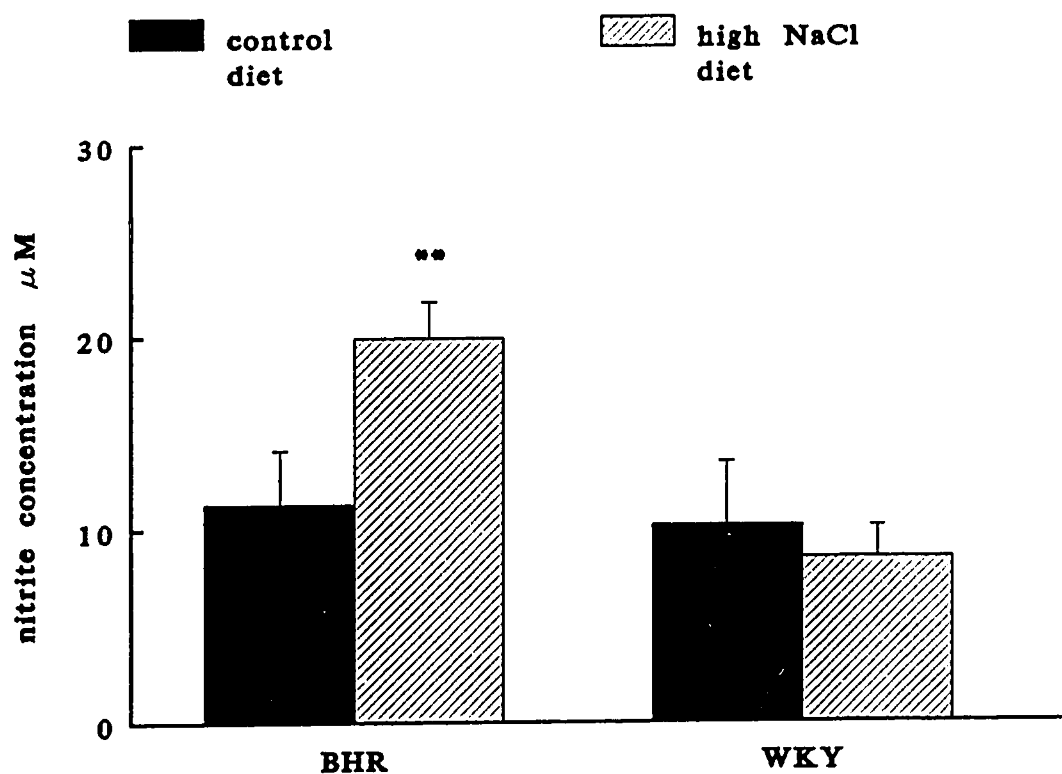


Figure VI-20. **Cytokine-induced nitric oxide production by VSMC of BHR and WKY fed a high sodium diet.** Quiescent VSMC (passages 3-5) were incubated with a cytokine mixture containing 5 units/ml IL-1β, 5 nM TNFα, 200 units/ml INFγ and 10 μg/ml LPS for 48 hours. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE (N=12) from 4 VSMC preparations, 3 experiments for each preparation. **: $P < 0.01$, compared with BHR fed a control diet.

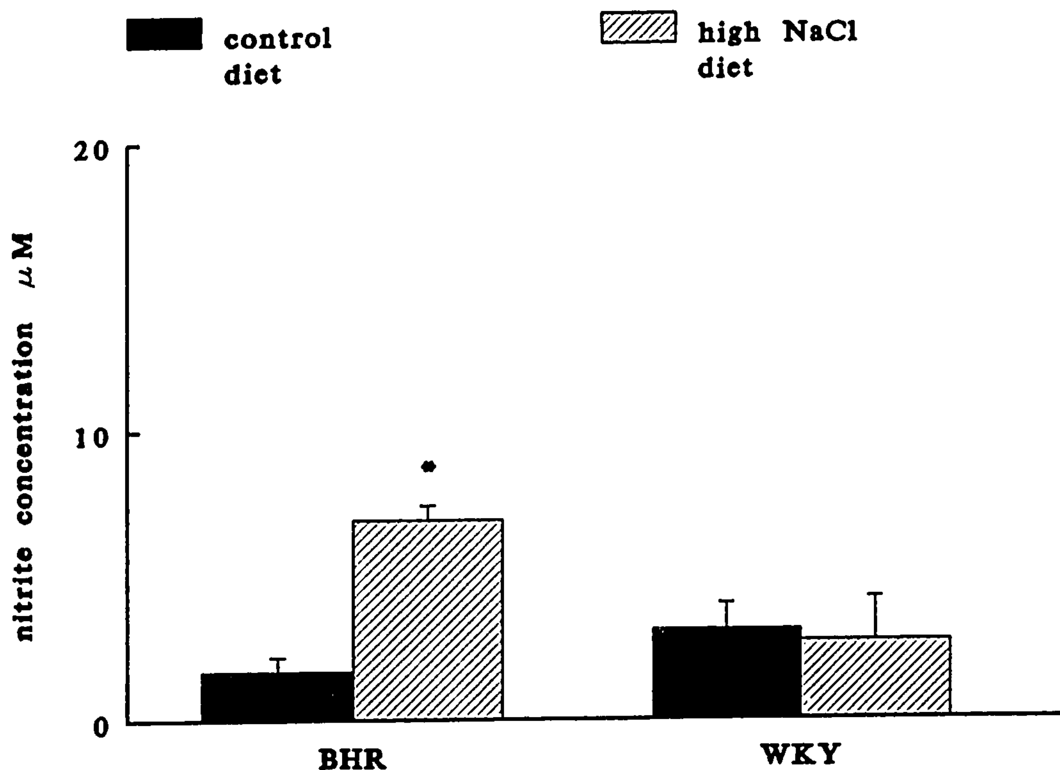


Figure VI-21. **LPS-induced nitric oxide production by VSMC of BHR and WKY fed a high sodium diet.** Quiescent VSMC (passages 3-5) were incubated with 40 μg/ml LPS for 48 hours. The nitrite concentration in the culture supernatant was determined by a colorimetric assay. The data represent the means ± SE (N=12) from 4 VSMC preparations, 3 experiments for each preparation. *: $P < 0.05$, compared with BHR fed a control diet.

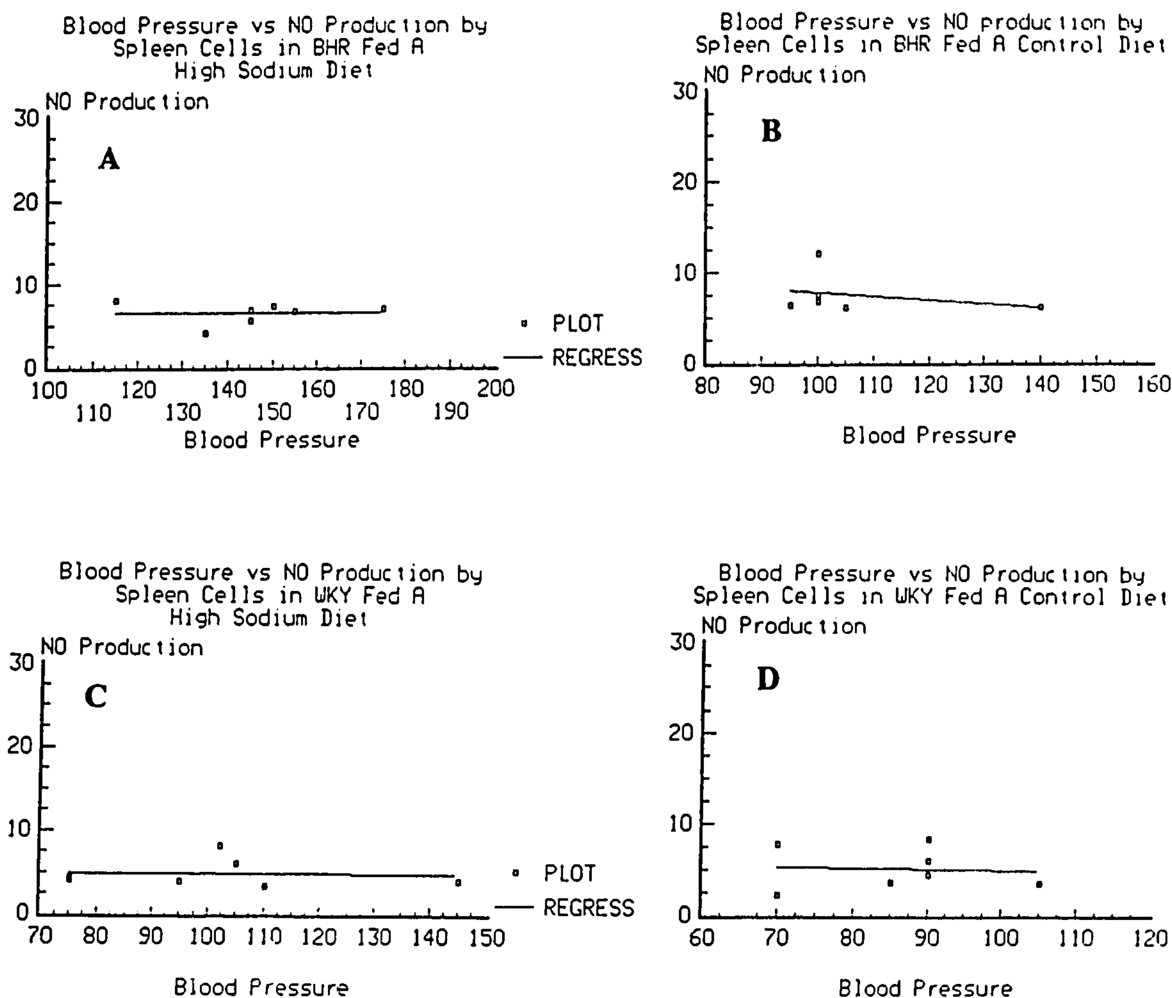


Figure VI-22. **Relationship between mean blood pressure and cytokine-induced nitric oxide production in spleen cells containing macrophages.** Panel A: blood pressure (mm Hg) *versus* NO production (nitrite concentration μM) by spleen cells of hypertensive BHR fed a high sodium diet (**r= -0.01**). Panel B: blood pressure *versus* NO production by spleen cells of normotensive BHR fed a control diet (**r= -0.37**). Panel C: blood pressure *versus* NO production by spleen cells of WKY fed a high sodium diet (**r= -0.03**). Panel D: blood pressure *versus* NO production by spleen cells of WKY fed a control diet (**r= -0.1**).

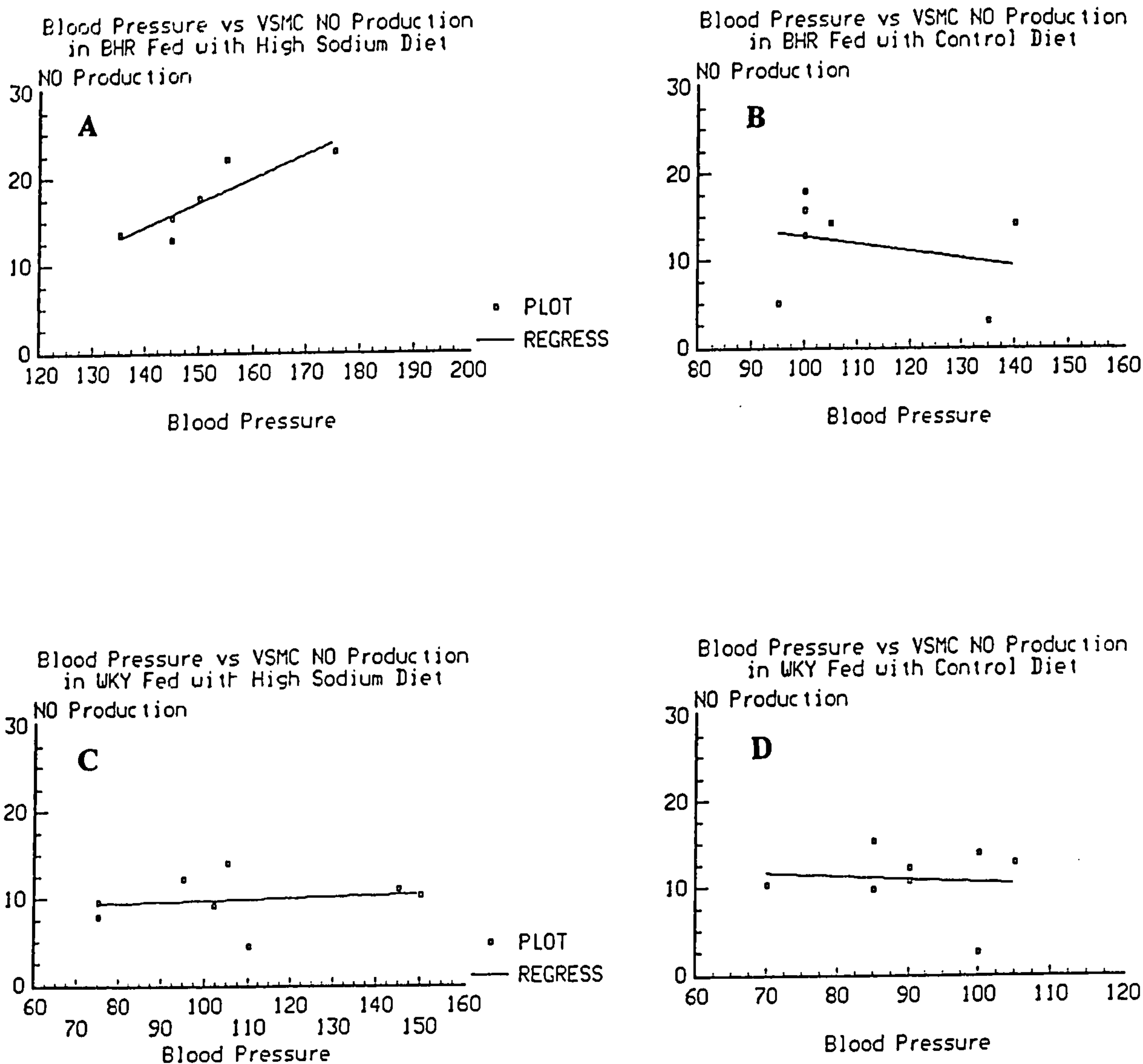


Figure VI-23. **Relationship between mean blood pressure and cytokine-induced nitric oxide production in VSMC.** Panel A: blood pressure (mm Hg) *versus* VSMC NO production (nitrite concentration μM) of hypertensive BHR fed a high sodium diet (**r= 0.87,** P= 0.02). Panel B: blood pressure *versus* VSMC NO production of normotensive BHR fed a control diet (r= **-0.28**). Panel C: blood pressure *versus* VSMC NO production of WKY fed a high sodium diet (**r= 0.14**). Panel D: blood pressure *versus* VSMC NO production of WKY fed a control diet (**r= -0.16**).

CHAPTER VII. THE ROLE OF T CELLS IN THE ALTERATION OF NO SYNTHESIS IN SHR MACROPHAGES

I. INTRODUCTION

In previous chapters the elevated NO synthesis in SHR macrophages and the resulting SHR lymphocyte depression were described. There is a positive correlation between activation of VSMC NO synthesis and the rise in blood pressure in SHR. Such a correlation, however, has not been observed between blood pressure and NO synthesis in SHR macrophages. Furthermore, the activation of NO synthase in VSMC was associated with elevated blood pressure induced by high salt intake. The activation of NO synthase in macrophages, however, was not associated with this salt-induced hypertension. These observations suggest that in the hypertensive state the mechanism involved in the activation of NO synthase in macrophages may differ from the mechanism for activation of NO synthase in VSMC. What is the mechanism for the activation of NO synthase in macrophages in SHR?

Several reports have shown that NOS mRNA and enzyme activity in macrophages could be induced by LPS and various T cell-derived cytokines (Stuehr and Marletta, 1987; Stout and Bottomly, 1989; Zhang *et al.*, 1994). It has also been demonstrated that there was synergistic cooperation between INFγ and either IL-2 or TNFα (Deng *et al.*, 1993). Recently, it has been reported that T cell mediated activation of NO synthesis in macrophages required cell-cell contact (Tao and Stout,

1993). Although the molecular mechanisms involved have not been defined, these results indicate that T cells play an important role in the regulation of the expression of NO synthase activity in macrophages. Therefore, it was of great interest to examine the involvement of T cells in the alteration of NO synthesis in SHR macrophages. An understanding of the mechanism responsible for NO synthesis alteration in SHR macrophages may provide evidence for the relationship between immune dysfunction and hypertension.

II. EXPERIMENTAL DESIGN

A. Antibody treatment. Anti-T cell monoclonal antibodies have been used *in vivo* to prolong allograft and xenograft survival time by depletion of certain T cells (Chavin *et al.*, 1992; Teramoto *et al.*, 1992). It is believed that anti-T cell antibody binds to a specific surface antigen on T cells. This antigen-antibody complex triggers a cascade of events which leads to activation of complement and other mechanisms causing depletion of these specific T cells. Since CD2 and CD5 antigens are expressed on most T cells (Jones *et al.*, 1986; Beyers *et al.*, 1989), anti-CD2 and anti-CD5 monoclonal antibodies were chosen to ablate T cells in this study.

Three groups of one week old SHR or WKY rats were injected (i.p.) with mouse anti-rat CD2 (1-1.5 mg/kg), mouse anti-rat CD5 (1-1.5 mg/kg) monoclonal antibodies (ascites) or mouse control ascitic fluid (1-1.5 mg/kg). Because single antibody treatment is not usually sufficient to deplete all T cells, the rats were

injected three times a week for two weeks. Three days after the last injection, the rats were sacrificed and the spleens were collected. Previous studies have shown that multiple treatments achieved an optimal depletion (Barlow and Like, 1992). It has also been shown that this schedule was well tolerated by animals and did not cause morbidity and mortality. A control mouse ascites fluid was used to ensure that there was no non-specific antibody effect.

B. Assessment of T cell ablation. It is important to ensure that anti-CD2 and anti-CD5 monoclonal antibodies can induce T cell depletion in SHR. Therefore, the proliferation response of spleen cells to T cell mitogens was examined. If T cell are ablated by these antibodies, the number of proliferative cells will be minimal. Since SHR already have a reduced lymphocyte proliferation response, WKY which have normal lymphocyte proliferation were used as a control to assess the level of T cell depletion.

C. NO production in SHR macrophages. After six antibody treatments, the rats were sacrificed and the spleens were collected. NO production by spleen macrophages was carried out as described previously.

III. RESULTS

A. The role of T cells in the alteration of NO synthesis in SHR spleen macrophages was examined by ablating T cells with anti-T cell monoclonal antibodies. Neither anti-CD2 nor anti-CD5 antibody treatment showed any

significant effect on the production of NO in SHR spleen macrophages (Figure VII-1).

B. After administration of anti-CD5 monoclonal antibody, the production of NO in WKY spleen macrophages was significantly reduced (Figure VII-2). Anti-CD2 monoclonal antibody did not exhibit a significant effect on NO production in WKY spleen macrophages.

C. Figure VII-3 demonstrated that control SHR spleen macrophages produced a significantly greater amount of NO than did control WKY spleen macrophages.

D. The efficiency of antibody treatment was demonstrated as proliferation responses of spleen cells. The proliferation responses were significantly decreased in SHR (Figure VII-4, left panel) and WKY (Figure VII-5, left panel) after treated with anti-CD5 monoclonal antibody. Anti-CD2 antibody treatment had no significant effect on the lymphocyte proliferation responses in either SHR or WKY. Once again, the reduced lymphocyte proliferation response was evident in newborn SHR compared with that in newborn WKY. SHR exhibited only one fifth to one seventh of the response observed in WKY.

E. L-NMMA markedly increased the proliferation responses of lymphocytes in control and anti-CD2 antibody treated SHR (Figure VII-4, right panel). L-NMMA also significantly increased the response of lymphocytes in control WKY (Figure VII-5, right panel). The lymphocyte proliferation response of anti-CD5 antibody treated SHR was increased slightly when L-NMMA was added to the

culture, but the response was significantly lower than that of control or anti-CD2 treated SHR. The lymphocyte proliferation response of anti-CD5 antibody treated WKY remained significantly lower even though L-NMMA was added to the culture.

IV. DISCUSSION

In an attempt to elucidate the mechanism for the activation of NO synthase in SHR macrophages, the role that T cells play in the alteration of NO synthesis in SHR macrophages was investigated. The reasons for choosing newborn SHR and WKY in this study were: 1) at 4 weeks of age, the altered NO synthesis in SHR macrophages was already evident, 2) immunity of the rat pups were not hindered by the process of T cell ablation because they were receiving immunoglobulins from the dam's milk, 3) at same dose, a rat with a small body weight consumes a lesser amount of costly monoclonal antibody.

It was important to ascertain that antibody treatment caused T cell depletion or inactivation in this study. Anti-CD5 antibody treatment markedly reduced the proliferation responses of lymphocytes in either SHR or WKY, suggesting that T cells possessing CD5 antigen were ablated. This was further supported by the results of L-NMMA study, in which L-NMMA failed to restore the lymphocyte proliferation responses in anti-CD5 treated rats, indicating that the reduced lymphocyte proliferation responses were not due to an inhibitory effect of NO. The proliferation studies confirmed that the T cells were either eliminated or inactivated by anti-CD5

antibody treatment. On the other hand, anti-CD2 antibody treatment did not produce any significant effect on the lymphocyte proliferation response, suggesting that this treatment did not cause cell elimination or inactivation.

The main observation arising from this study was that the activation of NO synthesis in SHR macrophages was $CD5^+$ T cell-independent because ablation of these T cells by anti-CD5 antibody *in vivo* did not affect the elevated expression of NO synthase activity in SHR macrophages. In normotensive rats, however, the ablation of $CD5^+$ T cells significantly decreased NO production by macrophages, suggesting that $CD5^+$ T cells were involved in the activation of macrophage NO synthase under normal condition.

CD5 antigen is a 69-KDa surface glycoprotein present on most peripheral T lymphocytes and thymocytes (Jones *et al.*, 1986). All mature resting T cells express this antigen (McAteer *et al.*, 1988). It was reported that treatment with anti-CD5 antibody prevented the development of experimental autoimmune encephalomyelitis (Sun *et al.*, 1992). Anti-CD5 antibody destroyed peripheral T cells when administered to rats (McAteer *et al.*, 1988; Strigard *et al.*, 1989). Consistent with these observations, the present study showed that anti-CD5 antibody treatment produced dramatic reduction in lymphocyte proliferation responses in both SHR and WKY. This suggested that the T cells which possess CD5 antigen were either physically eliminated or functionally inactivated in these treated rats. That the activated NO synthesis in SHR macrophages was not affected by ablation of $CD5^+$ T cells suggests that the activation of NO synthase in SHR macrophages is $CD5^+$ T

cell-independent. Considering that CD5 antigen is expressed on most, and all resting, T cells (Jones et al., 1986; McAteer et al., 1988), this CD5^{+} T cell-independency may suggest that the alteration of NO synthesis in SHR macrophages is not dependent on T cells at all. On the other hand, however, T cells were involved, at least in part, in NO synthesis in normal rats because anti-CD5 antibody treatment produced significant decrease in NO production by WKY macrophages.

Because most T cells also possess the CD2 antigen which functions both as an adhesion molecule and as a receptor involved in initiating T cell activation (Beyers *et al.*, 1989), anti-CD2 monoclonal antibody was used in an attempt to ablate T cells. In this study, however, anti-CD2 antibody treatment did not produce a significant effect on lymphocyte proliferation responses in either SHR or WKY, suggesting that the lymphocytes were not eliminated. In addition, anti-CD2 antibody did not affect NO synthesis in macrophages.

The mechanism by which anti-CD5 antibody treatment produces T cell depletion or inactivation is not clear. It may involve complement-mediated cytotoxicity and result in elimination of CD5^{+} T cells (Sun *et al.*, 1992). The modulation of the CD5 molecule through a receptor-mediated endocytosis of the CD5-antibody complex may also be involved (Strigard *et al.*, 1989). Recently, it has been shown that anti-CD5 antibody induced an increase in the kinase activity associated with the CD5 molecule (Alberola-Ila *et al.*, 1993) and that CD5 acted as a receptor and substrate for the protein-tyrosine kinase p56^{lck} (Raab *et al.*, 1994). These results suggest that the CD5 antigen may play a role in T cell signalling and

that CD5 and anti-CD5 antibody complex may cause inactivation of CD5$^+$ T cells. Whatever mechanisms underlie the effect of anti-CD5 antibody treatment, CD5$^+$ T cells do not appear to be involved in the elevated NO synthesis in SHR macrophages. The reason for the ineffectiveness of anti-CD2 treatment is unknown. It is possible that CD2 and anti-CD2 antibody complex do not activate the complement system and do not cause cell elimination. It has been reported that in association with inhibition of immunity by anti-CD2 antibody treatment there was a down-modulation of cell surface CD2 molecule without cellular depletion and without alteration of other T cell surface markers (Bromberg *et al.*, 1991; Guckel *et al.*, 1991). It is also possible that the unaffected lymphocyte proliferation response may be due to the signalling pathway activated by Con A, in which the CD2 antigen is bypassed.

It has been known for some time that T cells play a key role in the induction of NO synthesis in macrophages via synthesis and release of cytokines (Stuehr and Marletta, 1987; Stout and Bottomly, 1989; Martin *et al.*, 1992). Changes in T cell functions or numbers may result in changes in the cytokine profile (Arai *et al.*, 1990; Miethke *et al.*, 1993). An alteration in the production and release of cytokines may result in changes in expression of NO synthase and, therefore, changes in NO production by macrophages. However, whether T cells are involved in the alteration of NO synthesis in macrophages in hypertensive state is unknown. The present study provides evidence showing that CD5$^+$ T cells may not be involved in the NO synthase alteration in hypertension. That the ablation of T cells did not affect the

NO synthesis in SHR macrophages suggests that the alteration of NO synthesis in SHR macrophages is T cell-independent and this alteration may be due to some other mechanism.

The present study showed that without stimulation by Con A or LPS only a trace amount of NO was synthesized. SHR macrophages produced large amounts of NO only after stimulation by Con A or LPS, suggesting that the induction of NO synthase expression in SHR macrophages still requires signals provided by LPS or Con A. It was reported that the induction of NO synthase in macrophages required signals provided by LPS and T cell-derived cytokines such as IFNγ (Tao and Stout, 1993). In this study, since CD5$^+$ T cells, which are the majority of T cells, were depleted, the signals for induction of NO synthase must come from other sources. Macrophages could be activated via a T cell-independent mechanism by which IFNγ was thought to be released from non T cell sources (Bancroft *et al.*, 1987). IFNγ also augmented synthesis of TNF, a potent NO synthase inducer, in macrophages (Abbas *et al.*, 1991b). Recently, it was shown that NO was produced in macrophages via a T cell-independent pathway (Beckerman *et al.*, 1993). It is likely that LPS or Con A activates the expression of NO synthase in SHR macrophages via these T cell-independent mechanisms.

The physiological significance of this T cell-independent pathway, which induces NO synthase in SHR macrophages and results in overproduction of NO, is not clear at this time. The T cell-independent mechanism may also be involved in activation of NO synthase in WKY macrophages since anti-CD5 antibody treatment

did not completely abolish NO production by WKY macrophages.

The present study also demonstrated that the proliferation response of spleen cells was significantly reduced in newborn SHR. It supports the results of the age study which showed that SHR lymphocyte depression was not related to the maturation and the development of hypertension. L-NMMA markedly increased the lymphocyte proliferation response in newborn SHR, indicating that NO was responsible for the lymphocyte depression. NO production by macrophages in newborn SHR was significantly increased. This supports the finding that the elevated NO synthesis in SHR may not be related to the development of hypertension. The result that L-NMMA increased the lymphocyte proliferation response in anti-CD2 antibody treated SHR confirmed that anti-CD2 antibody treatment did not cause cell elimination or inactivation. On the other hand, the result that L-NMMA failed to increase the lymphocyte proliferation response in anti-CD5 antibody treated rats supports the finding that the lymphocytes were eliminated or inactivated by anti-CD5 antibody treatment, otherwise, the lymphocytes should regain ability to proliferate.

In summary, this study showed that the alteration of NO synthesis in SHR macrophages was T cell-independent. However, T cells were involved in the activation of macrophage NO synthase under normal condition. The lymphocyte depression as the result of overproduction of NO in macrophages is evident in newborn SHR, which unlikely have high blood pressure. This study further proves that the lymphocyte depression and hypertension are not related.

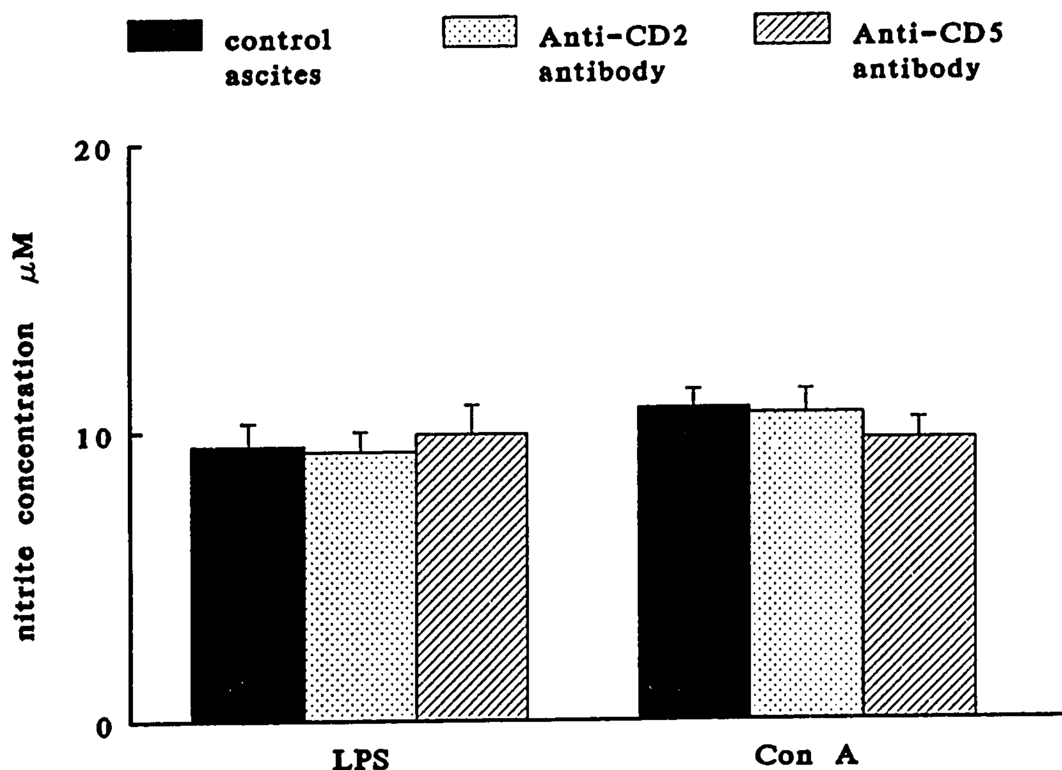


Figure VII-1. **Nitric oxide production by SHR spleen macrophages.** Newborn SHR were injected with either anti-rat CD2, anti-rat CD5 monoclonal antibody or control ascites fluid (1-1.5 mg/kg, i.p.) three times a week for two weeks. Three days after the last injection, the rats were sacrificed and the spleens were collected. The spleen cells were cultured with 2.5 μg/ml Con A or 40 μg/ml LPS for 72 hours. The nitric oxide concentration in the culture supernatant was determined by a colorimetric assay. The data represent the mean ± SE from 7 rats.

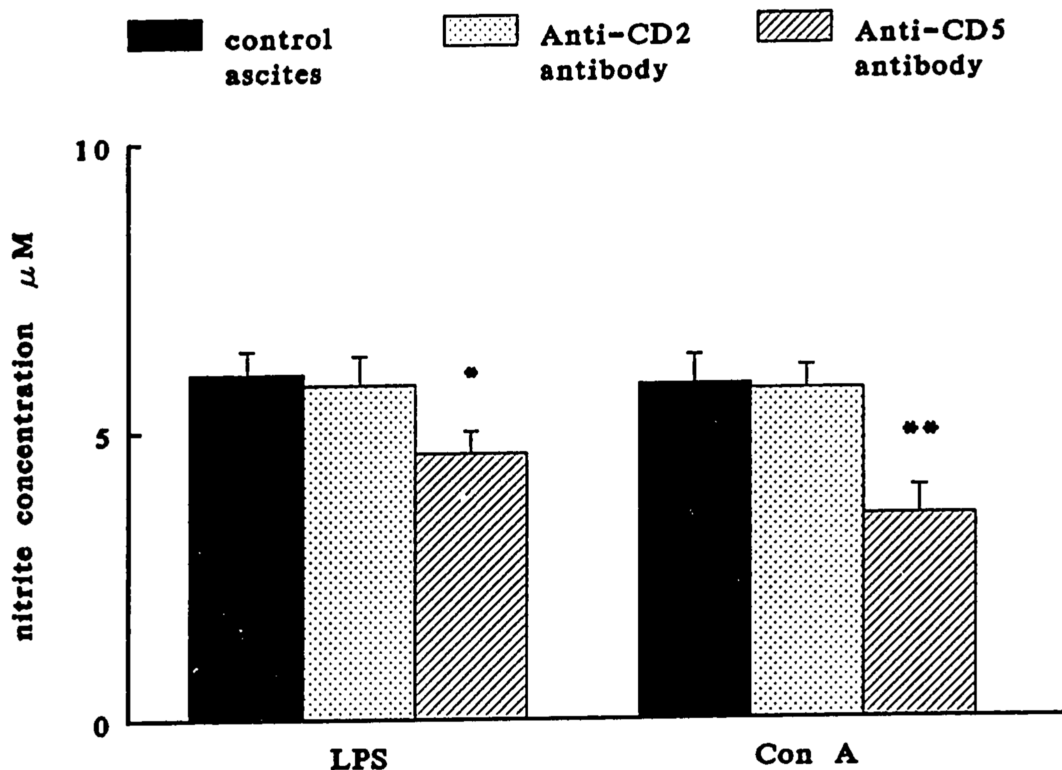


Figure VII-2. **Nitric oxide production by WKY spleen macrophages.** Newborn WKY were injected with either anti-rat CD2, anti-rat CD5 monoclonal antibody or control ascites fluid (1-1.5 mg/kg, i.p.) three times a week for two weeks. Three days after the last injection, the rats were sacrificed and the spleens were collected. The spleen cells were cultured with 2.5 μg/ml Con A or 40 μg/ml LPS for 72 hours. The nitric oxide concentration in the culture supernatant was determined by a colorimetric assay. The data represent the mean ± SE from 7 rats. *: $P < 0.05$, **: $P < 0.01$, compared to respective control ascites and anti-CD2 antibody treated rats.

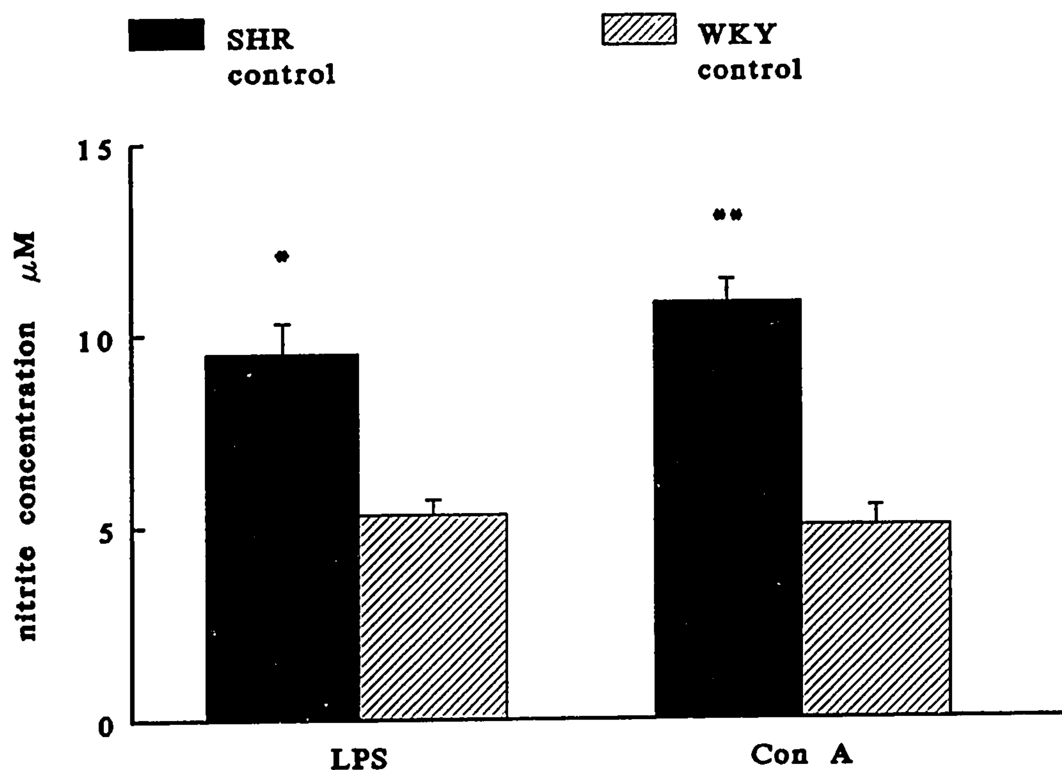


Figure VII-3. **Comparison of nitric oxide production by spleen macrophages between SHR and WKY.** Newborn SHR or WKY were injected with control ascites fluid (1-1.5 mg/kg, i.p.) three times a week for two weeks. Three days after the last injection, the rats were sacrificed and the spleens were collected. The spleen cells were cultured with 2.5 μg/ml Con A or 40 μg/ml LPS for 72 hours. The nitric oxide concentration in the culture supernatant was determined by a colorimetric assay. The data represent the mean ± SE from 7 rats. *: $P < 0.05$, **: $P < 0.01$, compared with respective control ascites treated WKY.

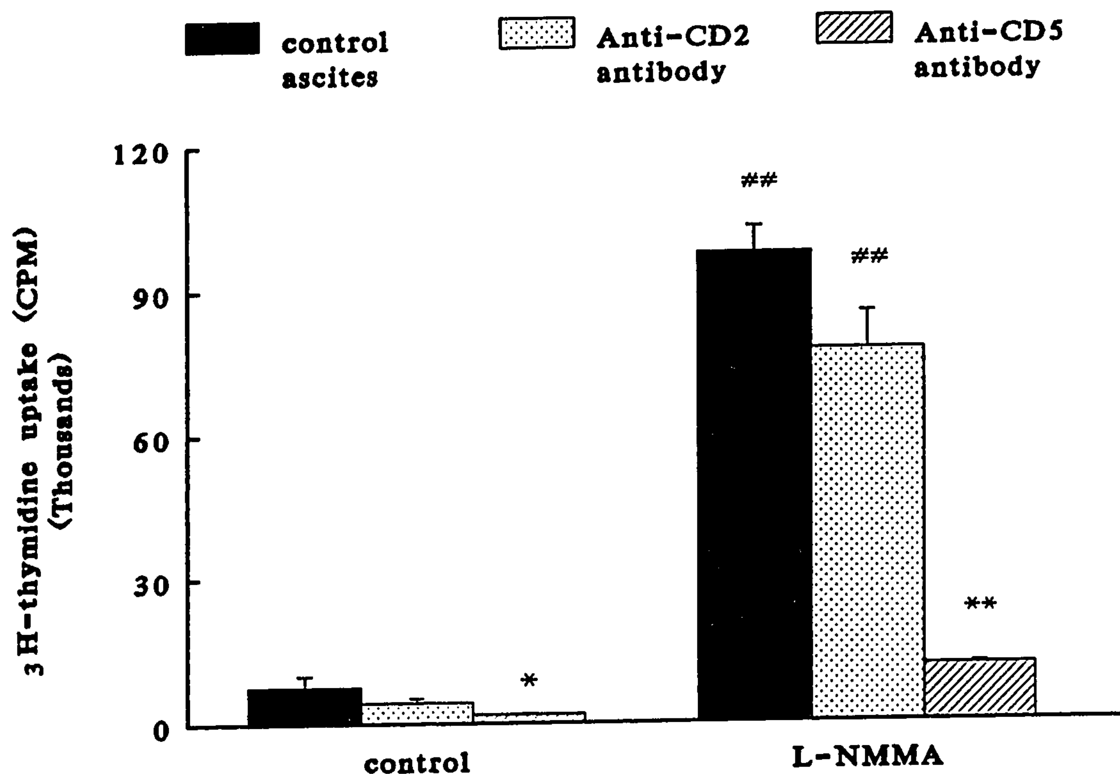


Figure VII-4. **The proliferation responses of spleen cells from SHR and effect of L-NMMA on the proliferation response.** SHR spleen cells (2 x 10^5cells/well) were dispensed in 96 well tissue culture plates in quadruplicate in the presence of 2.5 μg/ml Con A or 2.5 μg/ml Con A plus 0.05 mM L-NMMA. The cells were cultured for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from 7 rats. *: $P < 0.05$, **: $P < 0.01$, compared with respective control ascites and anti-CD2 antibody treated rats. ##: $P < 0.01$, compared with respective control group that without L-NMMA treatment.

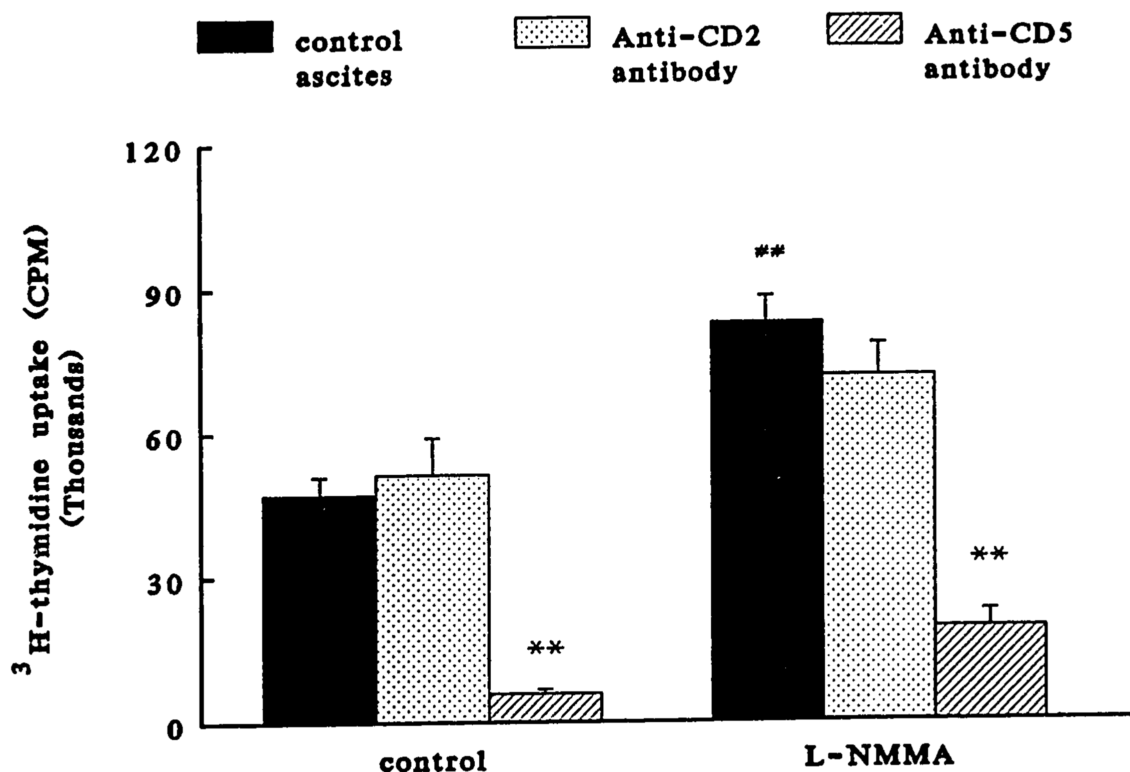


Figure VII-5. **The proliferation responses of spleen cells from WKY and effect of L-NMMA on the proliferation response.** WKY spleen cells (2 x 10^5cells/well) were dispensed in 96 well tissue culture plates in quadruplicate in the presence of 2.5 μg/ml Con A or 2.5 μg/ml Con A plus 0.05 mM L-NMMA. The cells were cultured for 72 hours. ^{3}H-thymidine was added to the culture for the final 18 hours. The data represent the mean of ^{3}H-thymidine uptake by the cells (CPM) per well ± SE from 7 rats. **: $P < 0.01$, compared with respective control ascites and anti-CD2 antibody treated rats. ##: $P < 0.01$, compared with control ascites treated rats that without L-NMMA treatment.

CHAPTER VIII. GENERAL CONCLUSIONS AND DISCUSSION

There are many reports on immune dysfunction and hypertension suggesting that a causal relationship may exist between immune abnormalities and hypertension. The overall hypothesis of this thesis, at the beginning, was that an immune defect may play a role in the pathogenesis of hypertension in SHR. The thesis work originally consisted of two major directions: 1) a description of immune abnormalities in SHR and of the underlying mechanisms, 2) the elucidation of the relationship between immune dysfunction and hypertension in SHR. As the studies progressed, a third theme emerged and that is 3) NO production by macrophages and VSMC as related to the development of hypertension.

In an attempt to investigate the relationship between immune dysfunction and hypertension, and to elucidate the mechanism of immune dysfunction, SHR immune function in comparison with that in WKY was first characterized. The results are as follows.

1. The proliferation response of spleen cells, a mixed population of cells, was significantly reduced in SHR.

2. The decreased proliferation response could not be corrected by IL-2 or IL-1.

3. The capacity of SHR spleen cells to produce IL-2 was normal.

4. The proliferation responses of T-enriched lymphocytes and thymocytes in SHR were not different from those in WKY.

5. After removal of macrophages, the proliferation response of SHR spleen cells increased dramatically, reaching the same, even a higher, level than those of WKY.

An important finding in this study is that SHR T cells are essentially normal as demonstrated by the fact that SHR T-enriched lymphocytes and thymocytes exhibited normal ability to proliferate and normal capacity to produce IL-2. Furthermore, this study shows that removal of macrophages from SHR spleen cells corrected the inhibited lymphocyte proliferation response, indicating that SHR macrophages are the cells involved.

To confirm the role of SHR macrophages played in SHR lymphocyte depression and to elucidate the mechanism of this inhibition, the effect of SHR macrophages on WKY lymphocytes and SHR macrophage-depleted lymphocytes was examined. Since macrophages play an important role in the regulation of immune responses via antigen presentation and the production of stimulatory or inhibitory substances (Unanue and Allen, 1987), one of the inhibitory pathways, the NO synthesis pathway, in SHR macrophages was investigated. The results are summarized as follows.

1. It was shown histochemically that the cells isolated by adhesion were indeed macrophages.

2. Isolated SHR macrophages significantly inhibited WKY lymphocyte proliferation response.

3. SHR macrophages also inhibited the proliferation response of SHR

macrophage-depleted lymphocytes.

4. Inhibition of NO synthase by L-NMMA fully reversed the depressed proliferation response of SHR spleen cells.

5. L-NMMA also corrected the inhibited WKY lymphocyte proliferation response caused by SHR macrophages.

6. Both SHR spleen cells, which contain macrophages, and the isolated macrophages from SHR spleen cells produced significantly higher amounts of NO than did those of WKY.

7. L-NMMA at the same concentration used to reverse the reduced lymphocyte proliferation response inhibited the elevated NO production in SHR macrophages.

8. The elevated NO synthesis in SHR spleen cells was not due to an increase in the number of macrophages because the number of cells possessing macrophage markers in SHR spleen cells was similar to that in WKY spleen cells.

This group of studies confirms that SHR macrophages are indeed the cells involved in the SHR lymphocyte depression. It also provides evidence that the activated NO synthesis pathway is responsible for SHR lymphocyte depression. It is likely that abnormal function of macrophages *per se*, and not the increased number of macrophages, is responsible for overproduction of NO in SHR. This elevated NO production in SHR macrophages mediates the lymphocyte proliferation inhibition.

In the last decade, an hypothesis that immune dysfunction might be involved in the etiology and pathogenesis of hypertension has been proposed (Khraibi *et al.*,

1984; Norman *et al.*, 1985). There is a growing body of evidence showing that immune function is abnormal in many forms of experimental and human essential hypertension. It has been documented that SHR and hypertensive patients have elevated humoral immune responses including increased serum concentrations of IgG and IgA, and a generation of autoantibodies against VSMC and thymocytes (Ebringer and Doyle, 1970; Kristensen, 1979; Takeichi *et al.*, 1981; Chen and Schachter, 1993). The cell-mediated immune response, however, is reduced. SHR exhibit a decreased T cell count, a depressed lymphocyte proliferation response, a reduced delayed-type hypersensitivity and a delayed allograft rejection time (Takeichi *et al.*, 1980; 1981; Fannon *et al.*, 1992; Purcell *et al.*, 1993). These abnormalities in the immune system could disrupt the immunoregulatory pathway. Associated with the depression in suppressor T cell activity, there is often an autoimmune process, including the generation of autoantibodies and an increase in immunoglobulin levels. An interesting feature of the immune dysfunction in hypertension is that there is an increased humoral response and a decreased cell-mediated response. It has been suggested that the primary defect observed in hypertension was in T cells. With this defect, especially the defect in the suppressor T cells whose function is to inhibit undesirable immune responses, the humoral immune response was elevated in hypertension (Takeichi *et al.*, 1981; Norman *et al.*, 1985; Fannon *et al.*, 1992). The increase in immunoglobulins and autoantibodies against VSMC could damage the vasculature, especially the blood vessels in the kidneys, resulting in increased vascular resistance and, thus, causing hypertension. Although these earlier studies are

suggestive, the mechanisms underlying immune dysfunction and the relationship between immune dysfunction and hypertension remain largely unknown.

The present study delineates the mechanism for the immune dysfunction in SHR. It provides the first evidence to suggest that the immune defect in SHR does not directly involve T cells (Xiao *et al.*, 1991). The T cell depression is the result of abnormal function of SHR macrophages. SHR macrophages overproduce NO which mediates this lymphocyte depression (Xiao and Pang, 1993). Pascual and co-workers (1993) reported a similar result also indicating that NO was responsible for immune dysfunction in SHR. It has been well documented that NO synthase in macrophages is an inducible isoform. The enzyme activity in macrophages is induced by various T cell-derived cytokines or LPS, indicating that T cells play an important role in the regulation of the expression of NO synthase activity. In order to elucidate the mechanism underlying the alteration of NO synthesis in SHR macrophages, the involvement of T cells in SHR NO synthesis was investigated. The results showed that NO synthesis in SHR macrophages was not affected by ablation of $CD5^+$ T cells, suggesting that the activation of NO synthesis in SHR macrophages is $CD5^+$ T cell-independent. However, the ablation of $CD5^+$ T cells significantly decreased the NO production in WKY macrophages, suggesting that the activation of NO synthase is T cell-dependent under normal conditions. Since the CD5 antigen is expressed in most of the active, and all of the resting T cells (Jones *et al.*, 1986; McAteer *et al.*, 1988), this study suggests that T cells may not play any significant role in the activation of NO synthesis in SHR macrophages. It is possible that other

mechanisms may be responsible for the alteration of NO synthesis in SHR macrophages, including genetic mechanisms.

Although the above studies characterized the mechanism responsible for the immune dysfunction in SHR, it did not address the question of how the immune dysfunction relates to hypertension. The hallmark of hypertension is an increase in vascular resistance. VSMC are responsible for controlling the lumen diameter of resistance vessels, and, thus, controlling vascular resistance. In hypertension, vascular smooth muscle exhibits abnormal growth (Lee, 1985; Mulvany, 1992) and responsiveness to vasoactive agents (Bohr *et al.*, 1991a; de Champlain *et al.*, 1991). It is of great interest to determine whether there is any interaction between VSMC and lymphocytes. An understanding of this possible interaction in SHR may provide evidence for a relationship between immune dysfunction and hypertension. Thus, the effect of SHR VSMC on the lymphocyte proliferation response was investigated. Once it was shown that VSMC caused inhibition of lymphocyte proliferation in SHR, the role of NO in this interaction and NO production by VSMC were also studied. The results are summarized as follows.

1. SHR VSMC but not WKY VSMC inhibited the proliferation response of lymphocytes from SHR and WKY.

2. L-NMMA reversed the inhibited lymphocyte proliferation response caused by SHR VSMC.

3. NO synthesis in SHR VSMC was significantly increased compared with that in WKY.

4. The expression of NO synthase activity in VSMC of SHR and WKY was induced by cytokines and LPS.

5. The stimulation of NO production in VSMC by inducers was dose-dependent and time-dependent.

No direct evidence is available concerning the interaction between lymphocytes and VSMC in hypertension. The present study provides the first evidence that VSMC can influence lymphocyte activity in SHR (Xiao and Pang, 1994a). The overproduction of NO in VSMC contributes to the lymphocyte depression in SHR. Increased NO synthesis in both VSMC and macrophages in SHR is an interesting and important phenomenon. It suggests that there may be a general activation of inducible NO synthesis in the hypertensive state. The present data also provide the first evidence that the overproduction of NO in SHR VSMC is biologically functional as SHR VSMC can inhibit lymphocyte proliferation in co-culture.

The generation of NO by NO synthase is an important autocrine and paracrine signaling pathway in the regulation of various cell functions and in communication (Culotta and Koshland, 1992). The importance of NO in the physiological control of blood pressure is now well established. The vasorelaxing effect of NO acts as a counter balance to the effect of vasoconstricting substances and also serves to maintain the blood pressure constant. In SHR as well as human essential hypertension, it has been well documented that the amounts of a variety of vasoconstricting substances and the responsiveness to these vasoconstrictors are

increased (de Champlain *et al.*, 1991; Dai *et al.*, 1992; Ferrier *et al.*, 1993; Jameson *et al.*, 1993). Although a considerable number of studies on the involvement of NO in the hypertensive state have been reported, the results are not clear and are often contradictory. It has been well documented that endothelium-dependent vasorelaxation was reduced in hypertension (Tesfamariam and Halpern, 1988; Deng *et al.*, 1993a; Malinski *et al.*, 1993; Panza *et al.*, 1993). This suggests that endothelium-derived NO synthesis by constitutive NO synthase was impaired in hypertension (Calver *et al.*, 1992; Luscher, 1992a). However, it was also reported that the constitutive endothelium-derived NO synthesis in SHR was not different from that in WKY (Ito and Carretero, 1992; Hayakawa *et al.*, 1993). Recently, it was demonstrated that light-activated release of NO from aortic rings was greater in SHR than in WKY (Kubaszewski *et al.*, 1994). The present study provides clear evidence for the first time that inducible NO synthesis is elevated in SHR VSMC and macrophages (Xiao and Pang, 1994a) . The activation of NO synthesis in macrophages and VSMC may reflect a general activation of the inducible NO synthase activity in SHR. This elevated inducible NO synthesis may play a compensatory protective mechanism against the increased vasoconstriction in hypertension. The increased NO synthesis in VSMC and macrophages may also be important for our understanding of the involvement of the NO synthesis system in hypertension.

At this time, however, it is not clear whether the activation of NO synthesis in SHR VSMC and macrophages is related to hypertension. An understanding of

the time sequence of the development of hypertension, changes in the NO synthesis system in macrophages and VSMC and lymphocyte depression may elucidate the relationship between immune dysfunction and hypertension. Therefore, an age related study was performed. The results are summarized as follows.

1. SHR had significantly higher blood pressures than did WKY at 4, 8, 12 weeks and 1 year of age. The blood pressure increased continuously from 4 weeks of age and reached a plateau value by 12 weeks of age.

2. The proliferation responses of SHR spleen cells were significantly reduced in all age groups. The lowest response occurred at 4 weeks of age.

3. In all age groups, the depressed proliferation response of SHR spleen cells was fully reversed by L-NMMA or removal of macrophages from the spleen cell population.

4. SHR spleen macrophages produced greater amounts of NO than WKY spleen macrophages in all age groups. The production of NO by SHR macrophages at 4 weeks of age tended to be higher than that of SHR macrophages at 8 or 12 weeks of age.

5. There was no correlation between lymphocyte depression and hypertension ($r= 0.03$) nor between NO production by macrophages and blood pressure in SHR ($r= -0.15$).

6. SHR VSMC produced significantly higher amounts of NO than did WKY VSMC in all age groups.

7. In SHR, a significant positive correlation was observed between blood

pressure and NO synthesis in VSMC (r= 0.71 or r= 0.69 by cytokines or LPS stimulation, respectively). No significant correlation was found in WKY (r= 0.13 or r= -0.07, respectively).

One of the important findings in this study is that SHR lymphocyte depression is not related to age or to the degree of development of hypertension, indicating a dissociation between lymphocyte depression and the development of hypertension in SHR. Because the lymphocyte depression is the result of excessive NO production by macrophages, this dissociation is supported by the finding that NO synthesis in SHR macrophages is not correlated to the blood pressure in SHR. These results showed evidence for the first time that immune dysfunction and hypertension may not be related (Xiao and Pang, 1994c).

This study confirms the previous observations that NO synthesis in SHR VSMC was elevated. This result agrees with a recent report showing that NO production in SHR aortae was enhanced by IL-1 (Junquero *et al.*, 1993). The importance of this study is that it shows for the first time that the increase in NO synthesis in VSMC was significantly correlated with the rise in blood pressure in SHR. This positive correlation suggests that the elevated blood pressure may be related to the expression of NO synthase in SHR VSMC or *vice versa.*

This age related study also supported the hypothesis that a general alteration of inducible NO synthesis may exist in SHR. Hypertension and lymphocyte depression appear to be two separate and parallel events that occur in SHR. Because the blood pressure of SHR was already significantly increased at 4 weeks

of age, it cannot be ruled out that changes in blood pressure before 4 weeks of age may cause the alteration in NO synthesis. In order to clarify this question, lymphocyte proliferation and NO synthesis in macrophages and VSMC were investigated in an induced hypertensive state for comparison with those parameters in the normotensive state. The results are summarized as follows.

1. The lymphocyte proliferation response was normal in rats with salt-induced hypertension.

2. NO production by the macrophages in rats with induced hypertension was very similar to that of normotensive rats.

3. In rats with induced hypertension, the NO production in macrophages was not correlated with blood pressure (r= -0.01).

4. The rats with high blood pressure exhibited an elevated inducible NO synthesis in VSMC.

5. The increase in VSMC NO synthesis was significantly and positively correlated with the rise in blood pressure in these hypertensive rats (r= 0.87).

This study supports the hypothesis that blood pressure is not associated with the increased NO synthesis in macrophages or lymphocyte depression in SHR. The high blood pressure *per se* dose not appear to influence the elevated expression of NO synthase activity in macrophages and cause the lymphocyte depression. This result confirms that hypertension and immune dysfunction are two separate concurrent phenomena in SHR. The elevated inducible NO synthesis seems to serve as a common denominator between these two phenomena.

This study also supports the second hypothesis that the activation of NO synthesis in VSMC, unlike that in macrophages, is associated with the rise in blood pressure. The increased blood pressure may activate the expression of inducible NO synthesis in VSMC through an undefined mechanism(s). Whatever the underlying mechanism is, the increased NO production in VSMC may serve a compensatory role against the elevated blood pressure in SHR.

As discussed earlier, the involvement of NO in hypertension is not completely understood. Inhibition of NO synthesis by treatment of NO synthase inhibitors *in vivo* increased blood pressure in normotensive rats (Ikeda *et al.*, 1992; Ribeiro *et al.*, 1992; Morton *et al.*, 1993). Administration of L-arginine to hypertensive patients (Hishikawa *et al.*, 1993) or animals (Patel *et al.*, 1993) lowered the blood pressure. These results suggested that NO synthesis may be impaired in the hypertensive state. The present study and the studies reported by others demonstrated that inducible NO synthesis is actually increased in SHR. It seems paradoxical that NO synthesis is elevated at the cellular level but is insufficient when the whole body is considered. This suggests, however, that increased NO production in VSMC may tend to compensate for the elevated blood pressure in hypertension but that this compensatory mechanism is not sufficient enough to combat the effect of increased vasoconstricting factors and structurally altered vasculature. Furthermore, the constitutive NO synthase may also be involved in the overall blood pressure regulation in hypertension. It is also possible that constitutive and inducible NO synthases may be affected differently in hypertension.

The significance of this thesis study is summarized as follows:

1. This thesis is the first report to show a dissociation between abnormalities in the immune system and hypertension.

2. This work delineates for the first time that the immune dysfunction in SHR is mediated by the overproduction of NO in macrophages and, possibly, in VSMC.

3. Solid evidence is provided to show that inducible NO synthesis in macrophages and VSMC is elevated in SHR, suggesting that a general activation of the inducible NO synthesis system may exist in this hypertensive animal model.

4. A significant positive correlation is shown in this study between the increased VSMC NO synthesis and the rise in blood pressure in SHR, suggesting that hypertension may influence the expression of inducible NO synthase in VSMC.

5. The mechanism of NO synthase activation in SHR macrophages and VSMC is explored. The activation of NO synthesis in SHR macrophages is T cell-independent and blood pressure-independent. The enhanced NO synthesis in SHR VSMC, however, is related to the increased blood pressure.

Although the physiological significance of this elevated expression of NO synthase in hypertension is not yet completely understood, this work provides a basis and direction for future investigations. The following questions need to be addressed:

1. Does the enhanced activation of NO synthesis occur in other types of cells or tissues in SHR?

2. What is the mechanism underlying this general activation of inducible NO

synthesis in hypertension?

3. What is the significance of the NO synthesis alteration in hypertension.

Understanding the nature of the NO synthesis pathways in hypertension may not only help to elucidate the pathogenesis of hypertension but may also provide a new approach to the therapeutic management of hypertension by manipulating the NO synthesis in the right direction at the right time. If this is accomplished, the immune abnormalities in hypertensive patients may also be corrected.

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