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"HYPERTENSION AND HYPERCHOLESTEROLEMIA: BIOCHEMICAL AND PHARMACOLOGICAL STUDIES IN THE RABBIT"

By

Yi-Tsau Huang ©

this being a thesis submitted for the degree of

Doctor of Philosophy

in the Faculty of Medicine

of the University of Glasgow

Department of Medicine and Therapeutics

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DECLARATION

The work described in this thesis was carried out in then Department of Materia Medica, later Department of Medicine and Therapeutics, University of Glasgow, under the supervision of Dr. C.A. Hamilton and Professor J.L. Reid.

I enjoyed the collaboration of Dr. N. Hassan, who undertook the Sudan Red staining of the arterial tissues. The remainder of the work and the writing of the thesis was entirely performed by myself. It has not been submitted previously for a higher degree.

Yi-Tsau Huang

Sept., 1991

SUMMARY

1. Four groups of rabbits were set up to investigate the separate and combined effects of hypertension and hypercholesterolemia on a number of cardiovascular parameters, including in vivo vascular reactivity, phosphoinositide metabolism in aortic tissue, and platelet $[Ca^{2+}]_i$ concentrations. The four groups were: a control group, a hypertensive group (perinephritis hypertension), a hypercholesterolemic group (0.3% cholesterol diet), and a hypertensive-hypercholesterolemic group.

2. As the basic pharmacology of one of the agonists used in this work, endothelin, was poorly understood at the time these studies were planned, preliminary investigations into its effects in normotensive-normocholesterolemic animals were undertaken before embarking on the main project. In addition, some phosphoinositide studies were done using rat aorta.

Endothelin -1 caused a dose-related increase in phosphoinositide hydrolysis in both rat and rabbit aorta. In rat aorta, endothelin-1-induced phosphoinositide hydrolysis increased with stimulation time for the first 30 minutes, and thereafter plateaued. The endothelin-stimulated effects were attenuated with endothelium removal, or with extracellular Ca^{2+} depletion. The endothelin-1-induced phosphoinositide hydrolysis was greater in rat aorta than in rabbit aorta.

In vivo endothelin-1 caused a short-lived depressor response followed by a long-lasting pressor response in the rabbit. The pressor response was dose-related, and could be attenuated by the calcium antagonist nifedipine.

3. The imposition of perinephritis hypertension caused an increase of about 40 mmHg in mean arterial pressure in the operated rabbits, which stabilized after 6-7 weeks, while feeding a 0.3% cholesterol diet induced a continual rise in plasma

cholesterol levels in the rabbits, which reached 30 mmol/L after 4 months.

In contrast to 0.3% cholesterol diet, perinephritis hypertension was a significant risk factor for cardiovascular deaths in the course of a 4-month study. There were significantly greater numbers of cardiovascular deaths in both the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group ($p = 0.038$ and 0.009, respectively), but no significant difference between the hypercholesterolemic and the control group, or between the hypertensive-hypercholesterolemic and the hypertensive group. However, it is noteworthy that when the hypertensive-hypercholesterolemic group was compared to the hypertensive group, 0.3% cholesterol diet tended to augment the cardiovascular deaths in these hypertensive animals.

4. The imposition of perinephritis hypertension enhanced the pressor responses to angiotensin II, and endothelin-1, as well as the depressor responses to acetylcholine, isoproterenol, and nitroprusside at 2-3, 6-7, and 13-16 weeks of study. The differences tended to increase with the duration of hypertension for the pressor responses, but not for the depressor responses. The patterns of changes in the duration of hypertension differed from one pressor agonist to another, suggesting that structural changes in the vessel wall were not the sole explanation for enhanced vascular reactivity. Similar conclusions held for the depressor agonists. In contrast, the imposition of 0.3% cholesterol diet had no effect on the in vivo vascular reactivity at any time point of the study, whether given to normotensive or hypertensive animals.

5. Neither perinephritis hypertension nor 0.3% cholesterol diet caused any changes in the basal platelet $[Ca^{2+}]_i$ at the 17th week of study.

6. At the 18th week of study, perinephritis hypertension tended to enhance the noradrenaline-stimulated, but not the endothelin-stimulated phosphoinositide hydrolysis in rabbit aorta, but the difference was significant only at 10^{-4} M noradrenaline for the hypertensive versus the control group. In contrast, 0.3% cholesterol feeding tended to decrease both noradrenaline- and endothelin-stimulated, phosphoinositide hydrolysis. The difference was significant at 10^{-4} M noradrenaline for the hypercholesterolemic versus the control group, as well as for the hypertensive-hypercholesterolemic versus the hypertensive group, and at 10^{-6} & 10^{-5} M endothelin-1 for the hypertensive-hypercholesterolemic versus the hypertensive group.

There was no difference in the basal [^{3}H]-inositol phosphates formation between any experimental group and the control group, suggesting that neither perinephritis hypertension nor 0.3% cholesterol diet for 18 weeks altered the basal phosphoinositide metabolism in rabbit aorta.

7. Overall, in our study no significant additive effects of the two disease states were observed in any of the parameters examined. However, the number of biochemical responses, vessels and animals examined were limited, and further studies might identify sites of interaction between the two parameters.

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CHAPTER 1

GENERAL INTRODUCTION

Chapter 1. General Introduction

1.1. Hypertension, hypercholesterolemia, and atherosclerosis: An overview

1.1.1 Atherosclerosis as the most important cause of cardiovascular morbidity and mortality

Cardiovascular diseases are the most common cause of death in modern western countries. Atherosclerosis is the principal underlying cause of cardiovascular diseases; it has been estimated that atherosclerosis accounts for 85% of deaths from cardiovascular diseases (Fraser, 1986). Moreover, clinical manifestations of cardiovascular diseases, such as angina pectoris, myocardial infarction, sudden death, and stroke, are chiefly attributable to atherosclerosis (Kannel & Sytkowski, 1987).

It is now recognized that atherosclerotic cardiovascular diseases are "multifactorial" in etiology. Age, sex, hypercholesterolemia, hypertension, diabetes mellitus, obesity, cigarette smoking, oral contraceptives, sedantary lifestyle, Type A personality, family history, and menopause have all been proposed as "risk factors" for atherosclerotic diseases (Kannel & Sytkowski, 1987). Among them, the major risks which are also amenable to intervention are hypertension, hypercholesterolemia, and cigarette smoking (Dal Palu & Zamboni, 1990; Stamler, 1988). The role of two of these factors, hypertension and hypercholesterolemia, will be discussed in more detail.

1.1.2. Hypertension as a risk factor for atherosclerosis

In the Framingham Study, the epidemiology of the potential cardiovascular risk factors of white Americans in a U.S. northeastern town was extensively followed for more than 20 years. The results from this study strongly indicated that hypertension is an important and independent risk factor for atherosclerosis (Dawber, 1980). The

incidence of coronary, cerebral, or peripheral arterial diseases increased significantly with the rise of systolic as well as diastolic blood pressure. There was no critical point of exemption from the diseases, and even in the "normotensive" population, the relative risk increased progressively with the rise in blood pressure. "Blood pressure elevation" as a risk factor for atherosclerotic diseases has also been identified from various prospective studies worldwide, including Seven Countries (Finland, Greece, Italy, Japan, Netherlands, U.S.A., & Yugoslavia) (Keys, 1980), the Pooling Project (U.S.A.) (The Pooling Project Research Group, 1978), and several epidemiological studies from Australia (Welborn et al, 1969), Japan (Shimamoto et al, 1989), Norway (Holme et al, 1980), Puerto Rico (Garcia-Palmieri & Costas, 1986), Sweden (Carlson & Bottiger, 1985), and United Kingdom (Reid et al, 1976).

In addition, when blood pressure is lowered by various anti-hypertensive drugs, there is a reduction in stroke and coronary events; this strengthens the status of high blood pressure as an important risk factor (Collins et al, 1990).

1.1.3. Hypertension in the pathogenesis of atherosclerosis

In human autopsy studies from worldwide sources, it has been reported that blood pressure is positively and significantly correlated with the severity of atherosclerotic lesions (Robertson & Strong, 1968; Solberg & Strong, 1983).

In animal studies, hypertension can induce a host of changes conducive to atherosclerosis, such as endothelial-cell shape changes (Chobanian, 1990), reduced endothelium-dependent vascular relaxation (Luscher, 1988; Vanhoutte, 1989), enhanced endothelial permeability (Chobanian, 1983), adherence of monocytes, lymphocytes, and granulocytes to the endothelial surface (Chobanian,1990), macrophage accumulation and smooth muscle cell migration into the intima (Chobanian, 1990), intimal thickening (Chobanian, 1990), hypertrophy, hyperplasia, hyperploidy of smooth muscle cells in the media (Chobanian, 1990), and

extracellular accumulation of connective tissue such as proteoglycans, collagen, and elastin in the media (Chobanian, 1990). One point of note from these studies is that while intimal and medial thickening are commonly observed in hypertensive animals, increased lipid accumulation or accelerated atherogenesis requires the co-existence of hypercholesterolemia (Chobanian, 1983; Chobanian, 1990).

1.1.4. Hypercholesterolemia as a risk factor for atherosclerosis

The Framingham Study (Dawber, 1980), the Pooling Project (The Pooling Project Research Group, 1978), and the Seven Countries study (Keys, 1980) all strongly indicated that in addition to hypertension, hypercholesterolemia is a potent and independent risk factor for atherosclerotic diseases. Epidemiological studies from Australia (Welborn et al, 1969), Finland (Pelkonen et al, 1977), Israel (Goldbourt & Yaari, 1990), Japananese emigrants in Hawaii (Kagan et al, 1981), Norway (Holme et al, 1980), Puerto Rico (Garcia-Palmieri & Costas, 1986), Sweden (Carlson & Bottiger, 1985), and United Kingdom (Reid et al, 1976) are all consistent with the hypothesis that serum cholesterol levels are significantly correlated with cardiovascular events. The clinical history of homozygous familial hypercholesterolemic patients also highlights the importance of hypercholesterolemia in the pathogenesis of atherosclerosis. These patients have six to ten times the normal concentrations of plasma low density lipoproteins (main carrier of plasma cholesterol), and often suffer severe coronary atherosclerosis and premature death in childhood, even in the absence of other major risk factors such as hypertension, diabetes mellitus, or cigarette smoking (Brown & Goldstein, 1986).

The impressive reduction of coronary events in hypercholesterolemic patients treated with cholesterol-lowering drugs in two major prospective studies (The Lipid Research Clinics Coronary Primary Prevention Trial and The Helsinki Heart Study) also confirms the significant role of hypercholesterolemia in atherosclerosis (Lipid Research Clinics Program, 1984; Frick et al, 1987).

1.1.5. Hypercholesterolemia in the pathogenesis of athersclerosis

In human autopsy studies from worldwide sources, it has been reported that plasma cholesterol was positively and significantly related to the severity of atherosclerotic lesions (Solberg & Strong, 1983). Results from in vivo coronary angiography also support this conclusion (Dahlen et al, 1986; Holme et al, 1981).

In animal studies, hypercholesterolemia has been reported to induce a host of changes in the blood vessels conducive to atherosclerosis, such as endothelial-cell shape changes, enhanced endothelial permeability, leukocyte adherence, macrophage accumulation and smooth muscle cell migration into the intima, smooth muscle cell proliferation in the intima and media, cholesterol deposition, extracellular matrix accumulation, foam cell accumulation, intimal thickening, and atherosclerotic plaque formation (Chobanian, 1990; Masuda & Ross, 1990a; Masuda & Ross, 1990b; Rosenfield et al, 1987a; Rosenfield et al, 1987b).

1.1.6. Hypertension and hypercholesterolemia as coexistent or interactive risk factors in atherosclerosis

Although the beneficial effect of a reduction in stroke events by means of lowering blood pressure in patients with hypertension is widely recognized, the outcome of treating mild to moderate hypertension is less obvious in terms of reduction in coronary events (MacMahon et al, 1986; Collins et al, 1990).

Results from clinical trials in mild-to-moderate hypertensive patients suggest:
(1) In a hypertensive population, lowering blood pressure results in a greater reduction in stroke events than in coronary events (MacMahon et al, 1986; Collins et al, 1990). One of the explanations proposed is that the incidence of hemorrhagic strokes, the direct complication of hypertension, can be reduced by lowering blood pressure, while it is more difficult to reduce the incidence of so-called indirect

complications such as myocardial infarction (Dal Palu & Zamboni, 1990). Moreover, the metabolic side effects of anti-hypertensive drugs might offset the benefits of blood pressure reduction for coronary risks. For example, β-blockers are known to reduce the "protective" plasma high-density lipoprotein levels, and thiazide diuretics are known to increase plasma cholesterol levels (MacMahon et al, 1986; Collins et al, 1990).

(2) The "Multiple Risk Factor Intervention Trial"--- a large-scale study to investigate the effect of blood pressure lowering, diet counselling to reduce plasma cholesterol, and advice to reduce smoking, showed that measures to reduce cholesterol and smoking did result in a significant reduction of coronary heart disease, whereas anti-hypertensive drug therapy did not (Multiple Risk Factor Intervention Trial Research Group, 1982).

It is particularly significant against this background that until recently, most efforts in combating atherosclerotic diseases have been directed towards blood pressure reduction. These studies suggest that a "multiple-risk-factorial" approach in combating atherosclerotic diseases should be considered. In the middle-aged and elderly population, generally there is a picture of several concomitant risk factors at play in atherogenesis. The relative risks are cumulative according to the number and extent of risk factors present in each patient (Kannel & Sytkowski, 1987). The possible interplay of risk factors such as hypertension and hypercholesterolemia in the atherosclerotic process warrants further investigation (Reid,1988).

1.2. Changes in vascular function in hypertension, hypercholesterolemia, and atherosclerosis

1.2.1. Changes in vascular function in hypertension

The cause(s) of essential hypertension has been in question for several decades. Furthermore, the mechanisms by which primary or secondary hypertension in humans or animals progresses to the established, sustained state of hypertension,

with elevated peripheral resistance as its hallmark, are far from clearly defined. Even in the established phase of hypertension, there are qualitative as well as quantitative differences between different types of hypertension in the vascular reactivity to various agonists. In this context, numerous researchers have investigated haemodynamic and functional changes in the vasculature in and after the development of hypertension. In addition, haemodynamic and functional studies have been carried out with regard to hypercholesterolemia and atherosclerosis.

Changes in vascular function can be categorized as follows:

(1) Changes in endothelial permeability.

(2) Changes in endothelium-dependent vascular relaxation or contraction.

(3) Changes in vascular smooth muscle reactivity to various agonists.

(1) Enhanced endothelial permeability may be observed in hypertensive animals before any structural lesions occur. In hypertensive animals, investigators have demonstrated increased vascular permeability to colloid particles (iron and carbon), small molecules such as horseradish peroxidase, plasma proteins including fibrinogen, red blood cells, and platelets (Wiener & Giacomelli, 1983). It has been proposed that the permeability change is due to the haemodynamic (wall shear) stress (Fry, 1973), the mechanically coupled enzymatic mechanisms for generating histamine (Huttner & Gabbiani, 1983), or the vasculotoxic effect of renin and angiotensin II (Wiener & Giacomelli, 1983).

(2) Changes in endothelium-dependent vascular relaxation or contraction

Ever since the seminal finding of Furchgott & Zawadzki (1980) that rabbit aortic strips in organ baths could respond to low concentrations of acetylcholine by "vaso-relaxation" only in the presence of intact endothelium, the crucial role of the vascular endothelium not only as a passive barrier, but also as an active participant in the regulation and modulation of local vascular tone has become increasingly appreciated (Vanhoutte, 1989). Vascular endothelium or cultured endothelial cells

can generate and release short-lived endothelium-derived relaxing factors. The major endothelium-derived relaxing factor is probably nitric oxide, which causes muscle relaxation by an increase in intracellular cGMP concentration (Furchgott, 1990; Vanhoutte, 1989). It has been reported that in cultured endothelial cells, the precursor of nitric oxide is L-arginine (Palmer et al, 1988). Estimated half-life values for endothelium-derived relaxing factor ranged from 6 to 50 seconds (Furchgott, 1990). Endothelium-derived relaxing factor is generated and released ubiquitously in large vessels as well as resistance arteries, and veins. In addition to basal release, release of endothelium-derived relaxing factor can be stimulated by flow-induced increase in shear stress, by vasoactive hormones (e.g. vasopressin), by neurotransmitters (e.g. acetylcholine and substance P), by autocoids that modulate local blood flow, especially during inflammation (e.g. histamine and bradykinin), and by substances involved in haemostasis (e.g. thrombin, serotonin, and adenosine diphosphate released from platelets). Endothelium-derived relaxing factor also possesses anti-aggregation and anti-adhesion activities toward platelets (Furchgott, 1990; Vanhoutte, 1989).

There are numerous reports showing that the endothelium-dependent vascular relaxation is impaired in large vessels as well as in resistance arteries, from animals with primary or secondary hypertension, compared to controls from normotensive animals (Luscher, 1988; Sunano et al, 1989; Tesfamariam & Halpern, 1988; Lockette et al, 1986). The impaired relaxation was associated with a decreased cGMP production. (Shirasaki et al, 1988) Impaired endothelium-dependent vascular relaxation was also demonstrated in forearm resistance vessels from patients with essential hypertension (Linder et al, 1990; Panza et al, 1990).

In addition to the production of the endothelium-derived relaxing factor, the vascular endothelium can also release vasoconstrictor substances. So far there are a number of types of endothelium-derived contracting factors described by various groups (Anggard et al, 1990):

(A) Hypoxia- or anoxia-induced endothelium-derived contracting factor in peripheral, coronary, and cerebral arteries. Its action is very rapid and readily reversible; it may be a superoxide anion or a closely related free radical species (Vanhoutte & Katusic, 1988).

(B) Endothelium-derived contracting factor induced by stretch, increased transmural pressure, high potassium, calcium ionophore A23187 and arachidonic acid. It has been reported that this type of contracting factor is sensitive to inhibitors of cyclooxygenase such as indomethacin and could be a prostaglandin or related factor (Anggard et al, 1990; Vanhoutte & Katusic, 1988).

(C) Endothelium-derived contracting factor generated by cultured endothelial cells. Yanagisawa et al (1988) were the first group to purify and determine the sequence of this peptide contracting factor. They called this 21-amino-acid peptide "endothelin"; it has been identified in human plasma and is believed to have pathophysiological relevance (Kohno et al, 1990). Three distinct endothelin genes have been demonstrated in humans and other mammals using gene probing techniques (genes for endothelin-1, -2, & -3). However, it appears that only endothelin-1 is expressed and released by endothelial cells (Yanagisawa & Masaki, 1989a). Endothelin-1 is a highly potent vasoconstrictor (EC_{50} values ranging $2x10^{-10}$ - $5x10^{-9}$ M), and its action is long-lasting (more than 30 minutes), difficult to wash out and resistant to antagonists of α-adrenergic, H_1-histaminergic, serotonergic, and muscarinic receptors, and the inhibitors of cyclooxygenase or lipooxygenase (Yanagisawa et al, 1988).

(D) Recently, it has been reported that another endothelium-derived contracting factor exists. This factor is sensitive to indomethacin and may contribute to the endothelium-dependent contractile response to acetylcholine in aorta or arterioles from spontaneously hypertensive rats as compared to control normotensive rats (Luscher & Vanhoutte, 1986; Koga et al, 1989; Cordellini et al, 1990; Fortes et al,

1990; Kato et al, 1990).

(3) Changes in vascular smooth muscle reactivity to various agonists.

In comparing the dose-response relationships of vascular beds to an agonist in hypertensive and normotensive men or animals, there are three aspects to be examined:

(A) The threshold concentration for a response in each group.

(B) The ED_{50}, the concentration at which half maximal response is achieved.

(C) The E_{max}, the maximal response achieved in each group.

It is customary to refer to (A) and (B) as "sensitivity", and (C) as "reactivity". In vessel strip studies in vitro, E_{max} can be obtained, while in clinical investigations or in studies conducted in vivo with conscious animals, E_{max} data are often not obtainable due to the high risk and ethical considerations.

In the established phase of hypertension (either primary or secondary in etiology), there are structural changes to the blood vessel wall which may account for the changes in ED_{50} and E_{max} in the dose-response curves (Folkow, 1982). On the other hand, changes in dose-response curves in response to some pressor or depressor substances, but not others, e.g., noradrenaline but not angiotensin II (Miyahara, 1966; Hamilton & Reid, 1983), or verapamil but not sodium nitroprusside (Hulthen et al, 1982; Robinson et al, 1982), argue for a "selective, functional factor" in the pathophysiology of hypertension. This point is also substantiated by in vitro vessel strip or perfused vascular bed studies. Changes in some specific receptor-mediated responses, e.g., α- or β- adrenoceptor-mediated responses, but not in other receptor-mediated or non-receptor-mediated responses, have been reported from various groups in hypertension of primary or secondary origin (Collis & Vanhoutte, 1977; Fink & Brody, 1979; Katovich et al, 1984; Aqel et al, 1986).

Thus there are different, but not mutually exclusive, grounds for support of

"structural" as well as "functional" components contributing to the changes in vascular responses in hypertension (Friedman, 1983).

Furthermore, changes in vascular responses are not always the products of sustained hypertension. When they precede or parallel the development of hypertension, these specific changes may have causative or ontological implications (Webb & Bohr, 1981; Lais & Brody, 1978).

There are many reports showing that the in vivo pressor responses to α-adrenoceptor agonists are enhanced in humans or animals with primary or secondary hypertension (Mendlowitz & Naftchi, 1958; Miyahara, 1966; Jie et al, 1986; Hicks et al, 1983; Finch & Haeusler, 1974; Hamilton & Reid, 1983). Results from in vitro studies are controversial in primary hypertension (Bhalla et al, 1989; Mulvany, 1984), while most in vitro studies on secondary hypertension have reported an enhanced responsiveness (Finch, 1971; Collis & Alps, 1975; Katovich et al, 1984).

There are many reports showing that in the established phase of hypertension, in vivo as well as in vitro β-adrenoceptor-mediated cardiovascular responses are attenuated in both humans and animals (For review, see Feldman, 1987), while an increased vasoconstrictor response to serotonin has been observed in animals with primary as well as secondary hypertension (Cheng & Shibata, 1980; Mecca & Webb, 1984; Huzoor-Akbar et al, 1989; Turla & Webb, 1990).

To summarize, there are a host of functional changes in the vascular endothelium, as well as in smooth muscle cells, associated with hypertensive states.

1.2.2. Changes in vascular function in hypercholesterolemia and atherosclerosis

(1) Changes in endothelial permeability

In cholesterol-fed rabbits, enhanced entry of albumin has been demonstrated in the aortic intimal surface 2 weeks after starting a cholesterol-enriched diet. At this time, no lipid lesion was visible (Adams & Bayliss, 1977). Stemerman (1981) also

found that hypercholesterolemia was associated with increased uptake of horseradish peroxidase even in the absence of endothelial loss. Increased permeability to plasma proteins such as albumin, fibrinogen, and most notably, cholesterol (visualized by [^{3}H]-cholesterol uptake) in the pig aorta has been seen during dietary cholesterol challenge (Somer & Schwartz, 1971). Recently, Schwenke & Carew (1989) reported that in cholesterol-fed rabbits, as early as 4 to 16 days when the vasculature was still morphologically normal, the uptake of [^{131}I]-low-density-lipoproteins has been increased markedly in all segments of aorta, as compared to untreated controls. Moreover, the fractional degradation rate of [^{131}I]-low-density-lipoproteins was also severely retarded, especially in lesion-prone sites such as the aortic arch and branch sites of the aorta.

(2) Changes in endothelium-dependent relaxation or contraction

The impairment of endothelium-dependent relaxation has been observed in vivo in human arteries or arterioles with hypercholesterolemia (Creager et al, 1990) as well as atherosclerosis (Ludmer et al, 1986; Cox et al, 1989), while the endothelium-independent relaxation in these vessels was largely preserved. Similar in vivo findings have been made in both large and resistance vessels in cholesterol-fed animals (Bossaller et al, 1987a; Yamamoto et al, 1988; Girerd et al, 1990). In vitro studies also highlighted the impairment of endothelium-dependent relaxation in large and resistance vessels from humans or animals with hypercholesterolemia or atherosclerosis (Bossaller et al, 1987b; Osborne et al, 1989; Guerra et al, 1989; Shimokawa & Vanhoutte, 1989; Merkel et al, 1990). Furthermore, some research groups have demonstrated that this impairment of endothelium-dependent relaxation in the arteries from hypercholesterolemic and atherosclerotic animals was mainly due to the decreased release or production of endothelium-derived relaxing factor (Guerra et al, 1989; Shimokawa & Vanhoutte, 1989; Sreeharan et al, 1986). Bossaller et al (1987b) also found that the impaired endothelium-dependent relaxation in the atherosclerotic arteries was associated with suppressed production

of cGMP. The relaxation apparatus, in terms of relaxation to nitroprusside or other agonists acting directly on smooth muscle cells, was generally intact. In line with this, it was also shown that L-arginine, which is the precursor for the synthesis of endothelium-derived relaxing factor, i.e., nitric oxide, could partially or mostly reverse this impaired endothelium-dependent relaxation in the atherosclerotic arteries, suggesting that a decreased production of nitric oxide was involved (Girerd et al, 1990; Rossitch et al, 1991).

(3) Changes in vascular smooth muscle reactivity to various agonists.

While studies on the vascular pressor/constrictor or depressor/dilator responses to α- or β- adrenoreceptor agonists, or angiotensin II in hypercholesterolemic or atherosclerotic humans or animals have given controversial results (for more detailed discussion, see the Introduction Section of the "Pressor and Depressor Responses in Hypertensive and Hypercholesterolemic Rabbits" Chapter), there are more consistent reports on the altered responsiveness to serotonin and ergonovine. The in vivo vasodilator response to serotonin in hindlimb vascular beds was reported to be impaired and converted to vasoconstriction in atherosclerotic animals (Heistad et al, 1984; Lopez et al, 1989a). An augmented in vivo vasoconstrictor response to serotonin was also observed in cerebral, ocular, and mesenteric vascular beds (Faraci et al, 1989; Lopez et al, 1989b). Many in vitro studies also showed that there was an enhanced vasoconstrictor response to serotonin in both large and resistance vessesls from humans and animals with hypercholesterolemia or atherosclerosis (Kalsner & Richards, 1984; Merkel et al, 1990; Chilian et al, 1990; Yokoyama et al, 1983).

An enhanced constrictor response to ergonovine has been observed in aorta from diet-induced as well as hereditary hypercholesterolemic rabbits (Henry & Yokoyama, 1980; Yokoyama et al, 1983).

Thus, hypercholesterolemia and atherosclerosis may both augment

vasoconstrictor responses and attenuate vasodilator responses (Lopez et al, 1989a), and thereby have pathological implications in coronary or cerebral vasospasm (Shimokawa & Vanhoutte, 1989; Shimokawa et al, 1988).

To summarize, hypercholesterolemia and athersclerosis can induce a variety of functional changes in the vascular endothelium as well as smooth muscle cells, and may thus predispose the vasculature to occlusive or vasospastic diseases.

1.3. Changes in second messenger systems in hypertension, hypercholesterolemia, and atherosclerosis.

1.3.1. The second messenger systems involved in contraction-relaxation cycles and cellular proliferation processes of vascular smooth muscle cells

The contraction-relaxation cycles of vascular smooth muscle cells are mediated chiefly by changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Karaki, 1989). There are three main second messenger systems involved in the regulation of $[Ca^{2+}]_i$ and thereby the contraction or relaxation of vascular smooth muscle cells (Karaki, 1989). They are:

(1) The phosphoinositide hydrolysis system (i.e., inositol 1,4,5-trisphosphate and 1,2-diacylglycerol system).

(2) The cAMP system.

(3) The cGMP system.

Agonists may stimulate the production of inositol trisphosphate and diacylglycerol by way of phosphatidylinositol 4,5-bisphosphate hydrolysis, increase $[Ca^{2+}]_i$, and cause the contraction of vascular smooth muscle cells. Firstly, inositol 1,4,5-trisphosphate causes an intracellular release of Ca^{2+} from endoplasmic reticulum after binding to the receptor site in the latter, which in turn causes a short-lived $[Ca^{2+}]_i$ increase, and a phasic contraction of vascular smooth muscle cells (Somlyo et al, 1988). Secondly, 1,2-diacylglycerol activates protein kinase C in the membrane, which in turn brings about the tonic contraction of vascular smooth

muscle and an increase of Ca^{2+} influx through the cell membrane (Rassmussen et al, 1987; Khalil & Van Breemen, 1988).

Agonists may elevate cAMP levels in vascular smooth muscle cells by way of receptor--G-protein--adenyl-cyclase activation. This decreases $[Ca^{2+}]_i$ as well as the Ca^{2+} sensitivity of contractile elements, and thus relaxes the smooth muscle (Karaki, 1989).

Agonists may elevate cGMP levels in vascular smooth muscle cells by way of receptor-activated (e.g., atrial natriuretic peptide), non-receptor-mediated (e.g., nitroprusside), or indirect endothelium-dependent (e.g., acetylcholine), activation of guanylate cyclase; this decreases $[Ca^{2+}]_i$ as well as the Ca^{2+} sensitivity of contractile elements, and thus relaxes the smooth muscle (Karaki, 1989). Guanylate cyclase activation by any of these pathways may also inhibit phosphoinositide hydrolysis and smooth muscle contraction in rat aorta (Rapoport, 1986).

In addition to their crucial roles in the regulation of contraction and relaxation of vascular smooth muscle cells, both the phosphoinositide hydrolysis and the cAMP messenger systems may be involved in the regulation of cellular proliferation in smooth muscle cells.

(1) The phosphoinositide hydrolysis system in cell proliferation

Studies on the biochemical changes induced by growth factors in a variety of cells have pinpointed some of the key signals at the early phase of cell proliferation. They include (A) increased phospholipase C activity and thereby increased phosphoinositide hydrolysis, (B) an increase in $[Ca^{2+}]_i$, (C) stimulation of the Na^+/H^+ antiport leading to cellular alkalization (an increase in cytosolic pH), (D) Enhanced tyrosine-specific protein kinase activity (Taylor, 1986; Berridge, 1986). In addition, as pointed out by Berk & Alexander (1989), growth factors for cultured vascular smooth muscle cells such as noradrenaline, adrenaline, platelet-derived growth factor, epidermal growth factor, and endothelin , are also capable of causing

smooth muscle contraction by way of phosphoinositide hydrolysis (Thyberg et al, 1990). In line with the suggestion that phosphoinositide metabolism is involved in cell proliferation processes, it has been observed that there is a higher rate of phosphoinositide hydrolysis, together with a higher proliferation rate, in cultured vascular smooth muscle cells from spontaneously hypertensive rats as compared to Wistar-Kyoto rats, under basal as well as agonist-stimulated conditions (Paquet et al, 1989; Scott-Burden et al, 1989a; Paquet et al, 1990).

(2) The cAMP system in cell proliferation

Franks et al (1984) showed that the ability of cultured vascular smooth muscle cells to grow correlated with adenyl cyclase activity in the cell lines. Also, adenyl cyclase activity increased after the addition of growth stimuli, and this increase preceded the rise in [^{3}H]-thymidine incorporation. In addition, cAMP has been reported to enhance cellular proliferation by epidermal growth factor or modulate the proliferation cycle (Olashaw & Pledger, 1988). Thus both the cAMP and phosphoinositide hydrolysis systems have been implicated in the regulation of cell proliferation, although potential interactions have yet to be elucidated (Nishizuka, 1986).

Recently, Garg & Hassid (1989) reported that vasodilator drugs that generate nitric oxide, which activates guanylate cyclase and increases cellular cGMP levels, inhibited the mitogenesis and proliferation of cultured vascular smooth muscle cells, suggesting a possible role for the cGMP messenger system in the regulation of cell proliferation. Notably, in contrast to the cAMP and phosphoinositide systems, cGMP appeared to inhibit growth.

1.3.2. Changes in the phosphoinositide second messenger system in hypertension, hypercholesterolemia, or atherosclerosis.

Many investigations into the phosphoinositide second messenger system in hypertension have been undertaken, but the results are controversial. (For review, see Heagerty & Ollerenshaw, 1990, and "Chapter 6. Phosphoinositide Hydrolysis in

Hypertensive and Hypercholesterolemic Rabbits" in this thesis.) These investigations can be broadly categorized into three types:

(1) Investigations into the content of phosphoinositides in the membrane, namely the amount of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate, in non-stimulated cells, measured after [^{3}H] or [^{32}P] radiolabelling.

(2) Investigations into the activity of membrane phospholipase C under basal, non-stimulated conditions, measured as radioactivity of various inositol phosphates or diacylglycerol after radiolabelling the phosphoinositides with [^{3}H] or [^{32}P], i.e., the basal rate of phosphoinositide hydrolysis.

(3) Investigations into the response of phosphoinositide hydrolysis to the challenge by agonists, namely the relative yield of inositol 1-monophosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate or diacylglycerol after stimulation as compared to non-stimulated, basal states. This gives a measure of the activity of membrane phospholipase C in response to agonists.

(1) The content of phosphoinositides in the membrane

A higher content of, or a higher [^{3}H] or [^{32}P] incorporation into, phosphatidylinositol 4,5-bisphosphate in primary hypertension has been reported in various tissues including vascular smooth muscle cells (Durkin et al, 1990; Resink et al, 1987), platelets (Dimitrov et al, 1986), and erythrocytes (Marche et al, 1985; Boriskina et al, 1978). Similarly, a higher content of radiolabelled phosphatidylinositol 4,5-bisphosphate in secondary hypertension was observed in erythrocytes from Goldblatt renal hypertensive rats (Boriskina et al, 1978). Some groups found that the higher content of phosphatidylinositol 4,5-bisphosphate was associated with the developing stage of hypertension (Durkin et al, 1990; Riozzi et al, 1987; Kiselev et al, 1981). In contrast, a decreased (Remmal et al, 1988) or unchanged (Ek et al, 1989; Koutouzov et al,1987; Riozzi et al, 1987) [^{32}P]

incorporation into phosphatidylinositol 4,5-bisphosphate in primary hypertension has also been observed.

(2) Basal, non-stimulated rate of phosphoinositide hydrolysis (or phospholipase C activity)

A higher rate of basal phosphoinositide hydrolysis or phospholipase C activity in vascular smooth muscle cells (Uehara et al, 1988; Resink et al, 1987), renal cortex and medulla (Kawaguchi et al, 1987), and erythrocytes (Tremblay et al, 1990) has been observed in primary hyertension. In secondary hypertension, a higher rate of basal phosphoinositide hydrolysis was also found in aorta (Jones et al, 1988) and cardiac atria and ventricles (Eid & de Champlain, 1988) In contrast, an unchanged rate of basal phosphoinositide hydrolysis in both primary (Ek et al, 1989; Paquet et al, 1989; Jeffries et al, 1988; Koutouzov et al, 1988; Feldstein et al, 1986; Zhu et al, 1990) and secondary hypertension (Nixon et al, 1990; Takata et al, 1989) has been reported. Thus, diverse results have been reported for basal phosphoinositide hydrolysis in hypertension.

(3) The effect of agonist stimulation on phospholipase C activity

Increased phosphoinositide hydrolysis in response to agonist stimulation in primary hypertension has been observed in various tissues including vascular smooth muscle cells (Huzoor-Akbar et al, 1989; Paquet et al, 1989), platelets (Koutouzov et al, 1987; Marche et al, 1989), and fibroblasts (Zhu et al, 1990) In secondary hypertension, an enhanced phosphoinositide hydrolysis response has also been shown in vascular smooth muscle cells (Jones et al, 1988; Takata et al, 1989), cardiac atria and ventricles. (Eid & de Champlain, 1988) Of note here, both Huzoor-Akbar et al (1989) and Jones et al (1988) found an enhanced contractile response with increased phosphoinositide hydrolysis in aortic strips from spontaneously hypertensive rats and aldosterone-salt hypertensive rats, respectively. On the other hand, a decreased phosphoinositide hydrolysis response in primary hypertension has been reported in aorta (Heagerty et al, 1986), renal cortex (Jeffries et al, 1988), and

cultured neurons (Feldstein et al, 1986). No change in phosphoinositide hydrolysis responses was also observed in secondary hypertension in aorta (Nixon et al, 1990) and platelets (Limon et al, 1990).

Recently, in addition to the inositol phosphate pathway, the activity of protein kinase C has also been studied. It has been proposed that protein kinase C plays a significant role in long-term regulation of smooth muscle cell functions such as tonus maintenance and cellular proliferation (Rassmussen et al, 1987; Nishizuka, 1986). An enhanced protein kinase C activity in primary as well as secondary hypertension was observed in vascular smooth muscle cells (Turla & Webb, 1987; Murakawa et al, 1988; Turla et al, 1990; Turla & Webb, 1991), platelets (Takaori et al, 1986), and erythrocytes (Kravtsov et al, 1988).

Thus there are many reports showing that the phosphoinositide messenger system is perturbed in both primary and secondary hypertension, although the picture may differ depending on the model or stage of hypertension, tissue examined, and agonists employed.

In contrast, little is known about phosphoinositide metabolism in hypercholesterolemic or atherosclerotic states, although Winocour et al (1990) reported that the phosphoinositide hydrolysis response was significantly greater in diet-induced as well as genetically-determined hypercholesterolemic rats.

1.3.3. Changes in the cAMP system in hypertension, hypercholesterolemia, and atherosclerosis

In cardiovascular tissues such as heart and large as well as resistance vessels, there are many reports showing that the responsiveness of the cAMP system is decreased in the established phase of primary and secondary hypertension (Feldman, 1987; Hamet & Tremblay, 1990). This decreased responsiveness can be manifested in any of the following ways:

(1) An exaggerated increase in tissue and extracellular cAMP levels at the

developing stage of hypertension, followed by a progressive diminution in the established phase of hypertension (Hamet & Tremblay, 1990).

(2) Enhanced adenyl cyclase activity prior to the development of hypertension, followed by a decrease in activity in the hypertensive state. It has been suggested that this stage-dependent decrease in adenyl cyclase activity may involve G-protein interaction with the catalytic subunit of adenyl cyclase (Hamet & Tremblay, 1990; Bhalla & Sharma, 1982; Sharma et al, 1982).

(3) An decrease in the activity of cAMP-dependent protein kinase (Hamet & Tremblay, 1990; Coquil & Hamet, 1980).

(4) An increased activity of calmodulin, which exerts multiple effects including changes in the activity of adenyl cyclase, cAMP phosphodiesterase, the cation ion transport system, and cellular proliferation (Hamet & Tremblay, 1990).

As reviewed earlier, the cAMP system is involved not only in vasodilation and cardiac inotropism and chronotropism, but also in the regulation of the cellular proliferation process. Indeed, an increased cAMP level preceding and accompanying an increase in vascular DNA and protein content has been observed in spontaneously hypertensive rats (Chatelain et al, 1985) and coarctation hypertensive rats (Chatelain, 1983).

Perturbations of the cAMP system have also been noted in diet-induced atherosclerosis. In atherosclerotic rabbit aorta, an increased cAMP level was observed as compared to adjacent non-lesioned areas or the aorta from normal-diet rabbits (Augustyn & Ziegler, 1975). In contrast, in pigs on an atherogenic diet, a reduced cAMP level and adenyl cyclase activity together with an increased content of protein and cholesterol was seen in atherosclerotic lesions as compared to non-lesioned areas. Moreover, the cAMP level was negatively correlated with the content of protein and cholesterol (Lundholm et al, 1980).

1.3.4. Changes in the cGMP system in hypertension, hypercholesterolemia, and atherosclerosis

cGMP-induced relaxation in vascular smooth muscle cells can be effected by endothelium-derived relaxing factor (nitric oxide) and nitro-vasodilators (e.g., nitroprusside), via soluble guanylate cyclase activation, or by atrial natriuretic peptide via particulate guanylate cyclase activation (Hamet & Tremblay, 1990).

Most studies showed that in hypertension, there is an impaired endothelium-dependent relaxation, whereas the endothelium-independent relaxation to nitro-vasodilators is generally preserved; this suggests that the cGMP-soluble guanylate cyclase relaxation apparatus is not impaired (Vanhoutte, 1989; Marshall & Kontos, 1990). Possible mechanisms for this impaired endothelium-dependent relaxation include: decreased release or production of endothelium-derived relaxing factor from the endothelium, the destruction of endothelium-derived relaxing factor by oxidants after its release in the extracellular space, or the release of endothelium-derived contracting factors from the endothelium (Marshall & Kontos, 1990; Vanhoutte, 1989).

On the other hand, increased vasorelaxant responses to atrial natriuretic peptide, which mediates its effects via cGMP-particulate guanylate cyclase, have been observed in primary hypertension (Hamet & Tremblay, 1990).

In hypercholesterolemia and atherosclerosis, most papers also indicate an impaired endothelium-dependent relaxation, with the endothelium-independent relaxation generally unchanged (Marshall & Kontos, 1990); it has been shown that the cGMP-soluble guanylate cyclase relaxation apparatus in response to exogenous nitric oxide or nitroprusside is largely intact, suggesting the lesion is outside smooth muscle cells (Guerra et al, 1989; Marshall & Kontos, 1990).

1.4. A comparison of hypertension and hypercholesterolemia in the development of atherosclerosis

In the previous discussion, it has been shown that both hypertension and

hypercholesterolemia can cause (1) an increase in endothelial permeability, (2) the impairment of endothelium-dependent vaso-relaxation, (3) an alteration in vascular responses to agonists, (4) changes in the membrane phosphoinositide or cAMP messenger systems. There are numerous additional lesions common to the two disease states.

Hypertension and hypercholesterolemia have been reported to be associated with more "membrane lesions", such as changes in cell membrane fluidity (Naftilan et al, 1986; Lurie et al, 1985), membrane lipid composition (Naftilan et al, 1986; Lurie et al, 1985), Ca^{2+} flux rates through cell membranes (Jones et al, 1973; Strickberger et al, 1988), and α-adrenergic receptor number (Michel et al, 1990; Nanda & Henry, 1982).

Secondly, an increased content of calcium in the arterial walls has been observed in hypertensive as well as hypercholesterolemic humans and animals (Fleckenstein et al, 1987; Phair, 1988). It has been speculated that this calcium overload in the arterial tissue might have deleterious effects on the physiological functions of smooth muscle cells and aggravate the atherosclerotic processes.

Furthermore, lesions associated with one pathology may exacerbate abnormalities associated with the other disease state. Increased levels of low-density lipoproteins are associated with hypercholesterolemia. These low-density lipoproteins may act adversely at sites where hypertension-related defects have been reported. Such actions include:

(A) Injury to cultured endothelial cells (Henriksen et al, 1979).

(B) Activation of platelets with an increase in inositol 1,4,5-trisphosphate, diacylglycerol, and $[Ca^{2+}]_i$ (Knorr et al, 1988; Block et al, 1988). Activation of arterial smooth muscle cells, endothelial cells, lymphocytes, and fibroblasts via the phosphoinositide hydrolysis pathway has also been observed (Block et al, 1988).

(C) Inhibition of endothelium-dependent relaxation in aorta, possibly by reducing the production or release of endothelium-derived relaxing factor (Andrews et al,

1987; Takahashi et al, 1990).

(D) Increases in $[Ca^{2+}]_i$, cellular alkalization, DNA synthesis, expression of proto-oncogenes, phosphoinositide metabolism, and cell proliferation in cultured vascular smooth muscle cells (Scott-Burden et al, 1989b; Sachinidis et al, 1990; Libby et al, 1985).

In epidemiological terms, the presence of hypertension and hypercholesterolemia is additive in increasing the relative risk of cardiovascular events (Kannel & Sytkowski, 1987). In animal studies, the presence of hypertension and hypercholesterolemia can aggravate each other in inflicting atherosclerotic lesions in arteries (Hollander et al, 1976; McGill et al, 1985.) Nevertheless, there are some differences in the picture of atherosclerotic lesions induced by hypertension and hypercholesterolemia, although proliferation of smooth muscle cells is one of the key common features. Firstly, the proliferative response in hypercholesterolemia is mainly confined to the intima, whereas the proliferative response in hypertension is in the media. Secondly, the intimal lesions in hypercholesterolemia are seen in large vessels, but not the small vessels, while the medial lesions in hypertension are seen in large as well as small vessels (Mulvany & Simonsen, 1989). Thirdly, in animal studies, lipid deposition seldom occurs in vascular walls in hypertensive animals without the presence of hypercholesterolemia (Chobanian, 1990). Finally, the vascular lumen in hypercholesterolemic animals tends not to be diminished despite the proliferative processes, while the structural remodelling process in chronic hypertension reduces the lumen diameter in hypertensive animals (Heistad et al, 1991).

1.5. Summary

1. Hypertension and hypercholesterolemia have been identified as major and independent risk factors for atherosclerotic diseases. In many subjects, more than

one risk factor are often present, and the relative risk is cumulative. In clinical trials of treating mild to moderate hypertension, results suggest anti-hypertensive drugs can reduce the incidence of stroke and heart failure, but the outcome in terms of reducing coronary events is dubiously modest. On the other hand, major prospective studies of lowering plasma cholesterol in hypercholesterolemic patients showed impressive reductions in cardiovascular events, including coronary incidents.

2. In a wide range of animal species, experimental hypertension and hypercholesterolemia can induce a host of atherosclerotic changes in the arterial intima and media. These include endothelial shape changes, enhanced endothelial permeability, impaired endothelium-dependent vascular relaxation, platelet and monocyte adherence, macrophage and lymphocyte accumulation, smooth muscle cell migration into the intima, smooth muscle cell proliferation, extracellular calcium and connective tissue accumulation, and intimal thickening. Hypercholesterolemia can also induce lipid accumulation and foam cell formation. In addition, changesd in vascular responses to vasoconstrictors as well as vasodilators are observed in both conditions.

3. Changes in $[Ca^{2+}]_i$ are crucial signals for the activity of many cell types, including vascular smooth muscle cells, platelets, and neurons. There are three major second messenger systems involved in the regulation of $[Ca^{2+}]_i$ in vascular smooth muscle cells and platelets: the phosphoinositide hydrolysis system, the cAMP system, and the cGMP system. Stimulation of the phosphoinositide hydrolysis system can increase $[Ca^{2+}]_i$ in vascular smooth muscle cells and bring about muscle contraction, whereas activation of either the cAMP or the cGMP can decrease $[Ca^{2+}]_i$ and bring about muscle relaxation. There are many reports showing that all three messenger systems can be perturbed by hypertension as well as hypercholesterolemia. Moreover, changes in cell membrane characteristics such as fluidity, lipid composition, Ca^{2+} flux rates, and receptor number, can be observed in hypertension and hypercholesterolemia.

4. Proliferation of vascular smooth muscle cells is the hallmark of the atherosclerotic lesions induced by hypertension and hypercholesterolemia. Early changes in cultured cells in response to growth factor stimulation include increased phospholipase C activity and phosphoinositide hydrolysis, an increase in $[Ca^{2+}]_i$, cellular alkalization, activated membrane Na^+/H^+ antiport, and enhanced tyrosine-specific protein kinase activity. These changes suggest that the phosphoinositide messenger system is involved in the regulation of cell proliferation processes. On the other hand, correlation of adenyl cyclase activity and DNA synthesis in cultured vascular smooth muscle cells also implicates the cAMP system in the regulation of cell proliferation.

5. The vascular endothelium has a crucial role in modulating local vascular tone and blood flow, in activating or degrading vasoactive hormones, in secreting endothelium-derived relaxing as well as contracting factors, and in maintaining a patent and thromboresistant circuit. In pathological states such as hypertension and hypercholesterolemia, it contributes to the atherogenetic processes by increasing paracrine growth-factors or decreasing growth-inhibitors for vascular smooth muscle cells.

Thus there is much evidence for increases in cardiovascular risk as well as for many potential sites of interactions when hypertension and hypercholesterolemia occur together. However, the relative importance of such interactions remains to be elucidated.

In this thesis, aspects of hypertension and hypercholesterolemia, both alone and in combination, will be investigated with the aim of identifying key areas of interaction.

CHAPTER 2

PRELIMINARY STUDY ON THE PHARMACOLOGY OF ENDOTHELIN-1

Chapter 2. Preliminary Study on the Pharmacology of Endothelin-1

2.1. Introduction

2.1.1. Endothelium-derived relaxing and contracting factors

Furchgott & Zawadzki reported in 1980 that the vasorelaxation response of isolated rabbit aorta to acetylcholine in organ baths depended on the presence of vascular endothelia cells, and this response was not mediated by prostacyclin or related substances (Furchgott & Zawadzki, 1980). Later on, the phenomenon of endothelium-dependent vascular relaxation has been found to be present ubiquitously in various vascular beds among several species including man. Both arteries and veins possess this capability (Vanhoutte, 1989). Reports from several sources strongly suggest that this "endothelium-derived relaxing factor" is nitric oxide, and its precursor is L-arginine. There may be some basal secretion of endothelium-derived relaxing factor in vascular beds, and this secretion can be stimulated by shear stress, hormones, neurotransmitter, or platelet products. Endothelium-derived relaxing factor is short-lived, with a half-life varying from 6 seconds to 2 minutes, depending on the assay conditions. It stimulates soluble guanylate cyclase and increases cGMP levels in vascular smooth muscle cells, thereby bringing about vascular relaxation (Furchgott & Vanhoutte, 1989; Marshall & Kontos, 1990). There is growing evidence that the endothelium-dependent vascular relaxation is impaired in humans and animals with disease states such as hypertension, hypercholesterolemia, and atherosclerosis (Marshall & Kontos, 1990; Cooke, 1990).

In addition, "vasoconstriction" dependent on, or enhanced by, intact endothelium has been observed in several vascular beds in response to various chemical and physical stimuli such as noradrenaline, thrombin, hypoxia, increased transmural pressure, and mechanical stretch (Yanagisawa et al, 1988). Several groups have

identified a peptide substance from cultured endothelial cells which causes vasoconstriction (Hickey et al, 1985; Gillespie et al, 1986; O'Brien et al, 1987). In 1988, Yanagisawa et al succeeded in purifying, determining the sequence, and also synthesising the peptide endothelin (Yanagisawa et al, 1988). This subsequently led to an explosive outburst of research throughout the world, investigating the pharmacological effects of endothelin in various tissues and its mechanisms of action.

So far, three types of endothelium-derived contracting factor have been identified, and endothelin is one of them (Anggard et al, 1990; Vanhoutte et al, 1991).

(1) Hypoxia- or anoxia-induced endothelium-derived contracting factor found in peripheral, coronary, and cerebral arteries. Its action is very rapid and readily reversible; it may be a superoxide anion or a closely related free radical (Vanhoutte & Katusic, 1988).

(2) Endothelium-derived contracting factor induced by stretch, increased transmural pressure, high potassium, calcium ionophore A23187 and arachidonic acid. It has been reported that this type of contracting factor is sensitive to inhibitors of cyclooxgenase such as indomethacin and meclofenamate, and could be a prostaglandin or related factor (Anggard et al, 1990; Vanhoutte & Katusic, 1988).

(3) Endothelium-derived contracting factor generated by cultured endothelial cells, namely endothelin (Yanagisawa et al, 1988). More detailed discussion on endothelin will be presented in the following sections.

2.1.2. Endothelins: subtypes, tissue autoradiography, receptors, and regulation

Using gene probing techniques, Inoue et al (1989) revealed that there are three distinct endothelin-related genes in human, rat, and porcine genomes. Each of the genes predicts a 21-residue peptide, similar to but distinct from each other, named

endothelin-1 (the original porcine and human endothelin), endothelin-2 (with two amino acid substitutions from endothelin-1), and endothelin-3 (with six amino acid substitutions), respectively. (Figure 2.1) It appears that vascular endothelial cells do not produce endothelin-2 or endothelin-3, and only endothelin-1 can be detected in endothelial cells or in the culture supernatant either at the peptide level or at the mRNA level (Yanagisawa & Masaki, 1989a). Further discussion of the characteristics of endothelin will be confined to endothelin-1.

Endothelin-1, an acidic 21-amino-acid peptide with two intrachain disulfide bridges, has a molecular weight of 2492. Structure-activity studies indicate that both the disulfide bonds, and the carboxyl-terminal residue L-tryptophan, are crucial to its potent vasoconstrictor activity (Yanagisawa & Masaki, 1989a).

Tissue autoradiography studies in humans, pigs, monkeys, guinea pigs and rats have shown that endothelin-1 binding sites are widely distributed, not only in arteries, veins, and heart (cardiac nerves, atria, ventricles, and coronary arteries, in decreasing order), but also in lung, kidney (cortical glomeruli and medulla, less in papilla), brain (especially the cerebellum), spinal cord, adrenal gland, liver, spleen, and the gastrointestinal tract (Lerman et al, 1990). MacCumber et al (1989) found that synthesis of mRNA occurred in close proximity to the binding sites of endothelin in many tissues, suggesting the role of a local hormone.

Results from radioligand binding studies suggested that there are at least two types of endothelin receptors: one is "endothelin-1-selective", and the other is "non-isopeptide-selective" (Takayanagi et al, 1991). This suggestion was further strengthened by the findings that two distinct types of endothelin receptors could be obtained using cDNA cloning and expression techniques (Arai et al, 1990; Sakurai et al, 1990). Both receptors contain seven membrane spanning domains, with similar

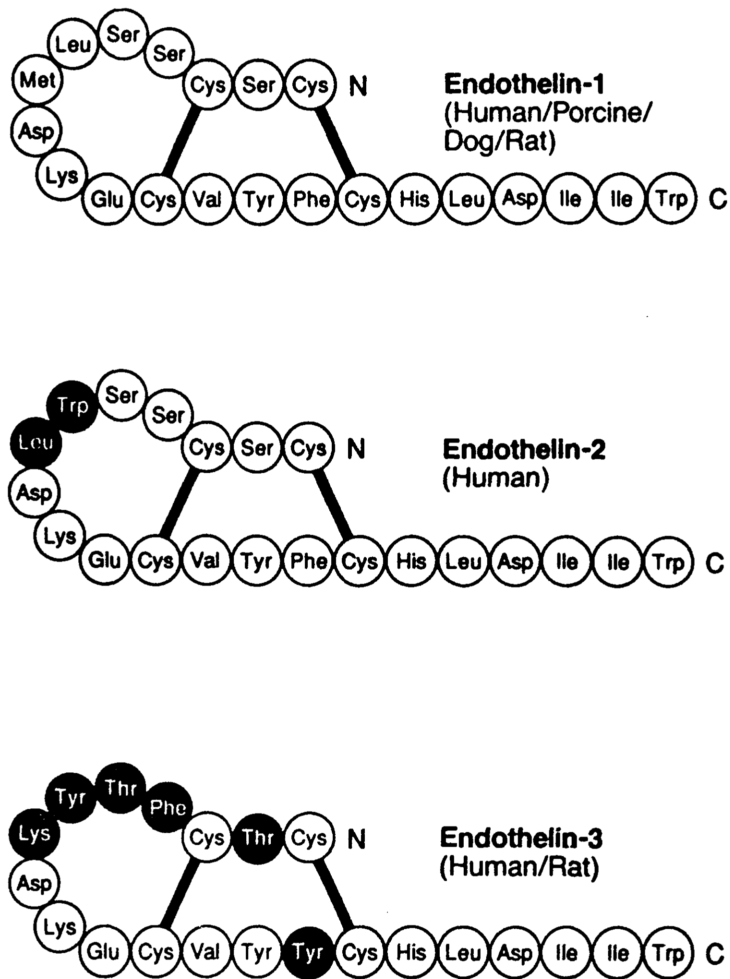


Figure 2.1: Amino acid sequences of the endothelin family.

(Filled circles: amino acid residues different from those in endothelin-1.)

(Source of figure: Yanagisawa M & Masaki T. Molecular biology and biochemistry of the endothelins. Trends Pharmacol Sci 1989; 10: 374-378.)

sequence and topology to other G-protein-coupled receptors. Northern blot analysis showed that mRNA for both receptors could be detected in brain, heart, lung, and kidney, but the "non-isopeptide-selective" receptor was not expressed in vascular smooth muscle cells (Sakurai et al, 1990; Arai et al, 1990).

It has been suggested that the production of endothelin seemed to be regulated at the level of messenger RNA transcription, since it was unlikely that endothelin was accumulated in endothelial granules and released in response to stimuli (Yanagisawa et al, 1988). Indeed, increases in messenger RNA for preproendothelin were observed within 1 hour of stimulation with agonists such as thrombin, adrenaline, or the calcium ionophore A23187. Increased secretion of endothelin-1 into the supernatant of incubating media for vessel strips or cultured endothelial cells has been reported when the media were stimulated with thrombin, angiotensin II, arginine-vasopressin, calcium ionophore A23187, glucose, transforming growth factor-β1, shear stress, or hypoxia (Emori et al, 1989; Boulanger & Luscher, 1990; Yamauchi et al, 1990; Ohlstein et al, 1990; Yoshizumi et al, 1989; Rakugi et al, 1990).

Yanagisawa et al (1988) proposed a possible route for endothelin biosynthesis. As shown in Figure 2.2, a 203-amino-acid peptide precursor translated from RNA, called preproendothelin, is cleaved by endopeptidase to a 39-amino-acid intermediate, called "big endothelin". Big endothelin is hydrolysed at Trp21-Val22 by some "endothelin-converting-enzyme" to the 21-amino-acid peptide, "endothelin". Some research groups have speculated that the physiologically relevant "endothelin-converting-enzyme" could be a metal-dependent protease, sensitive to phosphoramidon inhibition (Sawamura et al, 1991; McMahon et al, 1991). Taken together, the presence of mRNA encoding the preproendothelin in the endothelial cells indicates that endothelin is produced by de novo synthesis and is processed in a manner similar to that of many peptide hormones and neuropeptides.

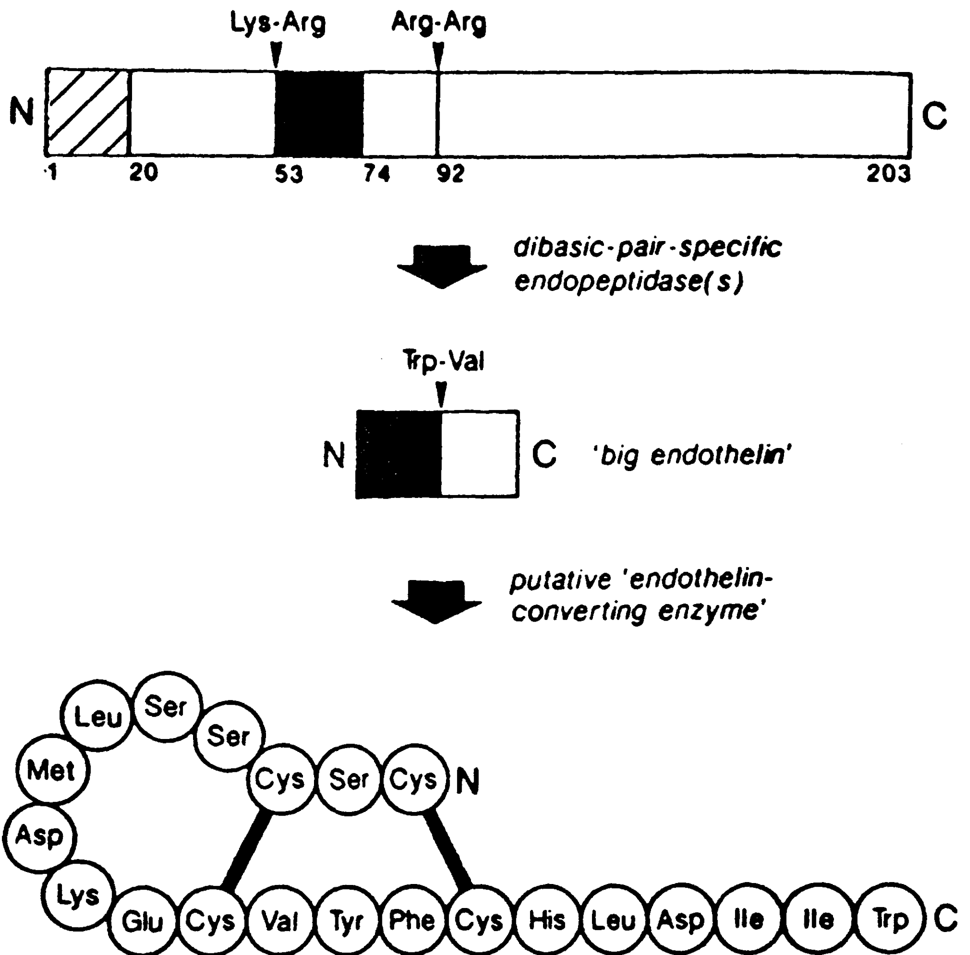


Figure 2.2: Possible pathway of endothelin-1 biosynthesis.

The putative secretory signal sequence and the endothelin sequence are shown by the hatched and the filled boxes, respectively. The rest of proendothelin sequence is indicated by an open box. A 39-amino-acid residue intermediate, so-called "big endothelin", is considered to be generated from proendothelin by the proteolytic cleavages at paired basic residues. Mature endothelin is then produced possibly through by a putative "endothelin converting enzyme".

(Source of figure: Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988; 332: 411-415.)

2.1.3. Cardiovascular effects of endothelin-1

The best defined characteristic of endothelin is its "highly-potent" (EC_{50} ranging between 2×10^{-10} and 5×10^{-9} M) and long-lasting (longer than 30 minutes) vasoconstrictor effect. Endothelin-1 is the most potent vasoconstrictor known so far (Yanagisawa et al, 1988; Yanagisawa & Masaki, 1989b). The vasoconstrictor activity is resistant to antagonists of α-adrenergic, histaminergic, serotonergic, and muscarinic receptors, as well as inhibitors of cyclooxygenase and lipooxygenase, but can be reversed by the addition of isoproterenol, forskolin, or glyceryl trinitrate (Yanagisaswa et al, 1988). Some groups have observed that endothelin-1 was more potent in producing contractile activity on veins than on arteries (Cocks et al, 1989; Miller et al, 1989). The presence of the endothelium can modulate the vasoconstrictor effect of endothelin-1, and removal of the endothelium caused a reduction in EC_{50} values or an increase in E_{max} (Toupouzis et al, 1991; Eglen et al, 1989; Kauser et al, 1990). In isolated rat aorta (Sakata et al, 1989), perfused mesenteries (de Nucci et al, 1988; Kitazumi et al, 1990), guinea pig aortic rings and perfused coronary resistance vessels (Folta et al, 1989), endothelin-1 has been reported to cause vascular relaxation in the pre-contracted vessels, which could be abolished by endothelium removal, methylene blue or oxyhaemoglobin treatment, suggesting that endothelin probably caused this relaxation via the release of endothelium-derived relaxing factor. On the other hand, the release of prostacyclin stimulated by endothelin-1 in the rat perfused mesentery arteries (Rakugi et al, 1989) and lungs (de Nucci et al, 1988), and the enhanced pressor response to endothelin-1 by indomethacin pre-treatment (de Nucci et al, 1988) suggested that prostacyclin was also a potential modulator of endothelin-induced vasoconstriction.

Intravenous injection of endothelin-1 (0.1 to 3.0 nmol/kg) into rats or other animals generally caused two phases of blood pressure changes: an initial, short-lived depressor response (lasting 0.5 to 2 minutes), followed by a potent, long-

lasting pressor response (lasting more than 30 minutes) (Yanagisawa & Masaki, 1989b). The pressor response was dose-related, and was mainly due to an increase in systemic resistance, with decreased cardiac output (Miller et al, 1989; Goetz et al, 1988; Yang et al, 1991; King et al, 1990). The depressor response was associated vasodilation and a decrease in systemic resistance, while cardiac output was increased or unchanged (Yang et al, 1991; King et al, 1990; Rohmeiss et al, 1990; Hoffman et al, 1989). Of note here is that differences exist with regard to the vascular responses to endothelin-1 among different vascular beds, as well as among different species. The percentage increase in vascular resistance was greater in renal or mesenteric arteries than in hindquarter vessels (Gardiner et al, 1990; Pernow et al, 1988; Hoffman et al, 1989), while Tippins et al (1989) observed that within the coronary circulation, resistance vessels were more sensitive to endothelin-1 than conductive arteries. An example of species differences is the effect of calcium antagonists on endothelin-1-induced vasoconstriction in porcine coronary arteries compared to canine coronary arteries (Masaki et al, 1990).

Recently, it has been reported that administration of low-dose endothelin-1 could induce a blood pressure reduction, with a reduction of total peripheral resistance and no secondary increase in blood pressure, in contrast to the better known depressor-pressor biphase response after higher doses of endothelin (Nakamoto et al, 1991; Minkes et al, 1989; Lippton et al, 1991). Indeed, renal vasodilation without subsequent constriction, induced after low doses of endothelin-1, has also been observed (Harris et al, 1991).

2.1.4. Mechanisms of action of endothelin-1 on the vasculature (Figure 2.3)

It was initially suggested that endothelin-1 acted as an endogeneous agonist of the dihydropyridine-sensitive Ca^{2+} channels, as its vasoconstrictor action depended on the presence of extracellular Ca^{2+} and could be inhibited by low doses of nicardipine (Yanagisawa et al, 1988). However, in cultured vascular smooth muscle

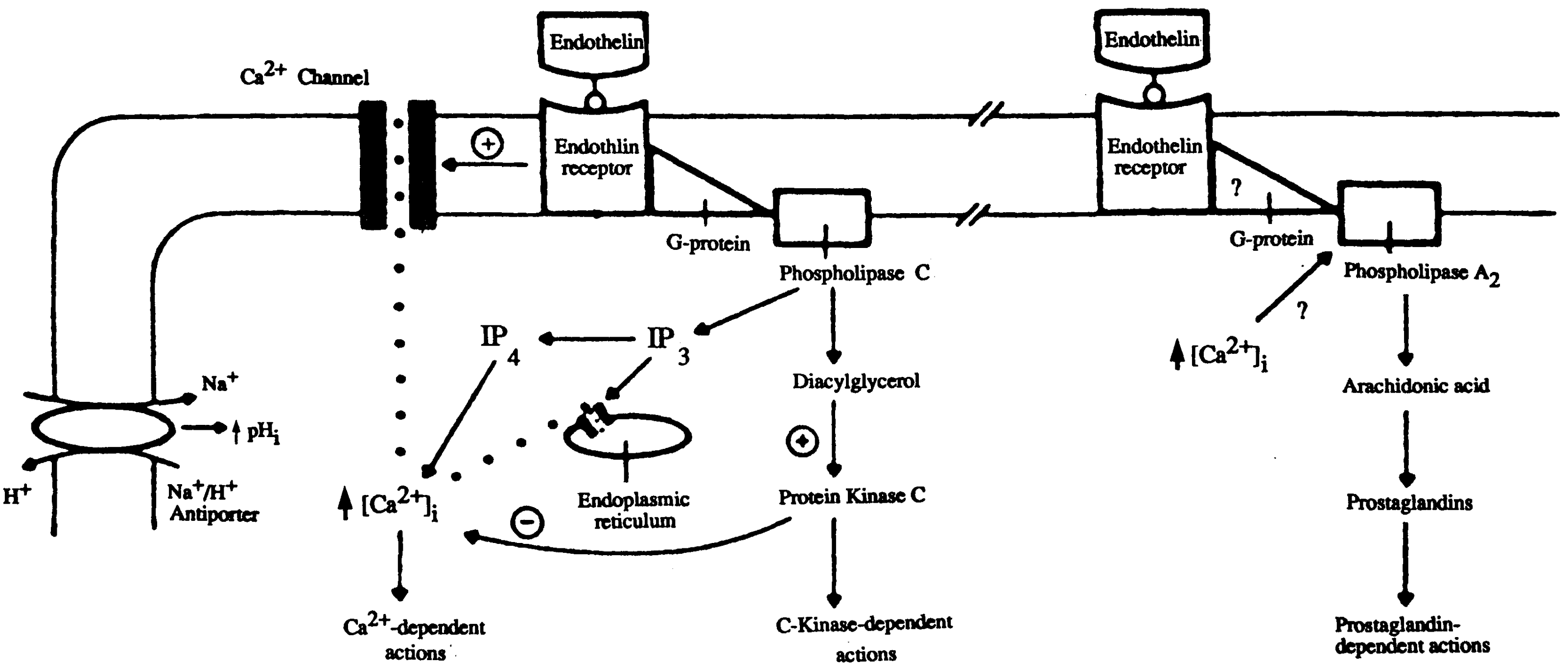


Figure 2.3: Proposed mechanisms of action of endothelin-1 on vascular smooth muscle cells. (See the text for detailed description.)

(Source of Figure: Simonson MS & Dunn MJ. Cellular signaling by peptides of the endothelin family. FASEB J 1990; 4: 2989-3000.)

cells, binding studies showed that endothelin-1 was bound to a specific, single-population binding site and could not be displaced by calcium antagonists such as nicardipine or diltiazem (Clozel et al, 1989). Studies on cardiac membranes also showed that the endothelin-1 receptor is different from dihydropyridine-sensitive channels, and the latter are not the primary sites of action for endothelin-1 (Miyazaki et al, 1990; Gu et al, 1989). Furthermore, there are many reports indicating that endothelin-1 stimulates the production of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol by way of phosphatidylinositol 4,5-bisphosphate hydrolysis. Inositol 1,4,5-trisphosphate causes intracellular Ca^{2+} release from endoplasmic reticulum, which initiates vascular smooth muscle cell contraction (Pang et al, 1989; Ohlstein et al, 1989; Wallnofer et al, 1989). After endothelin administration, both the elevation of intracellular Ca^{2+} concentration and the increase of inositol phosphates levels are sustained for more than 15 minutes, which may in part explain the sustained nature of its vasoconstrictor effects (Pang et al, 1989; Ohlstein et al, 1989).

The other product of phosphatidylinositol 4,5-bisphosphate hydrolysis, 1,2-diacylglycerol, may also contribute to the effects of endothelin-1 on vascular smooth muscle cells, such as tonic contraction, and increased influx of extracellular Ca^{2+},by activating membrane protein kinase C (Haller et al, 1990; Takuwa et al, 1990; Griendling et al, 1989). Recently, researchers have suggested that endothelin-1, though not binding to dihydropyridine-sensitive Ca^{2+} channels, may indirectly activate membrane calcium channels and facilitate the influx of extracellular Ca^{2+}, which may play some role in the tonic contraction (Van Renterghem et al, 1988; Inoue et al, 1990; Takuwa et al, 1990; Marsault et al, 1990). This indirect activation of membrane cationic channels may also explain the observation that the tonic vascular contraction induced by endothelin-1 is partly dependent on the presence of extracellular calcium (Yang et al, 1990; Takuwa et al, 1990; Marsault et al, 1990). However, the extent to which dihydropyridine-sensitive calcium channel antagonists may attenuate the vasoconstrictor effects of endothelin is open to controversy, and

may vary according to species, vascular beds, or experimental conditions (Masaki et al, 1990).

Recently, there is some evidence to suggest that a guanine nucleotide-binding regulatory protein (G-protein) is involved in the signal transduction of endothelin-stimulated phosphoinositide hydrolysis, with formation of a membrane complex of receptor--G-protein--phospholipase-C, reminiscent of α-adrenergic and other receptor-mediated transmembrane signalling pathways (Takuwa et al, 1990; Kelly et al, 1990; Galron et al, 1990; Arai et al, 1990). In addition, it has been shown that, apart from activating the phospholipase C-mediated phosphoinositide pathway, endothelin also stimulates phospholipase A_2 (Resink et al, 1989a; Reynolds et al, 1989) and phospholipase D (MacNulty et al, 1990), with the consequent release of arachidonic acid and choline, respectively, thus enlarging still further the list of potential secondary messengers involved in endothelin cellular action.

2.1.5. Other effects of endothelin-1

(A) Cardiac effects

In vitro studies showed that endothelin-1 at low doses was both an inotropic and chronotropic agent, and more distinctively, a potent coronary vasoconstrictor. At high doses, severe coronary vasoconstriction superseded by myocardial ischemia and a marked decline in cardiac performance was observed (Firth et al, 1990; Ishikawa et al, 1988; Ezra et al, 1989).

(B) Renal effects

At low doses of endothelin infusion, endothelin-1 was reported to cause renal vasodilation with concomitant diuresis and natriuresis, and no change in glomerular filtration rate (Harris et al, 1991). At high doses, endothelin-1 caused potent vasoconstriction in afferent as well as efferent arterioles, with decreases in glomerular filtration rate, renal plasma flow, urinary sodium excretion, and urine

volume (Firth et al, 1988; Miller et al, 1989; Tsuchiya et al, 1990).

(C) Pulmonary effects

Endothelin-1 has been shown to be a potent constrictor for both pulmonary vessels and tracheal-bronchial smooth muscle (Cardell et al, 1990; Touvay et al, 1990). Endothelin-1 also stimulates the angiotensin converting enzyme activity in pulmonary artery endothelial cells, which might have some physiological implications (Kawaguchi et al, 1991).

(D) Endocrine interactions

Endothelin-1 has been shown to stimulate the release of atrial natriuretic peptides in vitro and in vivo (Stasch et al, 1989; Garcia et al, 1990). In vitro studies indicate that endothelin-1 inhibits renin release (Rakugi et al, 1988), stimulates aldosterone biosynthesis (Cozza et al, 1989), and stimulates the release of noradrenaline and adrenaline release from adrenal chromaffin cells (Boarder et al, 1989).

(E) Neural effects

It has been suggested that endothelin-1 exerts effects at neuroeffector junctions in arteries, as it can potentiate the nerve-stimulated or noradrenaline-stimulated pressor responses (Tabuchi et al, 1990; Wong-Dusting et al, 1990). Nishimura et al (1991) also reported that endothelin-1 caused depolarization, activated an inward current, and facilitated a long-lasting voltage-dependent calcium current in ganglion neurons. Intra-cisternal injection of endothelin-1 has been shown to cause severe, long-lasting cerebral vasospasm (Asano et al, 1989) and affect central neural control of the circulation and respiration in animals (Kuwaki et al, 1991).

(F) Mitogenic effects

Endothelin-1 has been reported to stimulate mitogenesis, with proto-oncogene expression, DNA synthesis, and cell proliferation, in vascular smooth muscle cells (Komuro et al, 1988), fibroblasts (Takuwa et al, 1989), mesangial cells (Simonson et al, 1989), and neural glial cells (MacCumber et al, 1990).

2.1.6. Endothelin-1 in disease states

In view of its long-lasting vasoconstrictor effects on the vasculature, such as cerebral, coronary, and renal arteries, as well as the existence of mRNA and autoradiographic binding sites in many tissues throughout the body (MacCumber et al, 1989), people have speculated on the potential roles of endothelin-1 in pathophysiological states such as hypertension, cerebral vasospasm, Raynaud's phenomenon, and uremia (Lerman et al, 1990; Cooke & Dzau, 1989). These ideas were further strengthened by the detection of raised levels of endothelin-1 in the circulation of patients with cardiovascular diseases (Lerman et al, 1990) and reports of hypertensive patients with "endothelin-secreting" tumours (Yokokawa et al, 1991). In addition, the mitogenic effect of endothelin-1 (see Section I.5. for review) on vascular smooth muscle cells could indicate a possible role in hypertension and atherosclerosis. In the following, reports on endothelin-1 with respect to various disease states are reviewed and discussed.

(A) Endothelin-1 and hypertension

An increase in plasma endothelin-1 levels in essential hypertensive patients as compared to normotensive controls has been observed by some groups (Kohno et al, 1990; Shichiri et al, 1990), while others could not confirm this finding. (Davenport et al, 1990; Schrader et al, 1990) Suzuki et al (1990) reported that plasma endothelin-1 levels in genetically hypertensive rats were lower than those in Wistar-Kyoto rats. However, no change was observed in plasma endothelin-1 levels in humans and rats with secondary hypertension, as compared to their normotensive controls (Schrader et al, 1990; Suzuki et al, 1990).

Binding studies in cultured vascular smooth muscle cells showed that the binding affinity for endothelin-1 was similar between spontaneously hypertensive and Wistar-Kyoto rats, while a decreased or unchanged maximal binding capacity (B_{max}) in spontaneously hypertensive rats was observed (Clozel, 1989; Resink et al,

1990). In contrast, an increased binding affinity of endothelin-1 in kidneys from spontaneously hypertensive rats has been reported (Jeng et al, 1991).

Reports on the vasoconstrictor activity of endothelin-1 in hypertensive animals are inconsistent, partly depending on the model or stage of hypertension, experimental conditions (in vivo or in vitro; conscious or anaethetised) as well as the specific vascular beds examined.

In 12-16 week spontaneously hypertensive rats, the blood pressure response to intravenous endothelin-1 has been reported to be increased (Martel et al, 1991), similar (Eglen et al, 1989; Hirata et al, 1989), or decreased (Winquist et al, 1989; Watanabe et al, 1989), as compared to Wistar-Kyoto rats.

An enhanced pressor response in the perfused mesenteric arteries from spontaneously hypertensive rats has been reported by some groups (Criscione et al, 1990; MacLean & McGrath, 1990), while others (Tabuchi et al, 1990) observed no difference in responses. Similar pressor responses in the perfused tail arteries of spontaneously hypertensive and Wistar-Kyoto rats have also been reported (MacLean & McGrath, 1990).

An increased vasoconstrictor activity of endothelin-1, in terms of sensitivity or maximal tension development, has been observed in isolated aorta (Martel et al, 1991; Clozel, 1989), renal artery (Tomobe et al, 1988), and mesenteric artery (Miyauchi et al, 1989), from 10-16-week spontaneously hypertensive rats as compared to their proper controls.

In animals with secondary hypertension, Yokokawa et al (1990) observed a significantly greater pressor response in both systemic and renal vascular beds in deoxycorticosterone-acetate-salt hypertensive rats as compared to normotensive control rats. de Carvalho et al (1990) reported that an enhanced vasoconstrictor response to endothelin-1 occurred in microvessels but not in macrovessels, from both Goldblatt renal hypertensive and deoxycorticosterone-acetate-salt hypertensive rats, as compared to normotensive controls.

(B) Endothelin-1 and atherosclerosis

An enhanced vasoconstrictor response to endothelin-1 in cerebral arteries from atherosclerotic animals has been observed (Lopez et al, 1990; Rossitch et al, 1991).

(C) Endothelin-1 and other disease states

Increased plasma endothelin concentrations have been observed in various disease states such as chronic renal failure (Schrader et al, 1990), acute myocardial infarction (Schrader et al, 1990; Miyauchi et al, 1989), acute renal failure (Shibouta et al, 1990), subarachnoid haemorrhage (Masaoka et al, 1989), cardiogenic shock (Cernacek & Stewart, 1989), pulmonary hypertension (Stewart et al, 1991), pre-eclampsia (Greer et al, 1991), Raynaud's phenomenon (Zamora et al, 1990), and sepsis (Pittet et al, 1991). Increased endothelin concentrations in cerebral spinal fluids in patients with subarachnoid haemorrhage (Suzuki et al, 1990), or in urine in patients with renal diseases (Ohta et al, 1991) have also been reported. Nevertheless, whether or how these increased endothelin concentrations are related to the vasospastic or hypertensive states remains to be elucidated.

Although some of the reports are controversial, taken together there is substantive evidence that alterations in the production of or responses to endothelin may contribute to the development of cardiovascular diseases or help to maintain and amplify disease once established. We therefore decided to include studies with endothelin in our protocol. However, most work with endothelin had been undertaken in the rat and even here much controversy existed as to its mechanism of action. It was therefore necessary to establish baseline parameters in normal rabbits before embarking on the main study with hypertensive and hypercholesterolemic animals. At the time the studies on phosphoinositide metabolism were undertaken, little had been published on endothelin's effects on inositol phosphates production in vascular tissues. We therefore undertook some pilot studies in the rat, both for financial reasons, and also because at that time the rat was the only species in which

the effects of endothelin had been studied in any detail.

2.2. Materials and methods

2.2.1. In vivo studies in the rabbit

(A) Blood pressure changes after endothelin injection in normal rabbits (n=6)

An arterial line was inserted for measurement of mean arterial pressure and heart rate in one ear under local anaesthesia (2% lignocaine), and a venous line for drug administration in the other. After catheter insertion, the rabbit was allowed to rest in an individual cage for 60 minutes before any measurement was made. Mean arterial pressure and heart rate were measured with a Statham P23 1D transducer and displayed using a Grass model 7B polygraph. Four doses of endothelin-1 (0.03, 0.05, 0.10, & 0.20 nmol/kg) were given to each rabbit on two consecutive days, with a maximum of three dosages given in one day. The next dosage was not given until mean arterial pressure returned to baseline level, generally at least 30 minutes after the previous dosage. Mean arterial pressure and heart rate were followed continuously for the first 10 minutes after endothelin injection, and every 5 minutes thereafter.

(B) The effects of nifedipine on the endothelin-1-induced blood pressure changes in normal rabbits (n=7)

Another set of rabbits received first one dose of endothelin (0.10 nmol/kg), followed by a bolus injection of nifedipine (0.10 mg/kg) or vehicle followed 10 minutes later by a bolus injection of endothelin (0.10 mg/kg). Nifedipine (0.10 mg/kg) was used because it has been shown in our Department to cause marked falls in blood pressure in hypertensive rabbits and its interaction with other pressor agents has previously been studied. (Hamilton et al, 1987)

2.2.2. Studies of endothelin-1's effects on phosphoinositide hydrolysis in rat aortic

segments and rabbit aortic rings

(A) Studies in rat aortic segments

Sprague-Dawley rats were killed by cervical dislocation. Descending aorta were isolated and cleared of adherent tissue and then washed in Krebs Ringer bicarbonate buffer (KRB) solution for 10 minutes X 3 times, at 37°C with continuous bubbling of 95% O_2/5% CO_2. (KRB composition: NaCl 118.3 mM, KCl 4.7 mM, $CaCl_2$ 0.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, glucose 11.1 mM, bovine serum albumin 1.5%; pH 7.4.) The tissue was incubated for a further 10 minutes in another KRB solution containing 10 mM LiCl and 2 μM imipramine. Afterwards the rat aorta was cut into 5 mm segments. One thoracic and one abdominal segment were put into each tube (except blank tubes) for incubation. The incubation medium was 0.5 ml KRB solution containing 10 mM LiCl (inhibiting the breakdown of inositol-1-monophosphate) and 2 μM imipramine (blocking the neuronal uptake of noradrenaline) and 0.5 μCi [^{3}H]-myoinositol. The tubes were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 4 hours unless otherwise stated. Different sets of experiments were conducted to investigate: (A) the dose-response relationships for phosphoinositide hydrolysis by endothelin (10^{-9} to 10^{-5} M), (B) the time course of phosphoinositide hydrolysis stimulated by endothelin-1 (10^{-6} & 10^{-5} M) and noradrenaline (10^{-4} M), (C) the effect of endothelium removal on the endothelin (10^{-6} M)- and noradrenaline (10^{-4} M)-stimulated phosphoinositide hydrolysis, (D) the effect of extracellular calcium in the KRB on the endothelin (10^{-6} M)- and noradrenaline (10^{-4} M)-stimulated phosphoinositide hydrolysis.

For dose response studies, endothelin-1 (10^{-9} to 10^{-5} M) was added 30 minutes before the end of incubation. For time course studies, endothelin-1 or noradrenaline was added 5, 15, 30, and 60 minutes before the end of incubation. For endothelium removal, a cotton swab was gently applied on the aortic surface. For calcium

dependence studies, KRB containing no Ca^{2+}, as well as 0.5 mM Ca^{2+}, were employed to compare the agonist-stimulated phosphoinositide hydrolysis in each medium. In addition, in some studies, EGTA (0.5 mM, final) was added 5 minutes before the addition of agonists to ensure complete removal of Ca^{2+} from the medium.

At the end of incubation, the radioactive [^{3}H]-inositol mono-, bis-, and tris-phosphates were collected and counted after a series of separation procedures (see the "Materials and Methods" Section in "Chapter 6. Phosphoinositide Hydrolysis in Aorta from Hypertensive and Hypercholesterolemic Rabbits" for a detailed description). Background radioactivity in the blank tubes was deducted from both the basal, non-stimulated and agonist-stimulated radioactivity. Agonist-stimulated [^{3}H]-inositol phosphates formation was calculated as a percentage of basal values.

(B) Studies in rabbit aortic rings

Dose-response relationships for endothelin-stimulated phosphoinositide hydrolysis in New Zealand white rabbits were studied as in the rat aorta.

Aortic tissues were obtained from male Sprague-Dawley rats (Charles River Laboratory, Cheshire, UK.), weighing 150 to 250 g, and from male New Zealand white rabbits (Cheshire Rabbit Farms, Cheshire, UK.), aged 3-4 months, weighing 2.0-2.5 kg.

Endothelin-1 was purchased from Scientific Marketing (Barnet, UK), and prepared as a 10^{-4} M stock solution, in 50 μl aliquots, and stored at -20°C. Dilutions were prepared from the stock aliquot immediately before use. Nifedipine was obtained from Sigma Chemical Co. (St Louis, USA) and dissolved in ethanol/arachis oil (v/v, 1:1) and protected from light at all times. [^{3}H]-myoinositol was purchased from Amersham International (Amersham, UK). Noradrenaline was prepared in 0.1% ascorbic acid. All other laboratory reagents unless specified were from Sigma Chemical Co. (Poole, UK)

All results were expressed as mean ± S.D. Repeated measures analysis of variance was used to compare the various measurements. $p < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Blood pressure changes after endothelin-1 injection in rabbits

Baseline mean arterial pressure and heart rate were 68.0 ± 3.1 mmHg and 227 ± 4 bpm, respectively. Endothelin-1 (0.03, 0.05, 0.10, 0.20 nmol/kg) caused an initial depressor response (lasting less than 1 minute) followed by a long-lasting pressor response (generally at least 20 minutes). The pressor responses reached their peak levels between the 1st and 3rd minute after the injection of endothelin. There was a significant dose-dependent increase in the pressor response, while the depressor response tended to be dose-related, but did not reached significance overall possibly due to an insufficient number of data points. The maximal increases in blood pressure were 8.0 ± 2.8 mmHg, 10.0 ± 4.7 mmHg, 15.0 ± 2.1 mmHg, and 18.7 ± 6.5 mmHg for the doses 0.03, 0.05, 0.10, and 0.20 nmol/kg endothelin,respectively. (Figure 2.4)

2.3.2. Effects of nifedipine on the endothelin-induced blood pressure changes

Baseline mean arterial pressure decreased from 81.6 ± 2.6 mmHg to 77.1 ± 1.4 mmHg, 10 minutes after the injection of nifedipine, but this failed to reach statistical significance. Nifedipine (0.1 mg/kg) significantly attenuated the pressor response to endothelin in the first minute ($p < 0.05$). From 2 to 5 minutes after endothelin, the nifedipine-induced attenuation was smaller and not statistically significant. From 5 to 30 minutes, the endothelin-induced pressor response tended to return to baseline more slowly in the nifedipine-pretreated animals, but the difference was not significant (Figure 2.5). The depressor response was not affected by nifedipine pre-

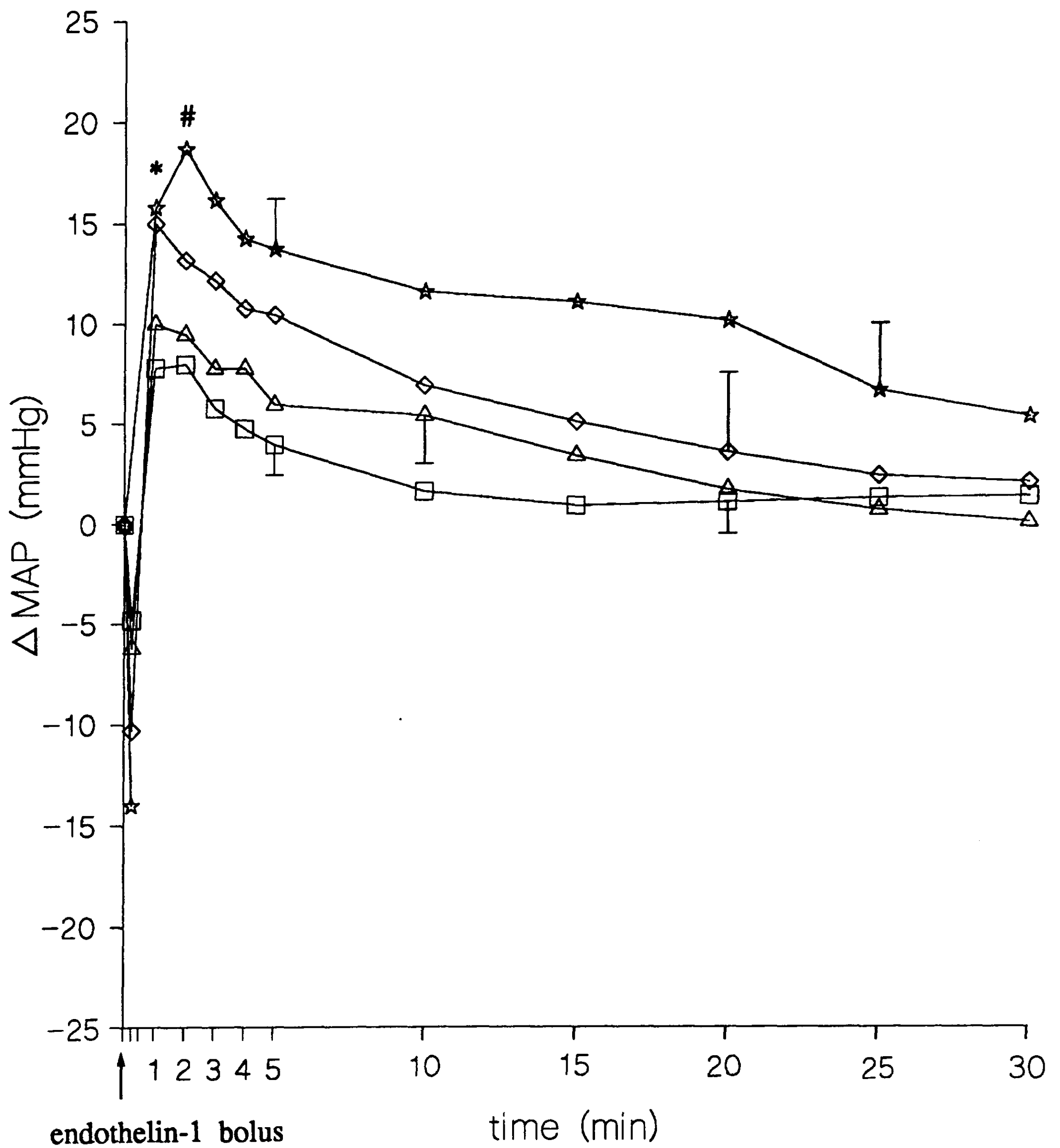


Figure 2.4: Changes in mean arterial pressure (Δ MAP) after endothelin-1 injection (0.03--0.20 nmol/kg, i.v.) in the rabbit.

Note the biphasic (depressor followed by pressor) responses. There was a significant dose-related pressor reponse to endothelin-1, and the maximum increases in blood pressure after 0.10 & 0.20 nmol/kg endothelin-1 were significantly greater than that after 0.03 nmol/kg (* & # : $p < 0.05$, respectively). (Mean ± S.D., n=6)

□—□ 0.03 nmol/kg endothelin-1.

△—△ 0.05 nmol/kg endothelin-1.

◇—◇ 0.10 nmol/kg endothelin-1.

☆—☆ 0.20 nmol/kg endothelin-1.

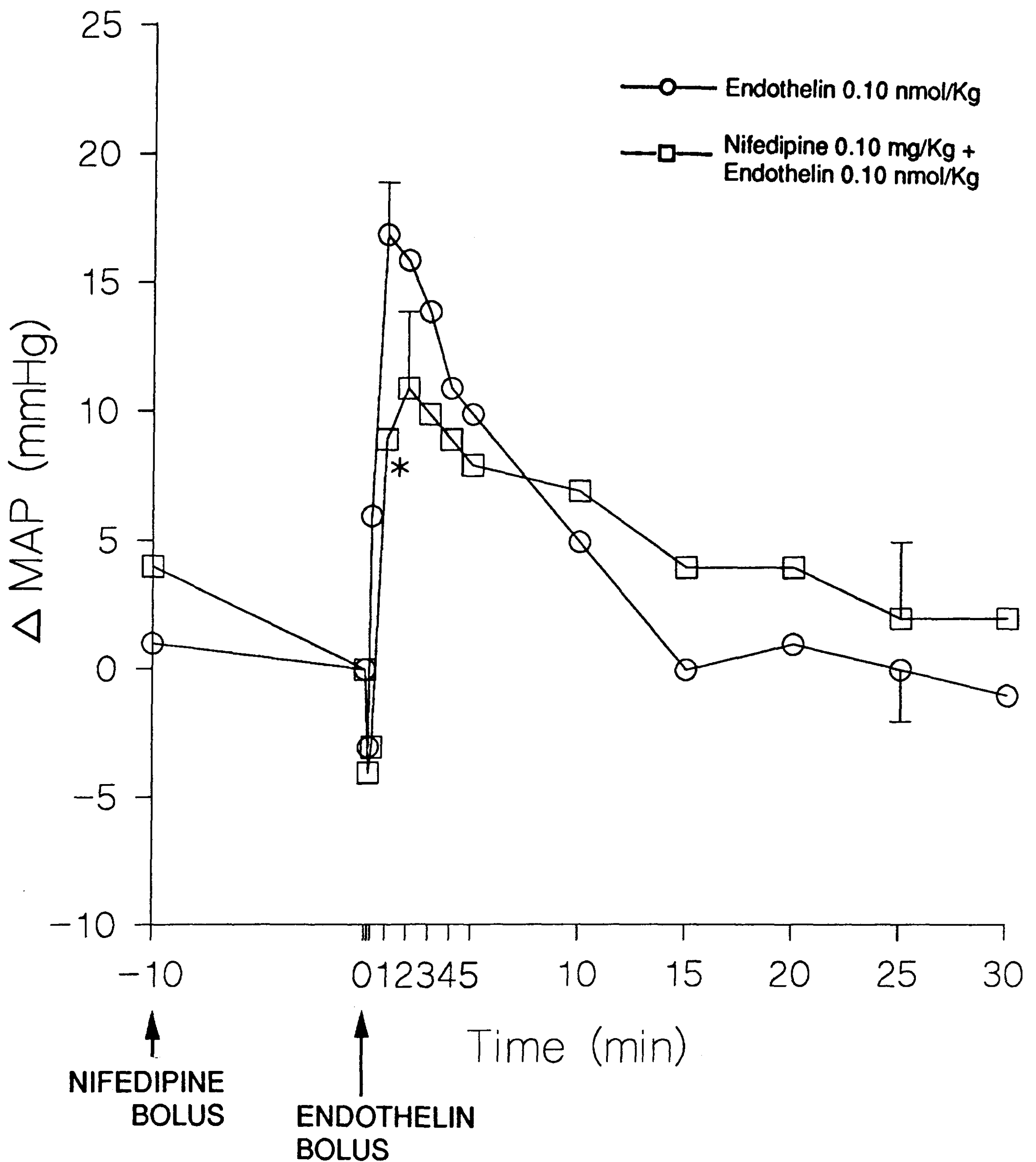


Figure 2.5: Attenuation of the pressor response (Δ MAP) to endothelin-1 by nifedipine in the rabbit.

Nifedipine bolus (0.1 mg/kg, i.v.) was given 10 minutes before the bolus injection of endothelin-1 (0.1 nmol/kg, i.v.; "time 0").(* $p < 0.05$ versus endothelin alone. Mean ± S.D., n=7)

treatment. Injection of vehicle had no effect on responses to endothelin, similar responses being observed after the first and second injection of endothelin under these conditions.

2.3.3. Endothelin-1-induced phosphoinositide hydrolysis in rat aortic segments

(A) Endothelin-stimulated phosphoinositide hydrolysis

Endothelin-1 (10^{-9} - 10^{-5} M) induced dose-dependent increases in phosphoinositide hydrolysis (i.e., [^{3}H]-inositol phosphates formation). The increase was significant from 10^{-8} M onwards. Noradrenaline also increased phosphoinositide hydrolysis; however, the maximum increase with endothelin (1040 ± 438)% at 10^{-5} M was significantly greater than that induced by 10^{-4} M noradrenaline (306 ± 110)%. (Figure 2.6)

(B) Time course of endothelin- and noradrenaline-induced phosphoinositide hydrolysis

After the addition of endothelin (10^{-6} or 10^{-5} M), there was an initial rapid increase in phosphoinositide hydrolysis. However, the rate of [^{3}H]-inositol phosphates formation declined with time and there was little increase after 30 minutes incubation with endothelin. On the other hand, noradrenaline (10^{-4} M) caused a continuous increase in [^{3}H]-inositol phosphates formation throughout 60 minutes incubation. (Figure 2.7)

(C) Effect of endothelium removal on agonist-stimulated phosphoinositide hydrolysis

Endothelium removal significantly attenuated, but did not completely inhibit, endothelin-induced phosphoinositide hydrolysis. [^{3}H]-inositol phosphates formation by 10^{-6} M endothelin decreased from (990 ± 201)% to (430 ± 229)% after the endothelium removal. In contrast, endothelium removal did not affect noradrenaline-induced [^{3}H]-inositol phosphates formation ((188 ± 56)% and (231 ± 68)% of basal levels, in the presence or absence of endothelium, respectively). (Figure 2.8)

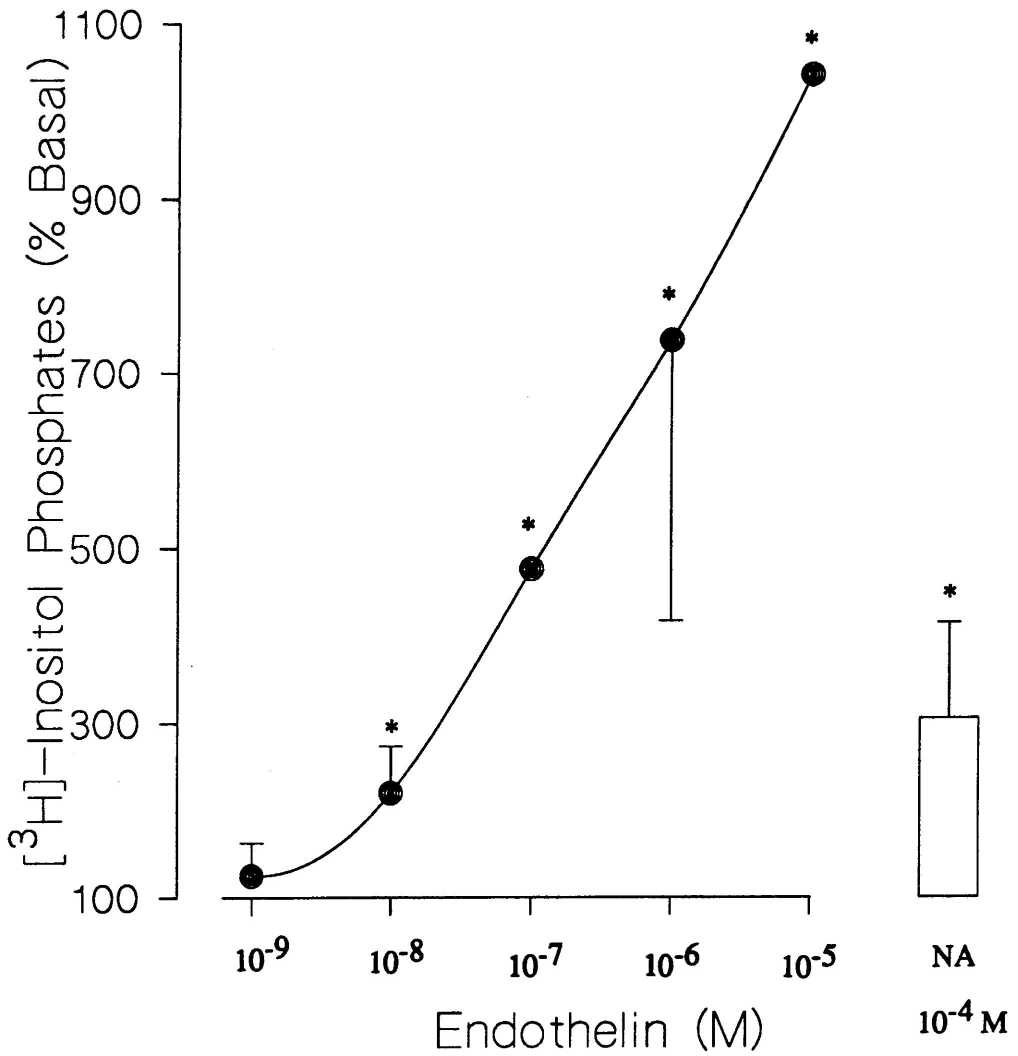


Figure 2.6: Endothelin-1-stimulated [^{3}H]-inositol phosphates formation in rat aorta.

(* $p < 0.05$ versus controls without endothelin-1. Mean ± S.D., n= 6 - 10.)

The histograph shows noradrenaline-stimulated [^{3}H]-inositol phosphates formation at 10^{-4} M for comparison.

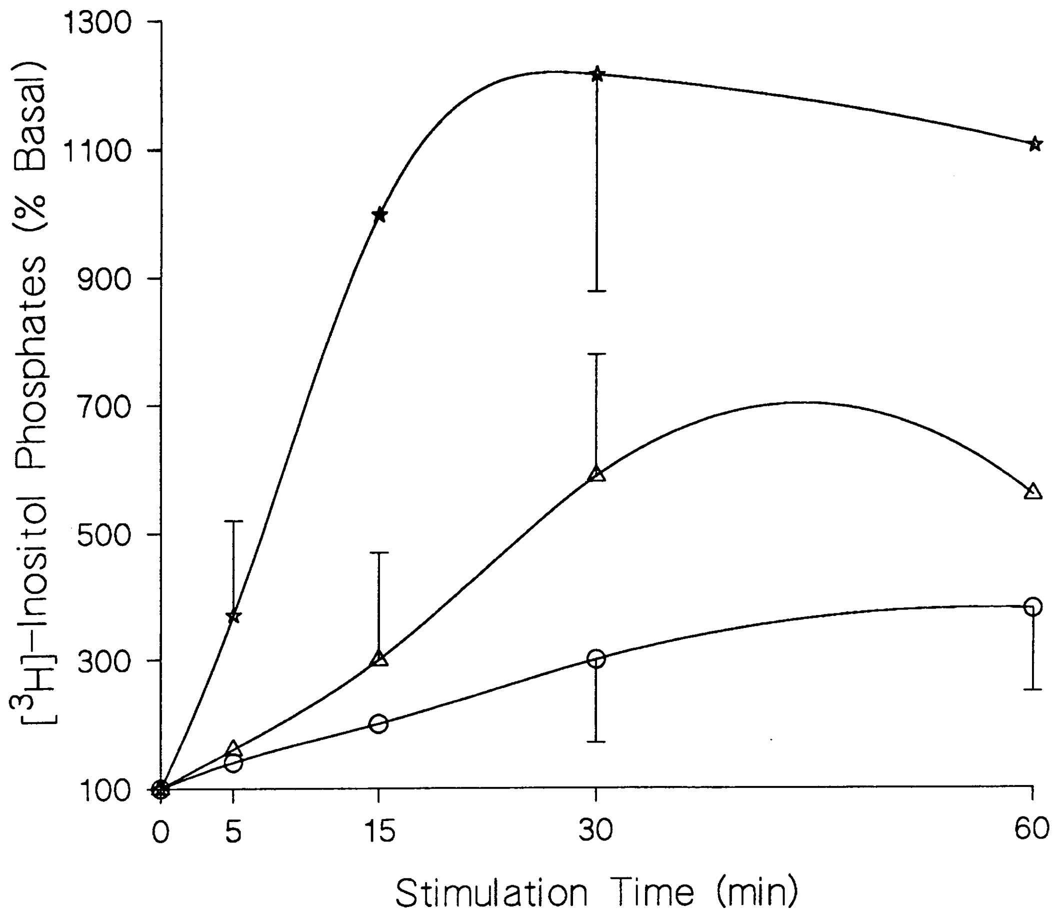


Figure 2.7: Time course of [^{3}H]-inositol phosphates formation stimulated by endothelin-1 or noradrenaline.

☆ 10^{-6} M Endothelin-1

△ 10^{-7} M Endothelin-1

○ 10^{-4} M Noradrenaline

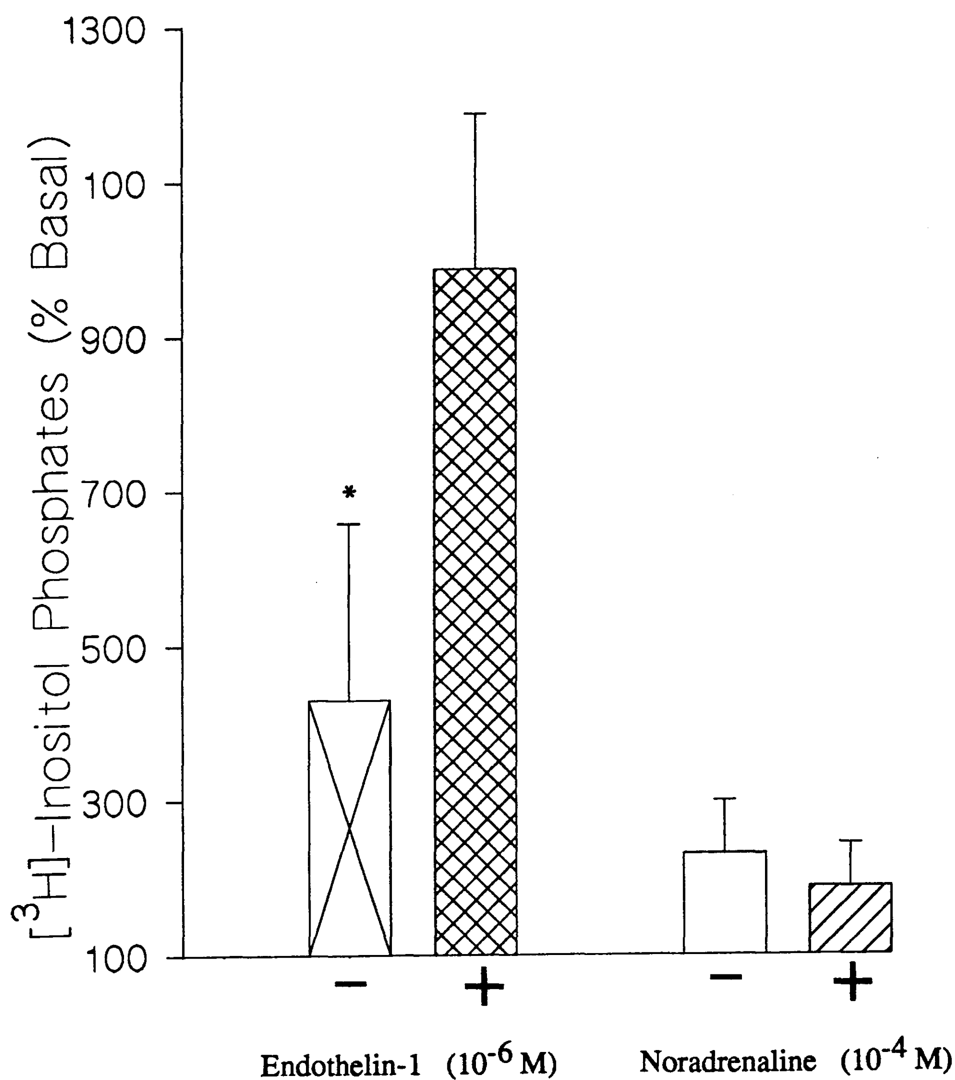


Figure 2.8: Effect of endothelium removal on the $[^3H]$-inositol phosphates formation stimulated by endothelin-1 (10^{-6} M) or noradrenaline (10^{-4} M).

(* $p < 0.05$ for - versus +. Mean ± S.D., n = 6.)

\+ With endothelium

\- Without endothelium

(D) Effect of extracellular calcium on the agonist-stimulated phosphoinositide hydrolysis

[^{3}H]-inositol phosphates formation by 10^{-6} M endothelin was (435 ± 166)% when the aortic segments were incubated in 0 mM Ca^{2+} KRB, as compared to (669 ± 211)% at 0.5 mM Ca^{2+} KRB ($p > 0.05$), and was further decreased to (231 ± 49)% when 0.5 mM EGTA was added into the 0 mM Ca^{2+} KRB ($p < 0.05$) 5 minutes before the introduction of endothelin. [^{3}H]-inositol phosphates formation by 10^{-4} noradrenaline was (225 ± 79)% when incubated in 0 mM Ca^{2+} KRB, as compared to 259 ± 49% at 0.5 mM Ca^{2+} KRB ($p > 0.05$), while [^{3}H]-inositol phosphates formation by noradrenaline was (180± 35)% when incubated in 0.5 mM EGTA and 0 mM Ca^{2+} KRB ($p > 0.05$). (Table 2.1)

2.3.4. Endothelin-1-induced phosphoinositide hydrolysis in rabbit aortic rings

Endothelin (10^{-8} - 10^{-5} M) also caused a dose-dependent increase in phosphoinositide hydrolysis in rabbit aortic rings, and the increase was significant from 10^{-7} M (204 ± 51)% to 10^{-5} M (270 ± 92)%. However, the magnitude and the slope of endothelin-stimulated phosphoinositide hydrolysis was lower than that in rat aortic segments. (Figure 2.9)

2.4. Discussion

1. Endothelin-induced blood pressure changes in rabbits

Our study showed that intravenous injection of endothelin-1 caused two phases of blood pressure changes: an initial, short-lived depressor response (lasting less than 1 minute), followed by a long-lasting pressor response (generally lasting for more than 20 minutes). A depressor/pressor profile has also been reported by others in anaesthetized or conscious rabbits (Cocks et al, 1989; Lippton et al, 1991), dogs (Tsuchiya et al, 1990), cats (Minkes et al, 1989) and rats (King et al, 1990; Miyauchi

Table 2.1. Effect of calcium depletion on phosphoinositide hydrolysis by endothelin-1 and noradrenaline in rat aorta. (data expressed as % Basal [^{3}H]-inositol phosphates)

KRB Conditions	Endothelin-1 (10^{-6} M)	Noradrenaline (10^{-4} M)
0.5 mM $[Ca^{2+}]_o$	669 ± 211%	259 ± 49%
0 mM $[Ca^{2+}]_o$	435 ± 166%	225 ± 79%
0 mM $[Ca^{2+}]_o$ + 0.5 mM EGTA	231 ± 49% *	180 ± 35%

(Mean ± S.D., n = 5, * $p < 0.05$ vs 0.5 mM $[Ca^{2+}]_o$)

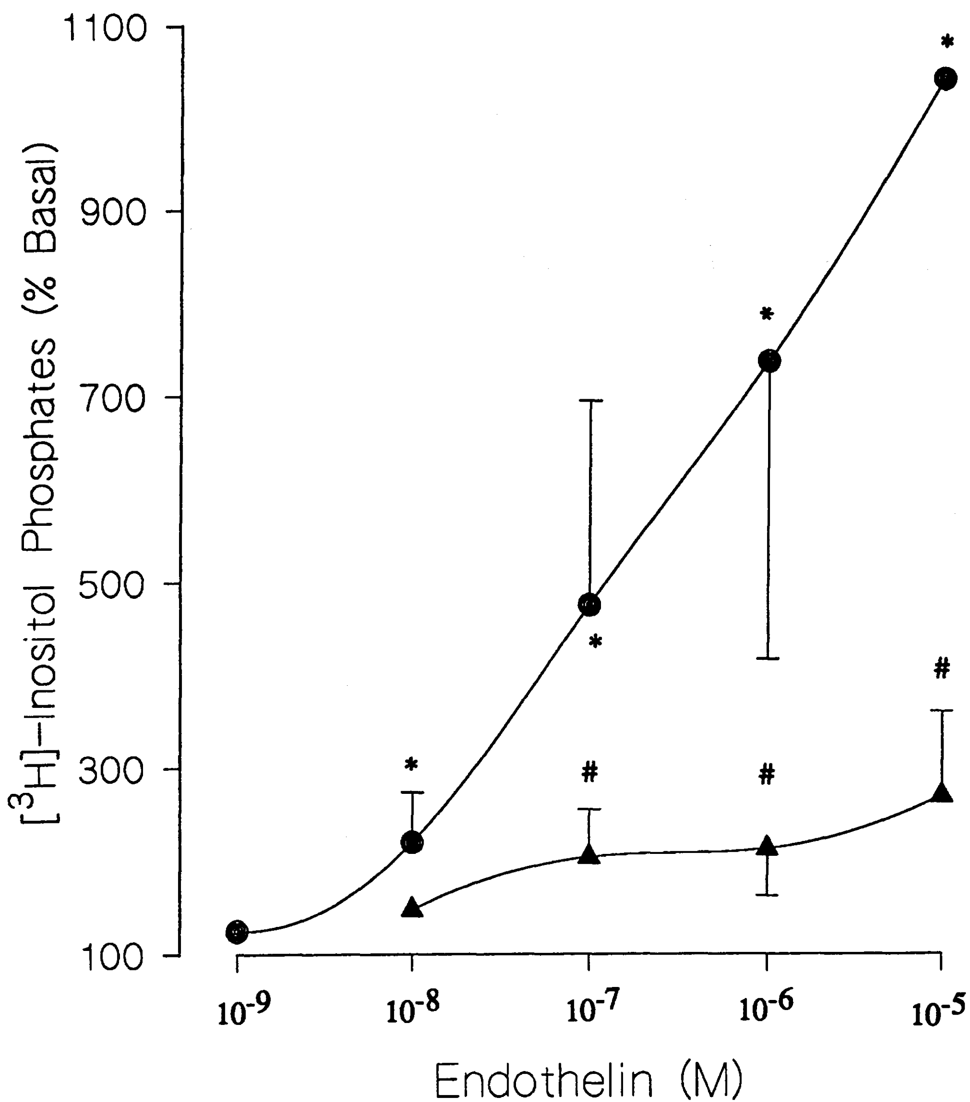


Figure 2.9: Endothelin-1-stimulated [^{3}H]-inositol phosphates formation in rabbit aorta.

(# $p< 0.05$ versus controls without endothelin-1. Mean ± S.D., n= 7)

Also included in the figure is the endothelin-1-stimulated response in rat aorta for comparison.

▲ in rabbit aorta.

● in rat aorta.

et al, 1989). The dose ranges for the pressor response were similar in this study to those reported by others in rabbits (Cocks et al, 1989; Lippton et al, 1991), dogs (Nakamoto et al, 1989), and rats (Miyauchi et al, 1989).

We also found that the dihydropyridine-sensitive calcium channel blocker, nifedipine, at the dosage of 0.1 mg/kg, could significantly attenuate, but not completely block, the pressor response to endothelin-1 (0.1 nmol/kg). The ability of a dihydropyridine-sensitive calcium channel blocker to reduce the in vivo pressor response was also demonstrated by others in rats (Knuepfer et al, 1989; Miyauchi et al, 1989), cats (Minkes et al, 1989), rabbits (Seino et al, 1990), and dogs (Otsuka et al, 1990). These findings are in line with the proposal that endothelin-1, though its binding sites are different from the dihydropyridine-sensitive calcium channels (Clozel et al, 1989; Gu et al, 1989; Miyazaki et al, 1990), may indirectly activate membrane calcium channels, and facilitate the influx of extracellular Ca^{2+} ions (Van Renterghem et al, 1988; Inoue et al, 1990; Marsault et al, 1990; Takuwa et al, 1990).

We also found that endothelin-1 dose-dependently stimulated phosphoinositide hydrolysis in rabbit aortic rings. The ability of endothelin-1 to induce both vasoconstriction and phosphoinositide hydrolysis in arterial tissues has also been observed by others (Ohlstein et al, 1989; Pang et al, 1989; Rapoport et al, 1990). Of note here is that in our studies, the dose-response curves for in vivo pressor responses were to the left of the dose-response curves for phosphoinositide hydrolysis. Several explanations for this can be put forward: (1) The phosphoinositide hydrolysis pathway is a major, but not exclusive, pathway, for endothelin-induced pressor or vasoconstrictor effects. Indeed, the susceptibility of the pressor response to calcium antagonists' attenuation, as discussed above, argues for a complex regulating mechanism to bring about the end results--pressor or vasoconstrictor effects. Moreover, endothelin can stimulate phospholipase C as well

as phospholipase A_2 in vascular smooth muscle cells (Resink et al, 1989a; Reynolds et al, 1989). The arachidonic acid produced via the phospholipase A_2 pathway may engender potential second messengers by way of cyclooxygenase or lipooxygenase pathways, which may contribute to the vasoconstrictor effects.

(2) A significant shift in the concentration-response curve for phosphoinositide hydrolysis to the right of that obtained for vascular contraction stimulated by other vasoconstrictors such as α_1-adrenoceptor agonists has been reported (Rapoport et al, 1987; Langlands & Diamond, 1990). The presence of spare receptors for phosphoinositide hydrolysis which are not necessarily involved in the contractile response may account for such a shift (Langlands & Diamond, 1990). Alternatively, as stressed by Karaki (1989), contractility of vascular smooth muscle may be regulated not only by $[Ca^{2+}]_i$ but also by the Ca^{2+} sensitivity of the contractile elements; i.e., the increase in $[Ca^{2+}]_i$, as well as the increase in the Ca^{2+} sensitivity, contributes to the contractile processes.

(3) Different vascular beds were examined, i.e., aorta for phosphoinositide studies vs resistance arterioles for the in vivo experiments, as the latter were the main determinant for systemic resistance and therefore blood pressure changes.

The depressor response, which was short-lived, tended to be dose-related as well. Recent studies have suggested that potential dilator mechanisms, such as the secretion of endothelium-derived relaxing factor, the secretion of prostacyclin, or the secretion of atrial natriuretic peptide, may all contribute to this depressor/dilator response, depending on the animal species, the presence of hypertension, or the vascular beds examined, and on the doses applied (de Nucci et al, 1988; Kitazumi et al, 1990; Garcia et al, 1990; Lippton et al, 1991; Harris et al, 1991). Further studies by us also suggested that the cyclooxygenase inhibitor--indomethacin, or the nitric oxide synthase inhibitor--Nw-Nitro-L-arginine-methyl ester, as well as the presence of perinephritis hypertension, all modulated the depressor response to endothelin-1

in the rabbit (Hamilton et al, personal communication).

2. Endothelin-stimulated phosphoinositide hydrolysis in rat and rabbit aorta

Our results demonstrate that in the dose range 10^{-9} to 10^{-5} M, endothelin-1 stimulated phosphoinositide hydrolysis in both rat and rabbit aorta, and the increase in [^{3}H]-inositol phosphates formation was greater in rat aorta than in rabbit aorta at all concentrations. Thus in terms of phosphoinositide hydrolysis stimulation, endothelin-1 is more efficacious in the rat aorta than in the rabbit aorta. So far no other comparative studies in the rat and rabbit aorta on endothelin-stimulated phosphoinositide hydrolysis are available in the literature. It remains to be elucidated whether the difference in these two species in our studies is due to differences in the receptor density, or the signal-transducing apparatus such as G-proteins or phospholipase C.

In the rat aorta, the effect of endothelin-1 on the stimulation of phosphoinositide hydrolysis was more potent as well as more efficacious than that of noradrenaline. This finding echoed the findings in the vessel strip studies showing endothelin-1 as the most potent among the common vasoconstrictors (Yanagisawa et al, 1988).

Our data also suggested that extracellular Ca^{2+} depletion by incubation in 0 mM Ca^{2+} KRB, and more distinctly, incubation in 0 mM Ca^{2+} KRB and 0.5 mM EGTA, could attenuate, but not completely abolish the endothelin-stimulated phosphoinositide hydrolysis in the rat aorta. The attenuation of stimulated phosphoinositide hydrolysis by Ca^{2+} depletion was more pronounced in the case of endothelin-1 than that of noradrenaline. The absence of statistical significance for some comparisons could be due to our small sample size (n= 5). (In the case of endothelin-stimulated phosphoinositide hydrolysis in 0 vs 0.5 mM Ca^{2+} KRB, a reduction was seen in 4 out of 5 experiments.) Rapoport (1987) also found that

noradrenaline-stimulated phosphoinositide hydrolysis in rat aorta was significantly attenuated in the Ca^{2+} free and EGTA incubation media as compared to the normal-Ca^{2+} KRB media. In contrast, Ohlstein et al (1989) reported that endothelin-stimulated phosphoinositide hydrolysis was not affected by Ca^{2+}-free plus EGTA incubation in the rabbit aorta. EGTA is a very strong chelator, and may modify levels of free intracellular as well as extracellular Ca^{2+}. Results with EGTA must therefore be treated with caution; different results may depend upon the exact assay conditions and chelation of intracellular Ca^{2+} could also contribute to the effects on agonist-induced phosphoinositide hydrolysis. In cultured rat aortic smooth muscle cells, Muldoon et al (1989) observed that endothelin-stimulated phosphoinositide hydrolysis was partially diminished by extracellular calcium depletion. Similarly, a partial inhibition in endothelin-stimulated $[Ca^{2+}]_i$ elevation was reported in cultured rat aortic smooth muscle cells (Danthuluri & Brock, 1990). Thus, in our and others' studies on rat vascular smooth muscle cells, endothelin-stimulated phosphoinositide hydrolysis was affected by extracellular Ca^{2+} depletion.

In the time course study of endothelin-stimulated phosphoinositide hydrolysis in the rat aorta, there was an attenuation of $[^3H]$-inositol phosphates formation with time after 30 minutes of stimulation. Various explanations for this are possible:

(1) Could it be due to substrate ($[^3H]$-phosphoinositides) exhaustion ?

Comparison of the radioactivities in the lipid phase, which comprised membrane $[^3H]$-phosphoinositides, in the non-stimulated tubes with the endothelin-stimulated tubes, showed that there was little reduction in the $[^3H]$-phosphoinositides even after 60 minutes of stimulation (Huang et al, 1989). Moreover, the time course studies were conducted using 10^{-6} and 10^{-7} M endothelin. In other experiments we showed that the $[^3H]$-inositol phosphates formation stimulated by 10^{-5} M endothelin was consistently greater than that by 10^{-6} or 10^{-7} M endothelin. Thus the possibility of substrate exhaustion is not substantiated.

(2) Could it be due to desensitization by protein kinase C which could be activated

via the phosphoinositide pathway ?

To test this hypothesis, we added the protein kinase C inhibitor, H7 (50 μmol/l) into the incubation medium 10 minutes prior to the administration of endothelin, and found that it had no effect on the [^{3}H]-inositol phosphates formation in our study (Huang et al, 1989), although this concentration of H7 has previously been shown to abolish the inhibitory action of one phorbol ester, 12-o-tetradecanoyl-phorbol 13-acetate, on the phosphoinositide hydrolysis (Berta et al, 1988).

(3) Could it be due to receptor down-regulation ?

Hirata et al (1988b) showed that in primary cultures of rat aorta, exposure to endothelin for 24 hours led to a substantial decrease in [^{125}I]-endothelin binding capacity, but not binding affinity, and to attenuated responses. Autoradiographic studies also revealed the internalisation of endothelin receptors within the cell and subsequent sequestration by the lysosomes. In our in vivo studies, no alteration of responses was observed in the rabbit on repeated dosing with endothelin, suggesting that in vivo prologed exposure may be required before down-regulation or desensitization is observed. However, repeated dosing may not have the same consequences as continued exposure to the agonist. Thus the attenuation of [^{3}H]-inositol phosphates formation after 30 minutes of endothelin stimulation might be due to receptor down-regulation, but this remains to be confirmed.

Phosphoinositide hydrolysis by endothelin in the rat aorta did not depend on the presence of the endothelium, although it was attenuated with endothelium removal. The possibility of damage or loss of smooth muscle by the procedure is not favoured since, in concurrent experiments, phosphoinositide hydrolysis by noradrenaline was not affected by endothelium removal. So far we have no satisfactory explanation for this interesting finding. On the other hand, studies in the vessel strips showed that the tension development, i.e., vasoconstriction induced by endothelin, could be enhanced by endothelium removal (Eglen et al, 1989; Kauser et al, 1990).

In summary, we have shown that endothelin-1 can induce a short-lived depressor response followed by a long-lasting pressor response in conscious rabbits. The pressor response was dose-related, and reached a maximum between 1 and 3 minutes after injection of endothelin. Moreover, this pressor response was attenuated by nifedipine.

Endothelin-1 also stimulated phosphoinositide hydrolysis in both rat and rabbit aorta; the effect was greater in the rat.

Endothelin-stimulated phosphoinositide hydrolysis was attenuated after 30 minutes stimulation, and also by extracellular Ca^{2+} depletion, and by endothelium removal. Endothelin-stimulated phosphoinositide hydrolysis was greater than that by noradrenaline.

The effects on phosphoinositide hydrolysis were only examined in aorta, but this work is consistent with the hypothesis that increased $[Ca^{2+}]_i$, via stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis, and an influx of extracellular calcium may both contribute to the vasoconstrictor response to endothelin.

CHAPTER 3

OVERALL DESIGN FOR STUDIES ON HYPERTENSIVE AND HYPERCHOLESTEROLEMIC RABBITS

Chapter 3. Overall Study Design

In this chapter, the study design is discussed, including selection of animals and measurements made. Overall effects of treatment on parameters such as body weight, blood pressure, and survival are presented.

3.1. Preparation of hypertensive and hypercholesterolemic rabbits

3.1.1. Animal groups

Male New Zealand white rabbits aged 3-4 months were obtained from Cheshire Rabbit Farms (Cheshire, UK) and allocated to one of four different groups:

(1) Control Group---sham operated, normal diet.

(2) Hypercholesterolemic Group---sham operated, 0.3% cholesterol diet.

(3) Hypertensive Group---renal wrapping operation, normal diet.

(4) Hypertensive-Hypercholesterolemic Group---renal wrapping operation, 0.3% cholesterol diet.

3.1.2. Renal wrapping operation

Perinephritis hypertension was induced by unilateral renal wrapping in cellophane and contralateral nephrectomy. Sham operated animals had one kidney removed and the other kidney manipulated but not wrapped. The operation was performed under barbiturate anaesthesia (sodium pentobarbitone, 30-40 mg/kg).

Perinephritis hypertension was first established by Page (1939) when he applied cellophane around the kidney in the dog, rabbit, or cat, and found that it induced an intensive inflammatory reaction around the kidney; as a result, sustained hypertension appeared in 3 to 4 weeks in the animals (Page, 1939; Graef & Page, 1940). Brace et al (1974) reported that in dogs the inflammatory reaction (scar tissue) around the wrapped kidney generated an increase of pressure (25-30 mmHg)

in the kidney, while the mean arterial pressure rose by an average of 66 mmHg. Several groups have put forward possible mechanisms for the generation of hypertension in this model. Page (1939) suggested that the pathophysiological mechanism for the rise in blood pressure in this model was similar to that of the Goldblatt renal hypertension model induced by constricting the renal arteries. Later, some divergent results emerged with regard to the importance of the renin-angiotensin system in the etiology of this model of hypertension. In dogs and rabbits, one-kidney wrapping and contralateral nephrectomy induced hypertension apparently independent of the renin-angiotensin system. Plasma renin activity was not elevated and chronic hypertension was not reduced by blockade of the renin-angiotensin system with angiotensin antagonists (Bumpus et al, 1973; Johnson et al, 1976; Ichikawa et al, 1977). In contrast, two-kidney cellophane wrapping induced hypertension with increased plasma renin activity. The chronic hypertension could be reduced by inhibitors of angiotensin converting enzyme (Denton & Anderson, 1985; DeForrest et al, 1989). Thus although renal wrapping is a reliable model for inducing secondary hypertension in animals, the mechanisms causing hypertension may differ with respect to operation procedures and remain to be clarified.

Hamilton & Reid (1983) reported that unilateral renal wrapping and contralateral nephrectomy in rabbits induced a rise of 35-50 mmHg in mean arterial pressure in the established phase of hypertension (3 to 6 weeks after operation and later on), but no significant changes in heart rate, plasma noradrenaline, adrenaline, sodium, potassium, or bicarbonate levels. Plasma renin activity and renal excretory function were not altered either. No change in body weight profile was observed due to renal operation.

3.1.3. 0.3% Cholesterol diet

A 0.3% cholesterol diet was prepared by mixing rabbit chow and appropriate

amounts of cholesterol dissolved in ether. Ether was then evaporated off. Cholesterol diet was started in the hypercholesterolemic and the hypertensive-hypercholesterolemic groups on the day after the renal operation.

The control and the hypertensive groups received normal rabbit chow (suppliers).

The rabbit, being herbivorous, is susceptible to hypercholesterolemia and atherosclerosis when challenged with a cholesterol diet, because it has great difficulty in metabolizing and excreting cholesterol. Thus the rabbit is widely used as an animal model of hypercholesterolemia and atherosclerosis induced by cholesterol diet (Stehbens, 1986). Nevertheless, researchers have found some important differences between experimentally-induced and human clinical hypercholesterolemia and atherosclerosis. Firstly, the correlation between the serum cholesterol level and the extent of lipid deposition is often poor in experimental animals, whereas human autopsy findings show a significant correlation between serum cholesterol and the severity of atherosclerotic lesions (Stehbens, 1986; Solberg & Strong, 1983). Secondly, in the cholesterol-fed rabbit aorta, the lipid-containing lesions are generally more extensive proximally than distally, in contrast to the enhanced severity in the abdominal aorta and iliac arteries in man (Stehbens, 1986). Despite these differences, cholesterol-diet feeding in the rabbit is an easily reproducible model of experimental hypercholesterolemia and atherosclerosis. Cholesterol feeding in rabbits can induce hypercholesterolemia and fatty streaks, fibrous plaques, and eventually, complicated atherosclerotic lesions, in aorta and other major conduit arteries. Early atherosclerotic lesions in the cholesterol-fed rabbit occur in predilection sites such as ascending aorta, aortic arch, and branch orifices in the descending thoracic and abdominal aorta. Monocyte adherence to the endothelial surface, lipid-laden macrophages in the intima, smooth muscle cell accumulation in the intima, lipid accumulation in the endothelial cells, intimal smooth muscle cells, as well as in the extracellular space, bulging of the intimal fatty lesions into the lumen, accumulation of extracellular collagen and fibrous matrix,

smooth muscle cell proliferation, and necrotic lipid lesions, have all been observed either in sequence or concurrently (Stehbens,1986; Rosenfeld et al, 1987). Moreover, the severity of atherosclerotic lesions can be aggravated by the imposition of experimental hypertension upon the cholesterol-fed rabbits (Stehbens, 1986). In primates, cholesterol-feeding and experimental hypertension have additive effects on the extent of atherosclerotic lesions (Hollander et al, 1976; McGill et al, 1985).

3.2. Study protocols:

3.2.1. Overall scheme of study

Four groups of animals were studied as shown in Table 3.1 in order to examine effects of hypertension, hypercholesterolemia, and hypertension-hypercholesterolemia on a number of cardiovascular parameters with a view towards identifying possible sites of interaction of the disease states.

Blood pressure, heart rate, body weight, plasma cholesterol levels and general health were monitored throughout the study. Responses to a range of vasoconstrictor and vasodilator drugs were studied at 2-3, 6-7, and 13-16 weeks. Platelet intracellular Ca^{2+} levels and aortic phosphoinositide metabolism were examined at the 17th and 18th weeks after surgery, respectively, as shown in Table 3.1.

3.2.2. Measurement of mean arterial pressure, heart rate, plasma cholesterol, and body weight

After 2-hours fasting, a polypropylene catheter was inserted into the central ear artery of the rabbit under local anaesthesia (2% lignocaine). 1 ml of blood was collected into a lithium heparin tube and centrifuged at 2500 rpm for 10 minutes. Plasma was collected and assayed for cholesterol levels using an enzymatic colorimetric method (Boehringer Mannheim Reagent).

Mean arterial pressure and heart rate were measured from the arterial line after

Table 3.1. Overall scheme of study in the rabbits

Time*	Procedures	Types of measurement				
Pre-Op. week	Recruitment & Allocation	A	B			
Day 0	Renal wrapping or sham operation					
Day 1	0.3% cholesterol or normal diet					
Week 1			B			
Week 2			B			
Week 3		A	B	C		
Week 4			B			
Week 5		A	B			
Week 6			B			
Week 7		A	B	C		
Week 8			B			
Week 9		A	B			
Week 10			B			
Week 11		A	B			
Week 12			B			
Weeks 13-16		A	B	C		
Week 17		A	B		D	
Week 18	Rabbits killed and aorta prepared					E

* Time in reference to renal operation date.

A: Measurement of mean arterial pressure, heart rate, & plasma cholesterol.

B: Measurement of body weight.

C: Measurement of vascular reactivity to pressor & depressor drugs.

D: Measurement of platelet $[Ca^{2+}]_i$.

E: Measurement of aortic phosphoinositide responses to endothelin-1 & noradrenaline.

the rabbit was allowed to rest for 60 minutes in an individual cage in a quiet warm laboratory. The arterial line was connected to a Statham P23 1D transducer and arterial pressure was displayed using a Grass Model 7B polygraph. Heart rate was determined from the pressure trace.

Mean arterial pressure, heart rate, and plasma cholesterol were measured before the renal operation (initial readings) and thereafter fortnightly.

Rabbit body weight was measured each week and the general wellbeing of the animal assessed.

3.2.3. Sudan Red staining of the atherosclerotic lesions

To demonstrate the extent of fatty deposition on the intimal surface of aorta and femoral artery in groups on normal and cholesterol diets, Sudan Red staining was carried out on the opened segments when the rabbits were killed for the phosphoinositide study at the 18th week. The staining method was adopted from published papers (Holman et al, 1958). In short, the opened aorta and femoral artery were pinned on a silicone bed and rinsed briefly in 70% alcohol, then immersed in the Sudan Red staining solution (5 g Sudan IV in 500 ml of 70% ethyl alcohol and 500 ml of acetone). After 15 minutes of staining with intermittent agitation, the specimens were differentiated in 80% alcohol for 20 minutes, then washed in running tap water for 1 hour.

3.3. Data analysis

1. All measurements of mean arterial pressure, heart rate, plasma cholesterol, and body weight at each time point were expressed as mean ± S.D. (n = 15-20 in each group) Repeated measures analysis of variance was used to compare each experimental group with the control group. $p < 0.05$ was considered significant.
2. Survival and causes of death analysis---All the rabbits that died prematurely

before the completion of the scheduled studies were recorded. Only the deaths which occurred 2 weeks or more after surgery were analyzed for probable causes of death, as early deaths were attributed to anaesthesia overdose, surgical stress, or related causes. Deaths were ascribed to cardiovascular causes when signs of hypertensive encephalopathy such as unstable gait, convulsion, oral or nasal bleeding, or autopsy findings of haemorrhagic spots in the brain or myocardial infarct lesions in the heart were observed. Deaths were ascribed to non-cardiovascular causes when specific reasons such as death due to lignocaine anaesthesia or drug injection, diarrhea, sacrifice due to severe ear infection or loss of appetite and weight, were identified.

"Life Tables and Survival Functions" (BMDP1L--BMDP Statistical Software, Inc., Los Angeles, U.S.A.) were employed to compare the patterns of total as well as cardiovascular deaths in each experimental group with those in the control group. Breslow analysis was used and $p < 0.05$ was considered significant.

3.4. Results

3.4.1. Mean arterial pressure profiles

As shown in Figure 3.1, there were no differences in mean arterial pressure at the initial, pre-operation time point between any of the groups. From 2-3 weeks on, mean arterial pressure was significantly higher in both the hypertensive and hypertensive-hypercholesterolemic groups as compared to the control group. There was no difference in mean arterial pressure between the hypercholesterolemic and the control groups or between the hypertensive and the hypertensive-hypercholesterolemic groups throughout the whole study. Mean arterial pressure in the hypertensive and the hypertensive-hypercholesterolemic groups tended to stabilize after 6-7 weeks. The mean arterial pressure in the hypertensive and the hypertensive-hypercholesterolmic groups rose from between 60-70 mmHg at the initial, pre-operation time point to 110-140 mmHg at the established phase of hypertension.

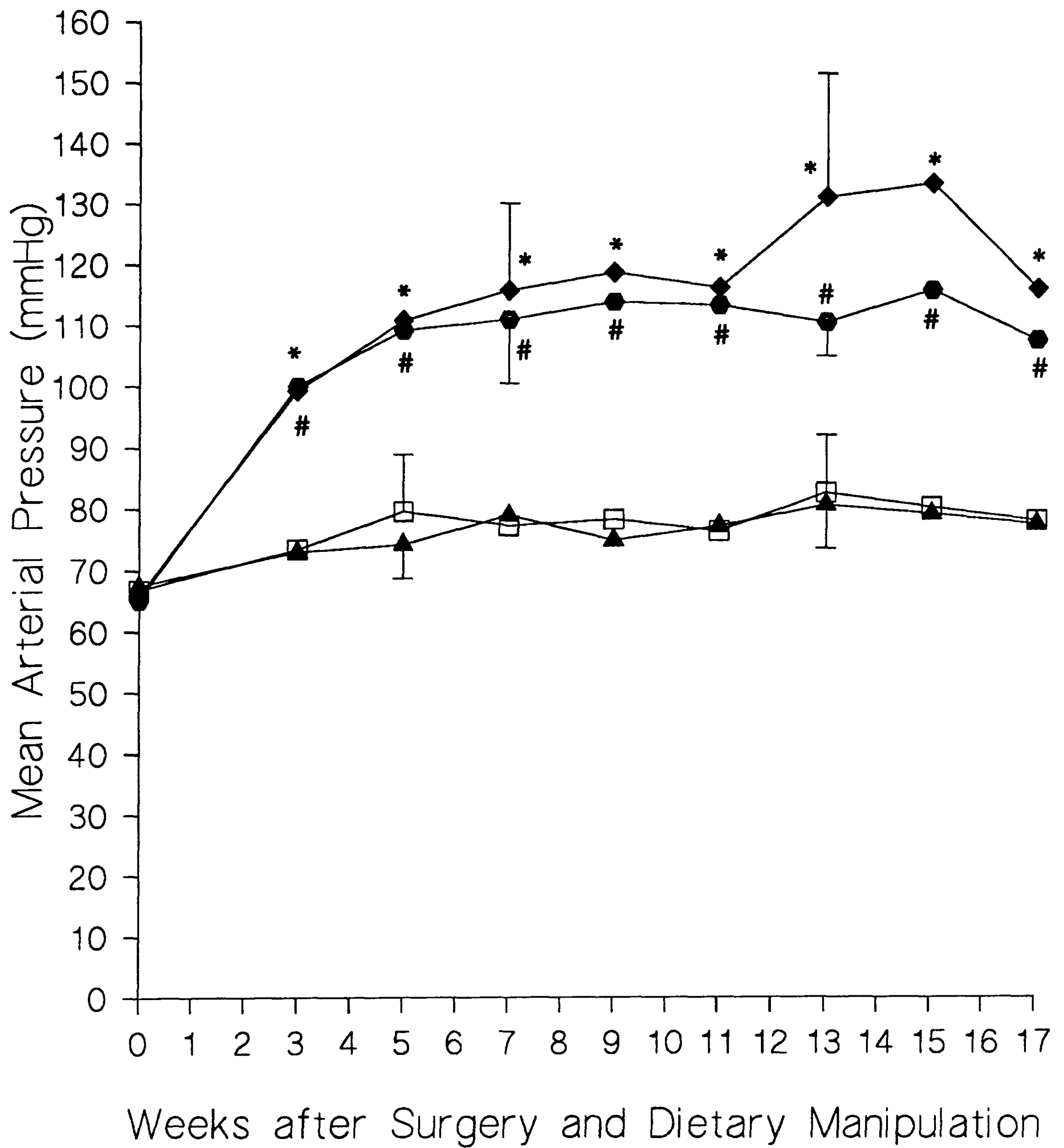


Figure 3.1: Mean arterial pressure profiles in each group of rabbits.

(* & # --- $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively. Mean ± S.D., n = 16 - 20.)

□ Control group.

▲ Hypercholesterolemic group.

◆ Hypertensive group.

● Hypertensive-Hypercholesterolemic group.

3.4.2. Heart rate profiles

As shown in Figure 3.2, there was no difference in heart rate between the experimental groups and the control group at either the initial or later time points up to 14-15 weeks. At 16-17 weeks, the heart rates in the hypercholesterolemic and the hypertensive-hypercholesterolemic groups were significantly less than that in the control group.

3.4.3. Plasma cholesterol profiles

As shown in Figure 3.3, initial plasma cholesterol levels were between 1.0 and 1.3 mmol/L, and there was no difference between groups. Later on, plasma cholesterol levels in the control and the hypertensive groups stabilized between 0.7 and 1.1 mmol/L; there was no difference between them at any time points. In the hypercholesterolemic and the hypertensive-hypercholesterolemic groups, plasma cholesterol levels rose sharply and continually with cholesterol feeding, and all the plasma cholesterol levels in both groups (except for the 2-3 week point in the hypertensive-hypercholesterolemic group) were significantly greater than those in the control group. They rose from 7.2-10.6 mmol/L at 2-3 weeks to 29.5-33.1 mmol/L at 16-17 weeks. Of note is that there was noticeable variation in individual susceptibility to cholesterol feeding among the rabbits in both the hypercholesterolemic and the hypertensive-hypercholesterolemic groups, hence the standard deviation was quite large at some points in both groups.

3.4.4. Body weight profiles

As shown in Figure 3.4, there was no difference in body weight between any of the experimental groups and the control group initially or at any time point up to 10-11 weeks. At 12-13, 14-15, and 16-17 weeks, the body weight in the control group

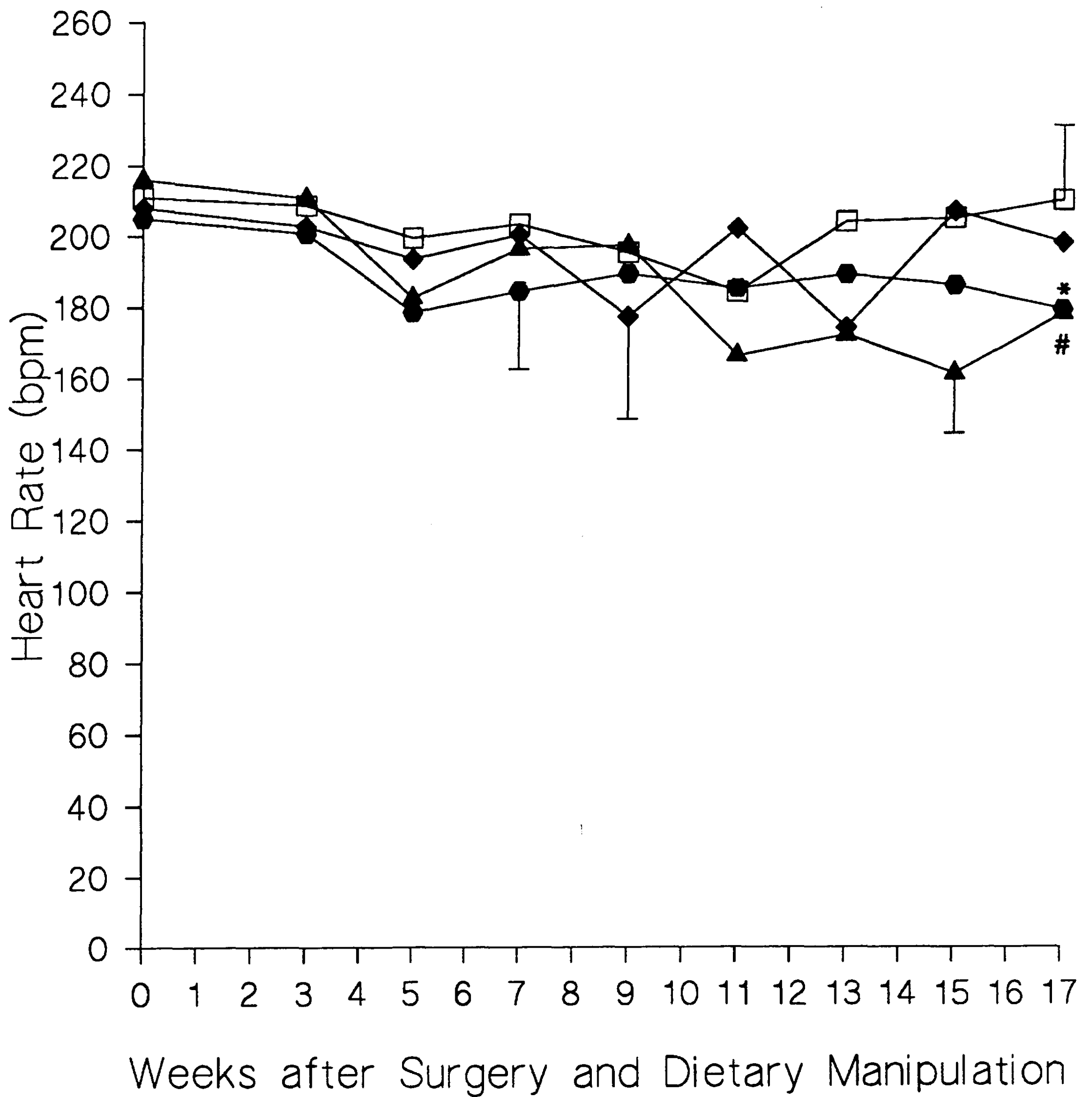


Figure 3.2: Heart rate profiles in each group of rabbits.

(* & # --- p < 0.05 versus the control group, respectively. Mean ± S.D., n = 15 - 20.)

- □ **Control group.**
- ▲ **Hypercholesterolemic group.**
- ◆ **Hypertensive group.**
- ● **Hypertensive-Hypercholesterolemic group.**

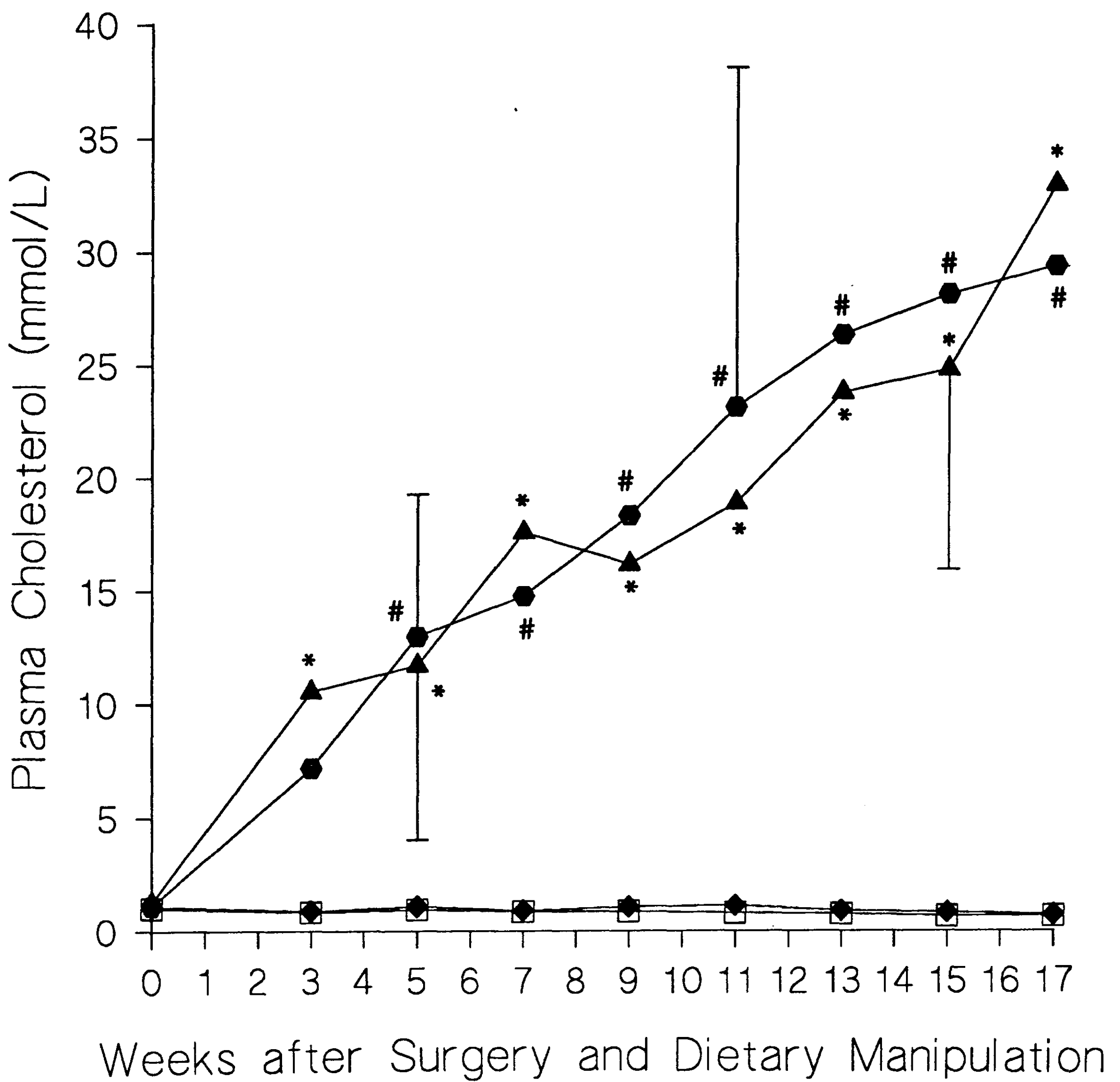


Figure 3.3: Plasma cholesterol profiles in each group of rabbits.

(* & # --- $p < 0.05$ for the hypercholesterolemic and the hypertensive-hypercholesterolemic groups versus the control group, respectively. Mean ± S.D., n = 16 - 19.)

- □ Control group.
- ▲ Hypercholesterolemic group.
- ◆ Hypertensive group.
- ● Hypertensive-Hypercholesterolemic group.

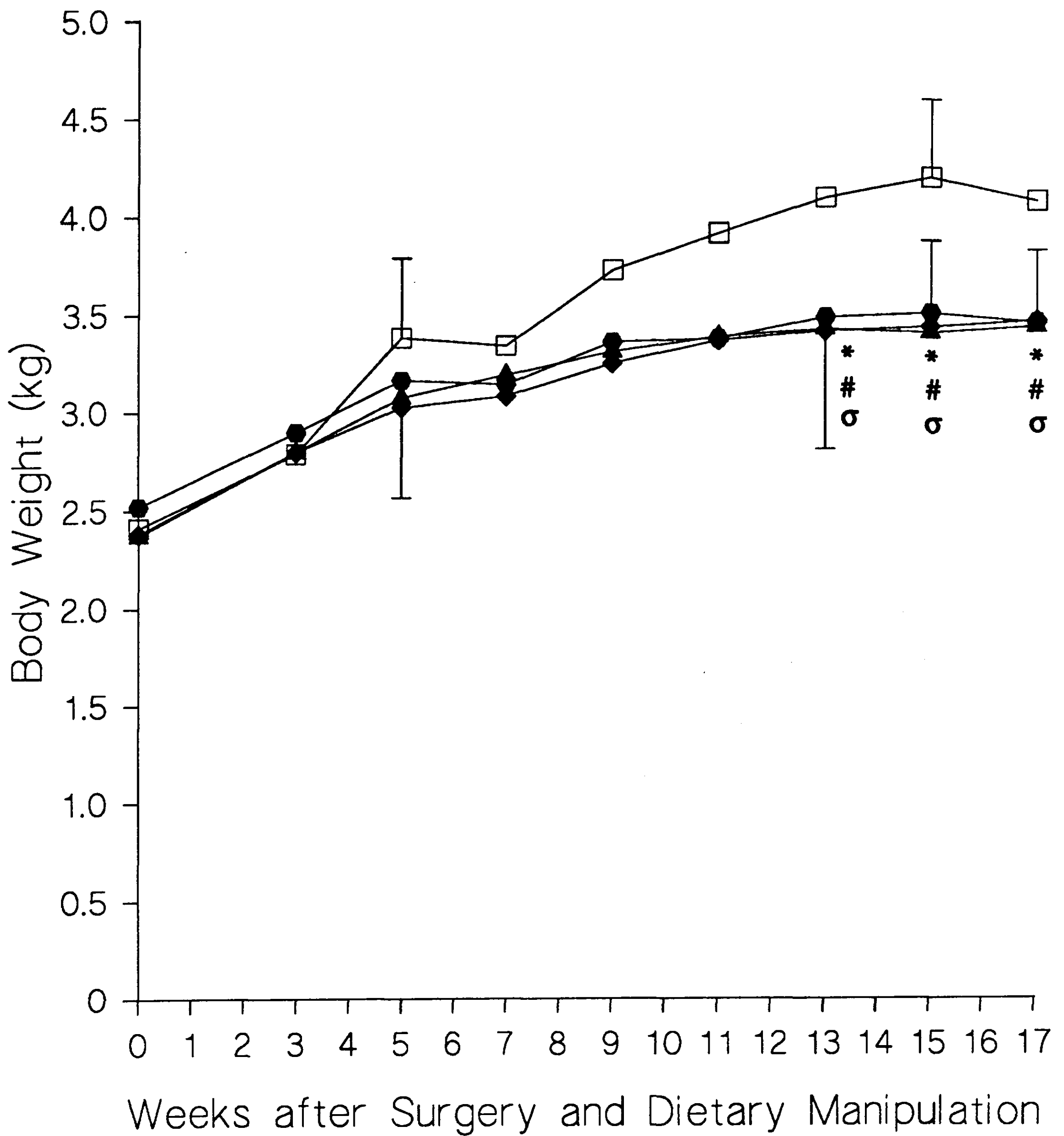


Figure 3.4: Body weight profiles in each group of rabbits.

(* & # & σ--- $p < 0.05$ for the hypercholesterolemic, the hypertensive, and the hypertensive-hypercholester olemic groups versus the control group, respectively. Mean ± S.D., n = 15 - 17.)

- □ **Control group.**
- ▲ **Hypercholesterolemic group.**
- ◆ **Hypertensive group.**
- ● **Hypertensive-Hypercholesterolemic group.**

was significantly greater than that in any experimental group.

3.4.5. Fatty deposition in the aorta and femoral arteries

Representative pictures of the aorta (at the thoracic and abdominal juncture) and the femoral artery (middle part) from each group are shown in Colour Plates 1 to 8.

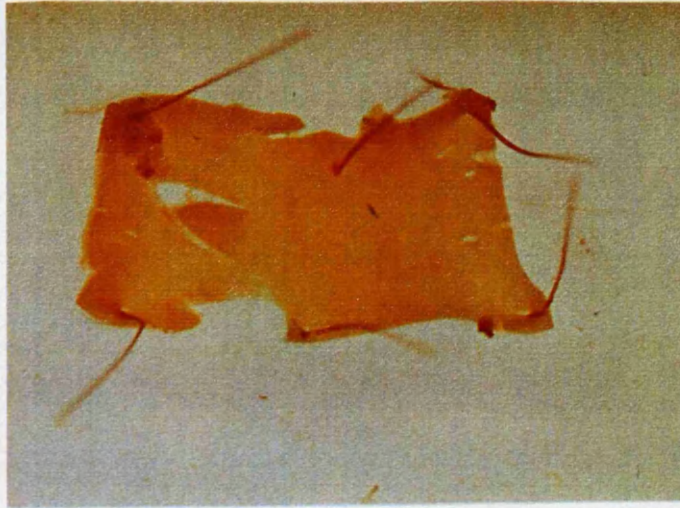
Of note here is that there was little or no Sudan Red staining in the aorta or the femoral artery from the control and the hypertensive groups, suggesting little fatty deposition in either group. However, the aortic intimal surface was grossly deposited with Sudan Red in both the hypercholesterolemic and the hypertensive-hypercholesterolemic groups, suggesting a high degree of fatty deposition in these two cholesterol-fed groups. To a lesser degree than the aortic surface, the femoral arterial surface was also stained with Sudan Red in both cholesterol-fed groups, suggesting that the fatty deposition was less diffuse or extensive in the femoral artery.

3.4.6. Survival and causes of death

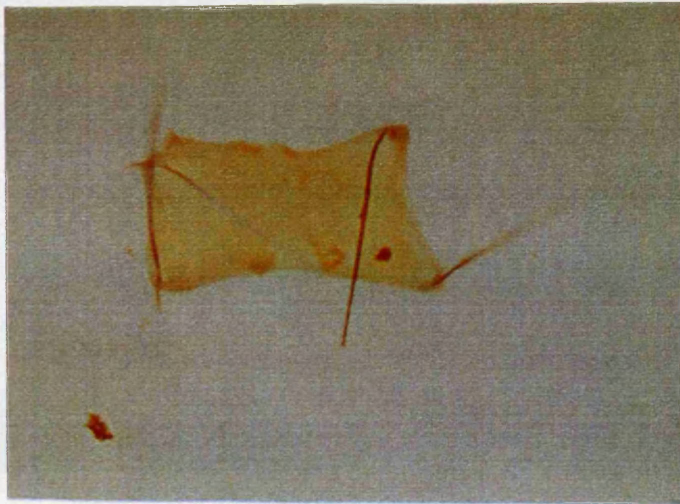
In the control group, there was one death related to lignocaine injection, one death to drug injection, one death as a result of sacrifice due to severe ear infection, and one death as a result of diarrhea, making up a total of four "non-cardiovascular deaths". One "cardiovascular death" was assigned because the rabbit exhibited cyanotic outlook, and on the previous day, blood pressure was only 50 mmHg at 16-17 weeks after operation.

In the hypercholesterolemic group, one death was related to drug injection, one death as a result of sacrifice due to severe ear infection, and one to severe weight loss, making up a total of three "non-cardiovascular deaths". There was no cardiovascular death in this group.

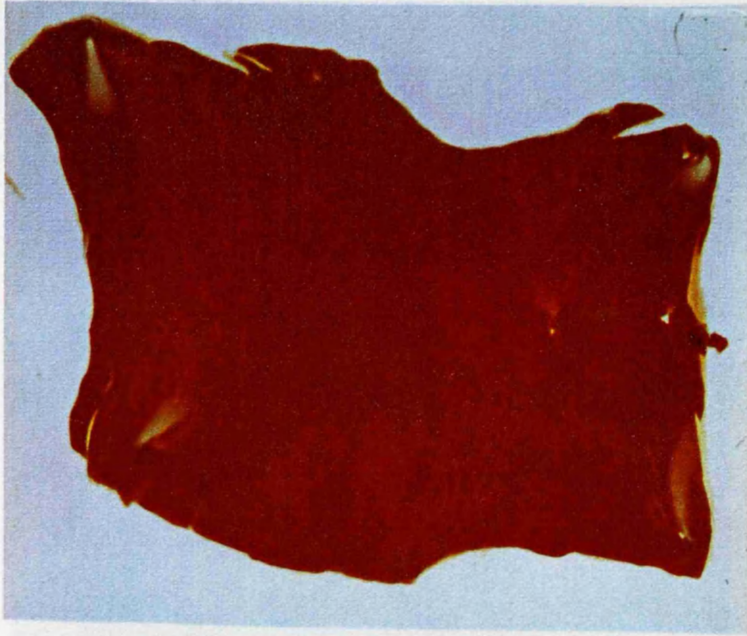
In the hypertensive group, there were three deaths related to drug injection, and



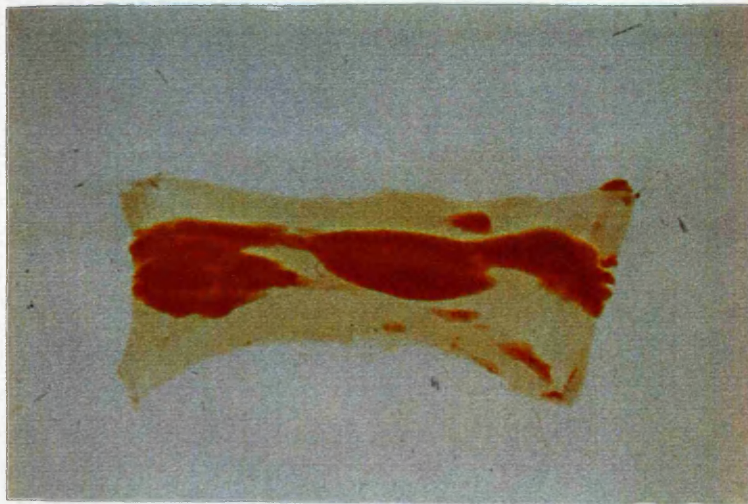
Colour Plate 1: Representative specimen of the aortic intima in the rabbit from the Control group, using Sudan Red staining technique.



Colour Plate 2: Representative specimen of the femoral intima in the rabbit from the Control group, using Sudan Red staining technique.

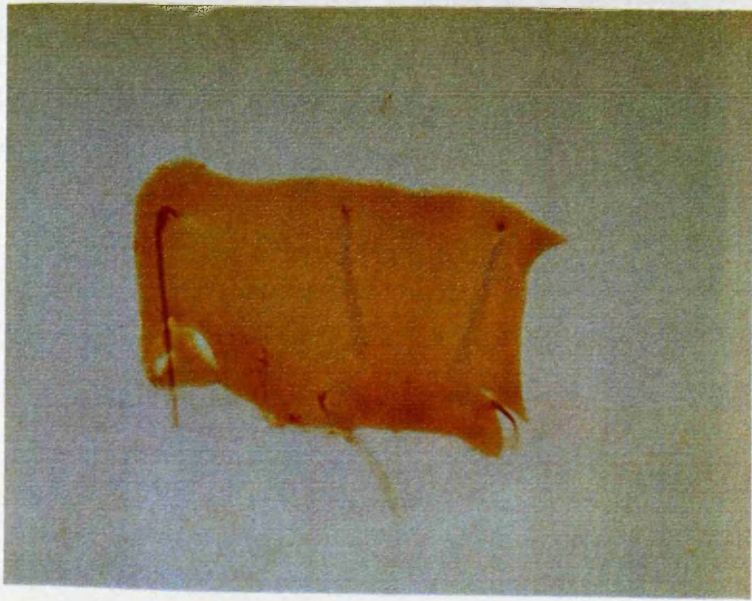


Colour Plate 3: Representative specimen of the aortic intima in the rabbit from the Hypercholesterolemic group, using Sudan Red staining technique.

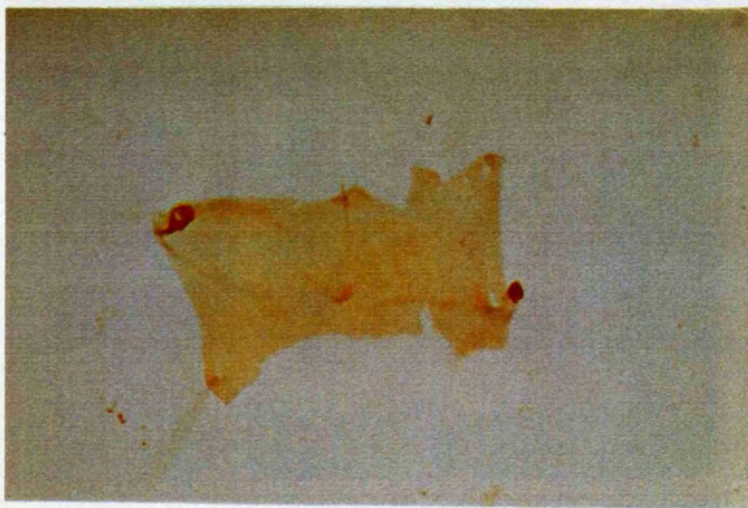


Colour Plate 4: Representative specimen of the femoral intima in the rabbit from the Hypercholesterolemic group, using Sudan Red staining technique.

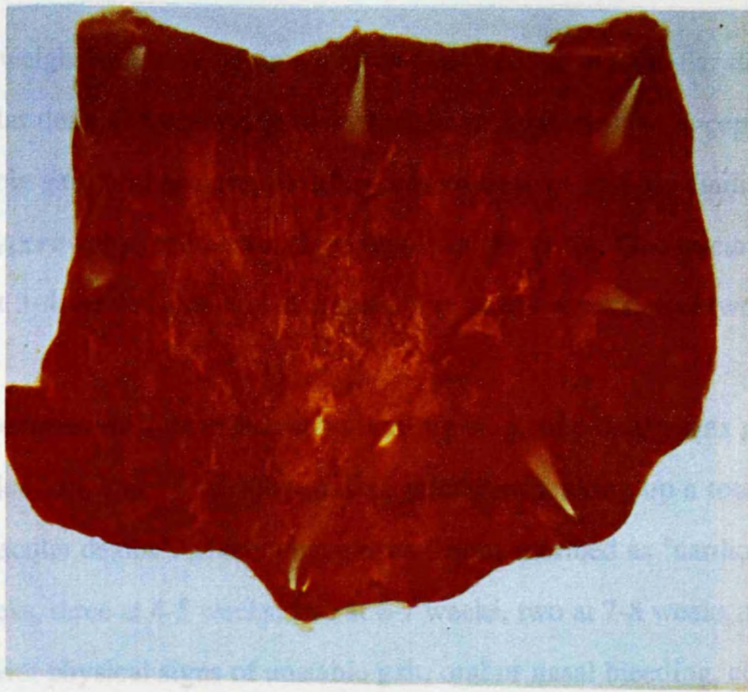
* Note: The fatty depositions were stained with Sudan Red.



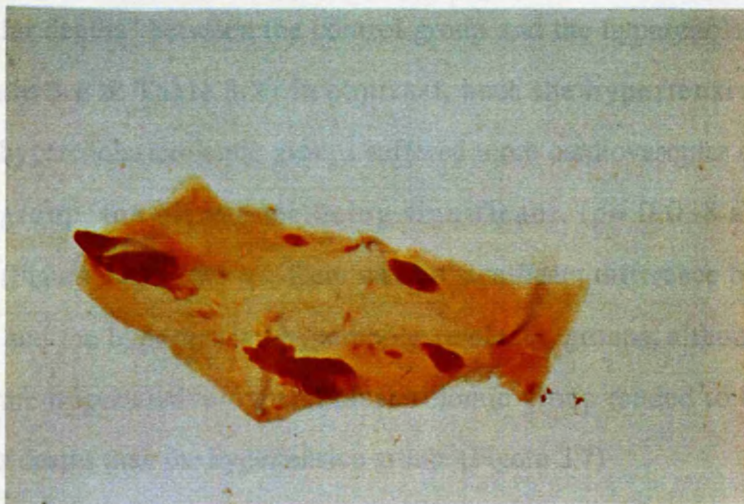
Colour Plate 5: Representative specimen of the aortic intima in the rabbit from the Hypertensive group, using Sudan Red staining technique.



Colour Plate 6: Representative specimen of the femoral intima in the rabbit from the Hypertensive group, using Sudan Red staining technique.



Colour Plate 7: Representative specimen of the aortic intima in the rabbit from the Hypertensive-Hypercholesterolemic group, using Sudan Red staining technique.



Colour Plate 8: Representative specimen of the femoral intima in the rabbit from the Hypertensive-Hypercholesterolemic group, using Sudan Red staining technique.

* Note: The fatty depositions were stained with Sudan Red.

one to severe weight loss, making up a total of four "non-cardiovascular deaths". Six "cardiovascular deaths" were ascribed with signs of hypertensive encephalopathy such as unstable gait, oral or nasal bleeding, convulsion, or autopsy findings of left ventricular hypertrophy, or haemorrhagic spots in the brain. One occurred at 2-3 weeks, one at 3-4 weeks, one at 4-5 weeks, one at 8-9 weeks, and two at 15-16 weeks.

In the hypertensive-hypercholesterolemic group, one death was related to lignocaine injection, and two deaths to drug injection, making up a total of three "non-cardiovascular deaths". There were eleven deaths ascribed as "cardiovascular", two at 3-4 weeks, three at 4-5 weeks, two at 6-7 weeks, two at 7-8 weeks, and two at 9-10 weeks, with physical signs of unstable gait, oral or nasal bleeding, convulsion, or autopsy findings of left ventricular hypertrophy or cerebral haemorrhage.

In the "Life Tables and Survival Functions" analysis, there was no significant difference in the profile of "total deaths" between the control group and any experimental group. (Figure 3.5) There was no significant difference in the profile of "cardiovascular deaths" between the control group and the hypercholesterolemic group. (Figure 3.6 & Table 3.2) In contrast, both the hypertensive and the hypertensive-hypercholesterolemic groups suffered more cardiovascular deaths than the control group, the difference being significant. ($p= 0.038$ and 0.009, respectively) (Figure 3.6) However, there was no significant difference between the hypertensive and the hypertensive-hypercholesterolemic groups, although at later time points, the hypertensive-hypercholesterolemic group tended to have more cardiovascular deaths than the hypertensive group. (Figure 3.7)

3.5. Summary and discussion

1. Hypertension and hypercholesterolemia were induced in the renal cellophane wrapping and 0.3% cholesterol diet models, respectively. Blood pressure rose and stabilized at about 6-7 weeks after operation in the two groups which had undergone

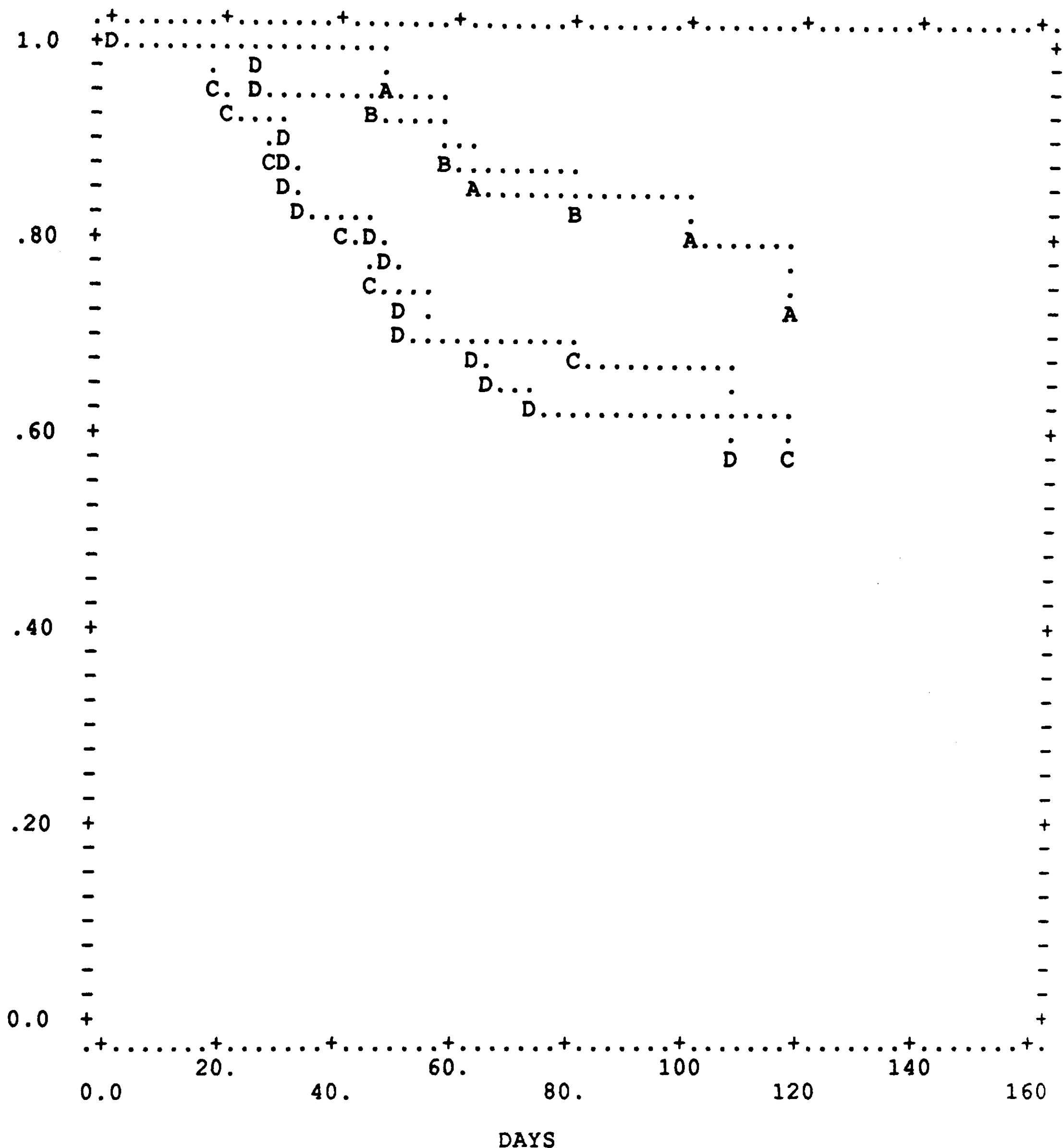


Figure 3.5: Profiles showing cumulative proportion of animals surviving in each group of rabbits when both cardiovascular and non-cardiovascular deaths were counted.

The abscissa axis is the number of study days in reference to the renal operation date as Day 0, and the ordinate axis is the ratio of survivors in reference to the starting point as 1.0. Note that although there were more total deaths in the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group, the difference did not reach significance.

A------ Control group.

B------ Hypercholesterolemic group.

C------ Hypertensive group.

D------ Hypertensive-hypercholesterolemic group.

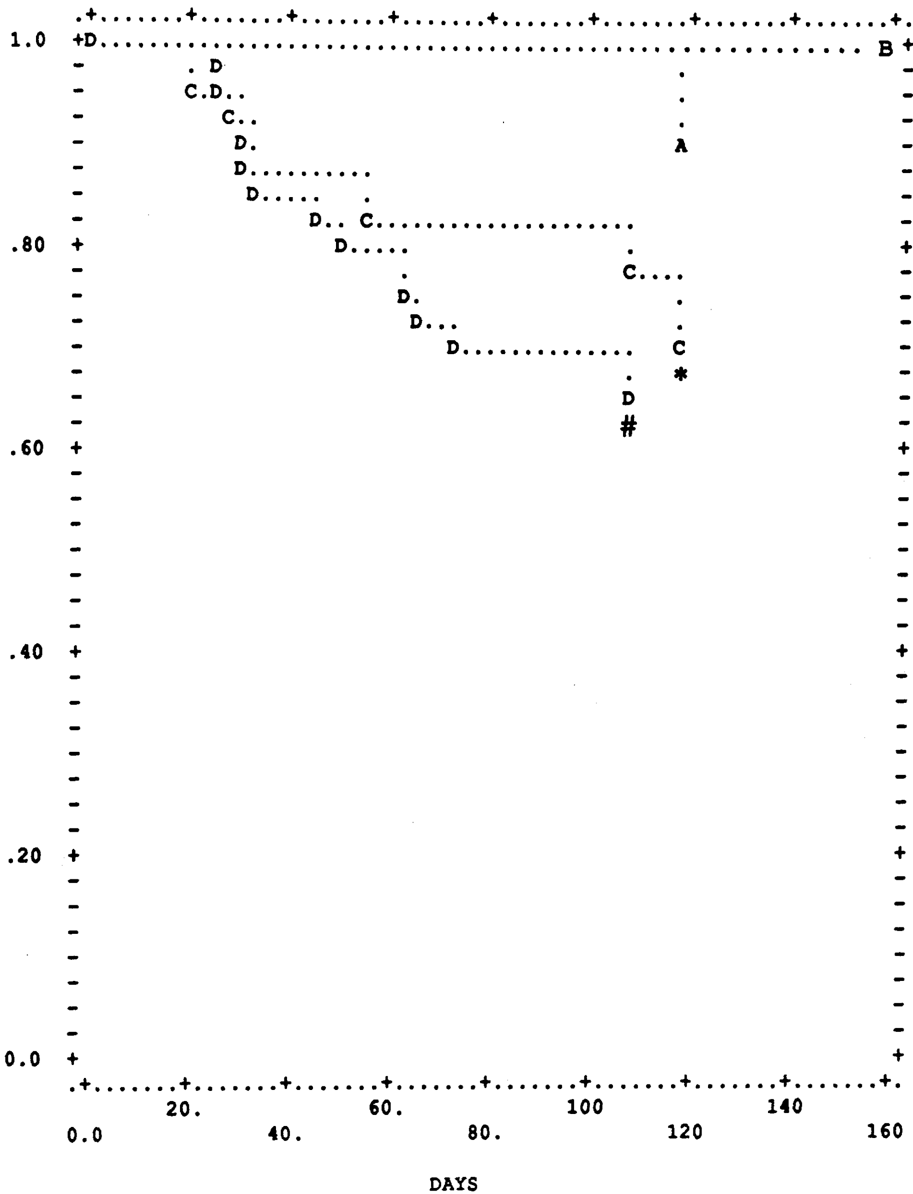


Figure 3.6: Profiles showing cumulative proportion of animals surviving in each group of rabbits when only cardiovascular deaths were counted.

The abscissa axis is the number of study days in reference to the renal operation date as Day 0, and the ordinate axis is the ratio of survivors in reference to the starting point as 1.0. There were significantly more cardiovascular deaths in the hypertensive (*--- p = 0.038.) and the hypertensive-hypercholesterolemic groups (# --- p = 0.009.)as compared to the control group, while there was no difference in cardiovascular deaths between the hypercholesterolemic and the control groups.

A----- Control group.

B----- Hypercholesterolemic group.

C------ Hypertensive group.

D------ Hypertensive-hypercholesterolemic group.

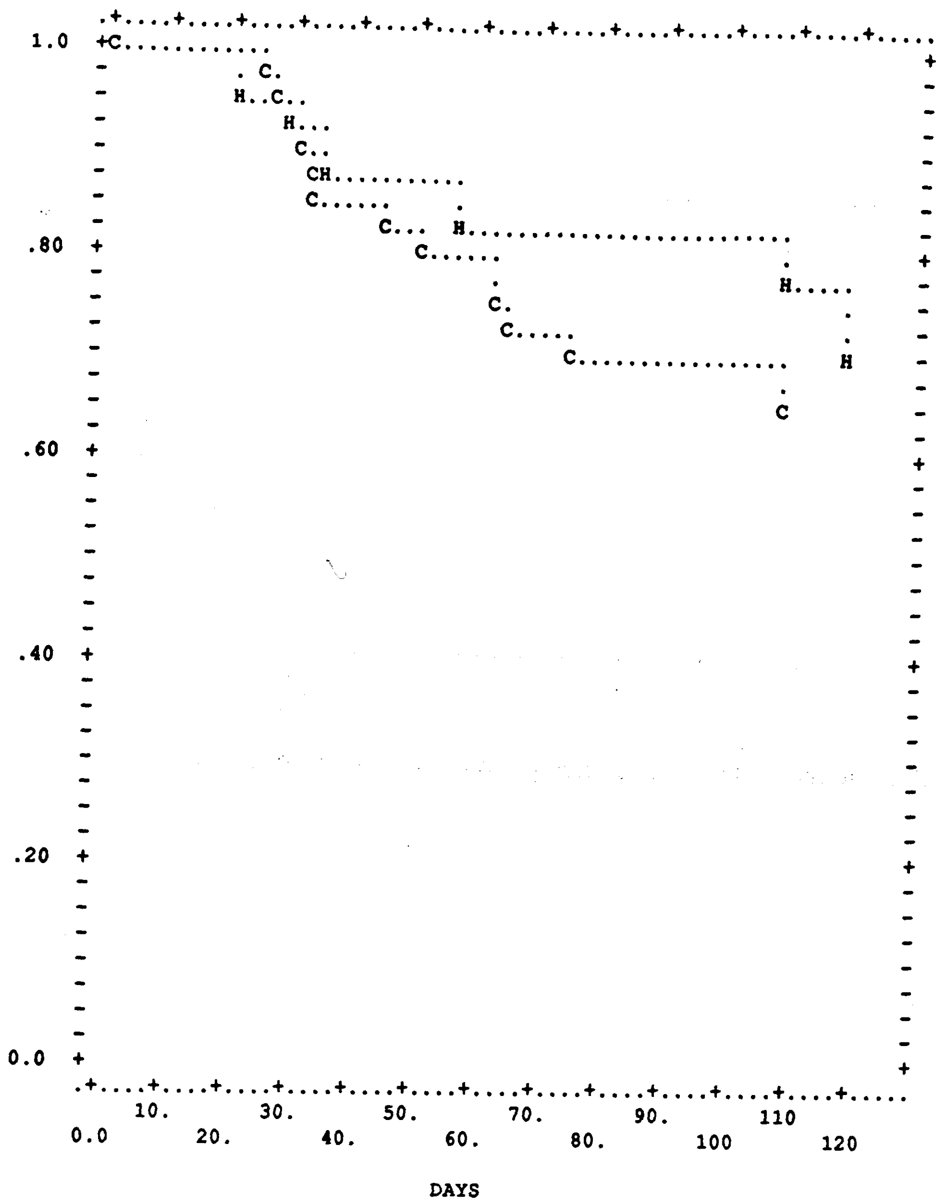


Figure 3.7: Profiles showing cumulative proportion of animals surviving in the hypertensive and the hypertensive-hypercholesterolemic groups when only cardiovascular deaths were counted.

The abscissa axis is the number of study days in reference to the renal operation date as Day 0, and the ordinate axis is the ratio of survivors in reference to the starting point as 1.0. Note that although there were more cardiovascular deaths in the hypertensive-hypercholesterolemic group as compared to the hypertensive group (32.4% vs 25.0%, respectively) , the difference did not reach significance.

H------ Hypertensive group.

C------ Hypertensive-hypercholesterolemic group.

Table 3. 2. Data of survivals and causes of death in each group of rabbits

Category	Control Gr.	Chol. Gr.	Ht. Gr.	Ht-Chol. Gr.
Total No. of rabbits	21	23	24	34
No. of CV deaths	1	0	6	11
% of CV deaths	4.8%	0%	25.0% *	32.4% *
No. of non-CV deaths	4	3	4	3
No. of survivals	16	20	14	20

* $p < 0.05$ vs Control group.

(Abbreviations: CV, cardiovascular; Chol, hypercholesterolemic; Ht, hypertensive.)

cellophane wrapping, while plasma cholesterol continued to rise from the time of introduction of the cholesterol diet, even up to the last sampling time (16-17 weeks).

2. Perinephritis hypertension did not change the plasma cholesterol profile, and vice versa, cholesterol feeding did not change the blood pressure profile, in the rabbit.

3. Cholesterol feeding or perinephritis hypertension generally did not change the profiles of body weight or heart rate.

4. Cholesterol feeding induced a marked degree of fatty deposition in the intimal surface of aorta and to a lesser degree, in the femoral artery. In contrast, there was little or no fatty deposition in the aorta or femoral artery in the "normal-diet" normotensive or hypertensive rabbits.

5. There was no difference in the likelihood of cardiovascular deaths between the control group and the hypercholesterolemic group. Both the hypertensive and the hypertensive-hypercholesterolemic groups had significantly more cardiovascular deaths than the control group. The hypertensive-hypercholesterolemic group tended to have more cardiovascular deaths than the hypertensive group, suggesting that hypercholesterolemia could augment cardiovascular mortality in the hypertensive animals. However, the difference between the hypertensive and the hypertensive-hypercholesterolemic groups was not significant in our study. The absence of significance could be due to small sample size. Much larger groups would be required to detect small differences between these groups.

Thus hypertension in this study was an important risk factor for cardiovascular deaths and hypercholesterolemia tended to augment this risk, although no synergism or interaction was detected in these two groups in terms of plasma cholesterol, blood pressure, heart rate, or body weight.

CHAPTER 4

PRESSOR AND DEPRESSOR RESPONSES IN HYPERTENSIVE AND HYPERCHOLESTEROLEMIC RABBITS

Chapter 4. Pressor and Depressor Responses in Hypertensive and Hypercholesterolemic Rabbits

4.1. Introduction

For several decades haemodynamic studies have played a crucially important part in hypertension research. Studies carried out on the offspring of hypertensive parents and in the early stages of hypertension in animal models have identified "predisposing characteristics" in terms of haemodynamic parameters which would subsequently lead to the established phase of hypertension, with increased peripheral resistance as its hallmark. Hence "neurogenic" and "volume" variants have been proposed as two main patterns of early haemodynamic events in primary hypertension (Folkow, 1982). On the other hand, studies on haemodynamic changes in established hypertension (either primary or secondary in etiology) are also essential in understanding the pathophysiology of hypertensive states. Investigations of the pressor or depressor responses to agonists such as noradrenaline, adrenaline, angiotensin II, or other neurotransmitters, hormones, or drugs have played a major part in these haemodynamic studies.

4.1.1 In vivo haemodynamic changes in response to various agonists in hypertension

a. α-Adrenoceptor agonists

In patients with essential hypertension, increased pressor responses to noradrenaline have been reported (Mendlowitz & Naftchi, 1958; Vlachakis, 1979). Similar observations have been made in several animal models of experimental hypertension, including spontaneously hypertensive rats (Hicks et al,1983), deoxycorticosterone-salt hypertensive and Goldblatt renal hypertensive rats (Finch & Haeusler, 1974), and perinephritis hypertensive rabbits (Hamilton & Reid, 1983).

Enhanced pressor responses to phenylephrine and other selective α_1-adrenoceptor agonists have also been shown in essential hypertensive patients (Jie et al, 1986), spontaneously hypertensive rats (Hicks et al, 1983), and perinephritis hypertensive rabbits (Hamilton & Reid, 1983). Thus there is an increased response to α_1-adrenoceptor agonists reported in various forms of hypertension. An enhanced α_2-adrenoceptor-mediated pressor response has also been observed in essential hypertensive patients (Jie et al, 1986), and perinephritis hypertensive rabbits (Hamilton et al, 1986).

b. β-Adrenoceptor agonists

Most published papers have reported a decreased responsiveness to bolus injections of isoproterenol in essential hypertensive patients in terms of heart rate changes (chronotropism) (Feldman, 1987); however, an increased or similar response to isoproterenol has been observed in borderline hypertensive patients (Messerli et al, 1976; London et al, 1976; Henquet et al, 1982). In spontaneously hypertensive rats, a decreased in vivo response to isoproterenol was seen in the "established phase", but not in the "developing phase" of hypertension (Toal & Leenen, 1984; Pfeffer et al, 1974). A reduced in vivo response to isoproterenol, measured as tail skin temperature changes, has also been observed in renal hypertensive rats (Katovich et al, 1978). Thus the picture with respect to β-adrenoceptor-mediated responses in vivo may vary with the stage of hypertension.

c. Angiotensin II

Enhanced pressor responses to angiotensin II have also been reported in essential hypertensive patients (Doyle & Black, 1955), spontaneously hypertensive rats (Hicks et al, 1983), and deoxycorticosterone-salt hypertensive rats (Finch, 1971). In contrast, Hamilton & Reid (1983) found similar pressor responses to angiotensin II

in perinephritis hypertensive and control normotensive rabbits, in the presence of increased pressor responses to noradrenaline and phenylephrine. Toal & Leenen (1985) observed a significantly decreased pressor response to angiotensin II in spontaneously hypertensive rats compared to Wistar-Kyoto rats. Thus the picture with regard to angiotensin II-mediated responses in vivo is confused.

d. Acetylcholine

Wright & Angus (1986) reported a significant reduction in "maximal dilatation" (but not sensitivity) to acetylcholine in the in vivo study of the iliac vascular beds in perinephritis hypertensive rabbits, whereas neither the sensitivity nor the reactivity to adenosine or serotonin was changed.

Recently, a loss of "endothelium-dependent" vascular relaxation to acetylcholine in essential hypertensive patients has been demonstrated in vivo, using forearm blood flow measurement, while the "endothelium-independent" vascular relaxation to sodium nitroprusside was not impaired (Panza et al, 1990; Linder et al, 1990). An impairment of endothelium-dependent vascular relaxation to acetylcholine was also reported in vivo in cerebral arterioles from stroke-prone spontaneously hypertensive rats (Mayhan et al, 1987).

e. Endothelin-1

Endothelin-1, a 21-sequence peptide isolated from cultured porcine endothelial cells, has been shown to be a highly potent and long-lasting vasoconstrictor in various vascular beds in a wide range of species (Yanagisawa & Masaki, 1989b). Conflicting results have been published with regard to in vivo pressor responses to endothelin in spontaneously hypertensive rats. Martel et al (1991) reported an increased pressor response, whereas Watanabe et al (1989) and Winquist et al (1989) observed a decreased response, while a similar response was found by others (Hirata et al, 1989; Eglen et al, 1989). In perinephritis hypertensive rabbits, we found a

greater maximum pressor response to endothelin-1 (Huang et al, 1990). Yokokawa et al (1990) also observed a greater pressor response to endothelin-1 in deoxycorticosterone-acetate-salt hypertensive rats.

f. Antagonists

There are also reports in which the in vivo dilator response to the calcium channel blocker verapamil (Robinson et al, 1982; Hulthen et al, 1982), or α-adrenoceptor antagonist prazosin (Amann et al, 1981) in the forearm resistance vessels was "enhanced" in essential hypertensive patients, whereas the response to sodium nitroprusside was similar in hypertensive patients and normotensive controls, suggesting a selective functional change in the arteries of essential hypertensive patients.

In summary, altered contraction or relaxation to a range of agonists and antagonists has been reported in established hypertension. However, although increased in vivo responsiveness is the most common finding, differences were observed depending both on the stimuli and the model of hypertension studied.

4.1.2. In vitro vascular responses to various agonists in hypertension

a. α-Adrenoceptor agonists

There are few in vitro vessel strip studies on human hypertension. Available data show a lack of evidence for "enhanced" α-adrenoceptor excitation-coupling in resistance arterioles or temporal arteries (Aalkjaer et al, 1987; Horwitz et al, 1974), with no change in sensitivity to noradrenaline or phenylephrine being observed.

Reports on the "reactivity" or "sensitivity" to α-adrenergic stimulation in arterial strips of spontaneously hypertensive rats have been inconsistent and contradictory (For review, see Bhalla et al, 1989). "Increased", "decreased" or "similar" maximal

tension development to noradrenaline has been reported by different groups. Similarly, reports on the sensitivity to noradrenaline are contradictory. Moreover, results from in vitro vessel strip studies are not always in line with in vivo studies or studies in perfused vascular beds (Triggle & Laher, 1985; Bhalla et al, 1989). It has been claimed that a specific α-adrenergic hypersensitivity in spontaneously hypertensive rats can be unmasked only after neuronal blockade (Mulvany et al, 1980; Bhalla et al, 1989).

In secondary, experimental hypertension, an "enhanced" α-adrenergic vasoconstrictor response in isolated strips or perfused vascular beds has been reported in various models of hypertension, including deoxycorticosterone/NaCl hypertension (Katovich et al, 1984; Finch, 1971), Goldblatt renal hypertension (Finch, 1971; Collis & Alps, 1975), and aldosterone-salt hypertension (Jones et al, 1988).

Thus the picture of in vitro vascular α-adrenergic responsiveness in primary hypertension is confused, whereas most studies on secondary hypertension show enhanced responsiveness.

b. β-Adrenoceptor agonists

A "decreased" vasodilator response to isoproterenol has been reported by most groups (For review, see Feldman, 1987). In contrast, Spector et al (1969) found an increased aortic relaxant response to isoproterenol in 7-to-10-week spontaneously hypertensive rats.

c. Angiotension II

Most studies on in vitro angiotensin II responsiveness in hypertension have been conducted in "isolated perfused vascular beds". A greater vasoconstrictor response to angiotensin II has been observed in mesenteric and renal arteries from spontaneously hypertensive rats (McGregor & Smirk, 1968; Collis & Vanhoutte, 1977), renal

arteries from stroke-prone spontaneously hypertensive rats (Berecek et al, 1980a), mesenteric arteries from Goldblatt renal hypertensive rats (McGregor & Smirk, 1968; Collis & Alps, 1975), and renal arteries from deoxycorticosterone hypertensive rats (Berecek et al, 1980b).

d. Serotonin

In animal studies, an increased in vitro vasoconstrictor response to serotonin was reported in aorta, mesenteric (Huzoor-Akbar et al, 1989) and hindquarter arteries (Cheng & Shibata, 1980) from spontaneously hypertensive rats, and aorta from stroke-prone spontaneously hypertensive rats (Turla & Webb, 1990), and femoral arteries from deoxycorticosterone-salt hypertensive rats (Mecca & Webb, 1984). In contrast, Spector et al (1969) found a decreased contractile response in aorta from spontaneously hypertensive rats.

e. Endothelin-1

Reports on vascular responses to endothelin-1 have also been controversial. Both Clozel (1989) and Martel et al (1991) found "an increase in sensitivity but a decrease in maximal tension" to endothelin-1 in aorta from 13-to-16-week spontaneously hypertensive rats, whereas Criscione et al (1990) showed "a similar sensitivity but decreased maximal tension" in aorta from spontaneously hypertensive rats of similar age. "An increase in sensitivity but similar maximal tension development" has been observed in mesenteric (Miyauchi et al, 1989) and renal (Tomobe et al, 1988) arteries from 12-week spontaneously hypertensive rats. In contrast, MacLean & McGrath (1990) reported " a similar sensitivity and maximal tension development" to endothelin-1 in isolated perfused tail artery from spontaneously hypertensive rats. In secondary hypertension, de Carvalho et al (1990) found " a similar sensitivity and maximal tension development" to endothelin-1 in aorta from Goldblatt renal

hypertensive, deoxycorticosterone-acetate-salt hypertensive, and control Wistar rats.

f. Acetylcholine

The impairment of "endothelium-dependent" vascular relaxation to acetylcholine has been shown in such vessel strips as (1) aorta from spontaneously hypertensive rats (Sunano et al, 1989; Konishi & Su, 1983), stroke-prone spontaneously hypertensive rats (Sunano et al, 1989), Dahl hypertensive rats (Luscher et al, 1987), and New Zealand genetic hypertensive rats (Winquist et al, 1984), (2) carotid arteries from spontaneously hypertensive rats (Hongo et al, 1988), and (3) resistance arteries from spontaneously hypertensive rats (Dohi et al, 1990) as well as stroke-prone spontaneously hypertensive rats (Tesfamariam & Halpern, 1988). The endothelium-independent vascular relaxation to sodium nitroprusside was not impaired in these studies.

In secondary hypertension, the impairment of endothelium-dependent vascular relaxation to acetylcholine has also been observed in aorta from coarctation hypertensive, renal hypertensive, and deoxycorticosterone-salt hypertensive rats. (Lockette et al, 1986; Van de Voorde et al, 1988), while the maximal relaxation to sodium nitroprusside was preserved.

4.1.3. Vascular reactivity and the duration of hypertension

Recently, more and more papers have been published on the effect of the duration of hypertension on vascular reactivity, in an attempt to understand the sequence of events in the evolution and development of haemodynamics from borderline hypertension to established hypertension in patients. Most longitudinal studies strengthen the observation that a primary increase in cardiac output is followed after a time by a secondary increase in total peripheral resistance, while cardiac output decreases (Weiss & Safar, 1989; Lund-Johansen & Omvik, 1990).

Weidmann et al (1980) observed that both the threshold dose and the mean

pressor dose (for increasing blood pressure by 20 mmHg) in noradrenaline dose-response curves were decreased slightly in borderline hypertensives and markedly in established essential hypertensives, as compared to normotensive subjects.

In spontaneously hypertensive rats, Toal & Leenen (1985) found that the the pressor responses to noradrenaline and angiotensin II changed in the direction of "increased" responsiveness at 4 to 6 weeks of age, "similar" responsiveness at 8 weeks, to "decreased" responsiveness at 14 to 16 weeks, as compared to age-matched Wistar-Kyoto rats. On the other hand, Smeda et al (1988) reported that there was a "decreased" sensitivity but enhanced contractile response to noradrenaline in the perfused kidneys of pre-hypertensive (4 to 5 weeks of age) spontaneously hypertensive rats, while at the established stage (21 weeks of age) of hypertension, there was a similar sensitivity but enhanced contractile response to noradrenaline in spontaneously hypertensive rats, as compared to their age-matched Wistar-Kyoto controls. Berecek et al (1980a) observed that there was a significant shift to the left of the dose-response curves to noradrenaline, angiotensin II, serotonin, and vasopressin in perfused kidneys of stroke-prone spontaneously hypertensive rats at 4 weeks, 2 months, and 4 months of age, as compared to their age-matched controls from Wistar-Kyoto rats, and the slopes and maximum responses of the dose-response curves became greater with age in the hypertensive rats.

Vanhoutte et al (1980) found an "increased" pressor response to exogenous noradrenaline in perfused kidneys of adult spontaneously hypertensive rats (4 to 6 months old), and a similar response to sympathetic nerve stimulation, as compared to Wistar-Kyoto rats; however, a similar response to exogenous noradrenaline but a greater pressor response to sympathetic nerve stimulation was observed in young spontaneously hypertensive rats (7 weeks old).

In secondary hypertension, Berecek et al (1980b) reported that enhanced renal

vascular reactivity to noradrenaline, angiotensin II, and vasopressin occurred at the pre-hypertensive stage (4 days after deoxycorticosterone-salt administration), and the enhanced vascular reactivity was further augmented at the established stage (61 days after). Collis & Alps (1975) also found an enhanced contractile response to noradrenaline and angiotensin II in perfused mesenteric arteries from the pre-hypertensive stage of Goldblatt renal hypertensive animals, while an increased contractile response to KCl occurred only at the established stage of hypertension.

On the other hand, it has been reported that there is a progressive decrease in the β-adrenoceptor (isoproterenol) sensitivity both with increasing blood pressure and increasing age in essential hypertensive patients (Bertel et al, 1980; London et al, 1976). A progressive impairment of relaxation response to isoproterenol from the pre-hypertensive stage, through the developing stage, to the established stage of hypertension has also been observed in aorta from both spontaneously hypertensive rats (Cheng & Shibata, 1981), and deoxycorticosterone-salt hypertensive rats (Katovich et al, 1984).

In comparing the endothelium-dependent vascular relaxation in spontaneously hypertensive rats versus Wistar-Kyoto rats, both hypertension and age were reported to be the determining factors of the degree of impaired relaxation (Hongo et al, 1988; Dohi et al, 1990; Sunano et al, 1989). Sunano et al (1989) also found that the age-related impairment of relaxation was greater in hypertensive rats.

In summary, there are many reports suggesting that the duration of hypertension is an important factor in aggravating the impairment of endothelium-dependent vascular relaxation and β-adrenoceptor-mediated responses. The effect of the duration of hypertension on vasoconstrictive responses to noradrenaline or angiotensin II reported so far is not a consistent picture.

4.1.4. In vivo haemodynamic changes in response to various agonists in hypercholesterolemia or atherosclerosis

There are numerous reports of altered vascular reactivity to agonists in hypercholesterolemic or atherosclerotic humans and animals, and it has been suggested that such changes could contribute to vasospasm and sudden death (Lopez et al, 1989a; Shimokawa et al, 1988; Shimokawa & Vanhoutte, 1989).

a. α-adrenoceptor agonists

An enhanced in vivo pressor response to α-adrenoceptor agonists such as noradrenaline and phenylephrine has been reported in atherosclerotic rabbits (Hof & Hof, 1988). However, when specific vascular beds are examined, different results are observed. In hypercholesterolemic but not yet atherosclerotic animals, a greater in vivo vasoconstrictor response to noradrenaline was observed in the coronary artery from the dog (Rosendorff et al, 1981) and the hindlimb small arteries from cynomolgus monkeys (Heistad et al, 1984). Whereas, in atherosclerotic cynomolgus and rhesus monkeys, the vasoconstrictor response to noradrenaline and phenylephrine in hindlimb arteries was similar in atherosclerotic and control animals (Heistad et al, 1984; Armstrong et al, 1985). Lopez et al (1989b) observed a greater in vivo vasoconstrictor response to phenylephrine in colon vascular beds from atherosclerotic cynomolgus monkeys. Thus the picture with α_1-adrenergic vasoconstrictor response may differ with regard to the duration of cholesterol feeding and extent of atherosclerosis (Heistad et al, 1984) as well as the vascular beds examined (Heistad et al, 1984; Lopez et al, 1989a).

b. Angiotensin II

Hof & Hof (1988) reported an increased in vivo pressor response to angiotensin II in atherosclerotic rabbits, whereas Armstrong et al (1985) found a similar response to angiotensin II in perfused hindlimb arteries from atherosclerotic and control monkeys.

c. Serotonin

In hindlimb arteries from normal cynomolgus monkeys, serotonin caused vasodilation, but this may be impaired and converted to vasoconstriction in atherosclerotic animals (Heistad et al, 1984; Lopez et al, 1989a). Potentiation of the vasoconstrictor response to serotonin was also observed in cerebral, ocular, and mesenteric arteries (Faraci et al, 1989; Lopez et al, 1989b). Duration of the atherogenic diet was a factor in altering the serotonin response in hypercholesterolemic monkeys (Heistad et al, 1984; Lopez et al, 1989a). Wright & Angus (1986) reported an almost twofold increase in sensitivity for vasodilation to serotonin in hindlimb arteries from rabbits fed on 1% cholesterol diet for 4 weeks, but the maximal dilation was unchanged.

d. Thromboxane A_2

Potentiation of the vasoconstrictor responses to thromboxane A_2 has also been shown in hindlimb, cerebral, and ocular arteries from atherosclerotic monkeys (Lopez et al, 1989a; Faraci et al, 1989). Lopez et al (1990) also observed the potentiation of vasoconstrictor response to endothelin-1 in hindlimb arteries from atherosclerotic monkeys.

e. Acetylcholine

Impairment of endothelium-dependent vascular dilatation to acetylcholine (Ludmer et al, 1986) and increased blood flow (Cox et al, 1989) was observed in vivo in human atherosclerotic coronary arteries, while endothelium-independent vascular dilation to nitroglycerin was preserved. Similarly, impaired vasodilation to acetylcholine but not nitroprusside was demonstrated in vivo in hindlimb arteries and resistance arteries from atherosclerotic rabbits fed on 1% or 2% cholesterol diet for 8-12 weeks (Girerd et al, 1990; Bossaller et al, 1987a; Yamamoto et al, 1988). Lopez et al (1989a) also found that endothelium-dependent vascular dilation to

adenosine 5'-diphosphate was impaired in hindlimb arteries from hypercholesterolemic as well as atherosclerotic monkeys. Creager et al (1990) reported that in forearm resistance vessels from hypercholesterolemic men, both endothelium-dependent and endothelium-independent vasodilation (to metacholine and nitroprusside, respectively) were impaired, while the vasoconstrictor response to phenylephrine was not different from that in normocholesterolemic controls.

4.1.5. In vitro vascular responses to various agonists in hypercholesterolemia and atherosclerosis

a. α-Adrenoceptor agonists

Kalsner & Richards (1984) reported an enhanced contractile response to noradrenaline in coronary arteries from patients who died of coronary artery disease as compared to those who died of non-cardiovascular disease. In cholesterol-fed hypercholesterolemic rabbits, contractile responses to noradrenaline in aorta have been reported to exhibit "increased" (Heric & Tackett, 1985), "similar" (Simonsen et al, 1991; Kishi & Numano, 1984), or "decreased" responsiveness (Ibengwe & Suzuki, 1986; Verbeuren et al, 1986). Wines et al (1989) found a decreased contractile response to noradrenaline in aorta from both Watanabe heritable hyperlipidemic rabbits and cholesterol-fed New Zealand white rabbits as compared to normal-diet rabbits. In other vascular strips, the picture is also inconsistent. In cholesterol-fed rabbits, an increased contractile response to noradrenaline in carotid arteries (Tesfamariam et al, 1989), or an increased sensitivity but similar maximal response to noradrenaline in basilar arteries (McCalden & Nath, 1989), or a decreased sensitivity but similar maximal response to noradrenaline in femoral arteries (Simonsen et al, 1991), have been reported by different groups. As for the other α-adrenergic agonist, phenylephrine, a similar contractile response in aorta from cholesterol-fed atherosclerotic rabbits (Henry & Yokoyama, 1980) or a

decreased response in aorta from Watanabe heritable hyperlipidemic rabbits (Kolodgie et al, 1990) has been reported.

b. Serotonin

Vascular responses to serotonin or ergonovine in hypercholesterolemic or atherosclerotic coronary arteries have aroused particular interest because of their potential pathophysiological implications in coronary vasospasm and sudden death (Schroeder et al, 1977). Kalsner & Richards (1984) found a distinctively enhanced contractile response to serotonin and histamine in coronary arteries from patients who died of coronary heart disease. In animal studies, an enhanced contractile response to serotonin has been observed in (A) aorta from diet-induced hypercholesterolemic or atherosclerotic rabbits (Merkel et al, 1990; Kishi & Numano, 1984; Heric & Tackett, 1985), (B) aorta from Watanabe heritable hyperlipidemic rabbits (Yokoyama et al, 1983; Wines et al, 1989), (C) coronary arteries from diet-induced hypercholesterolemic-atherosclerotic pigs (Shimokawa & Vanhoutte, 1989).

In contrast, similar contractile responses to serotonin were reported in aorta, or small cerebral, mesenteric, and femoral arteries from cholesterol-fed atherosclerotic rabbits as compared to their controls from normal-diet rabbits (Verbeuren et al, 1986; Simonsen et al, 1991).

c. Ergonovine

An enhanced contractile response to ergonovine has been observed in aorta from cholesterol-fed New Zealand white rabbits (Henry & Yokoyama, 1980) as well as from Watanabe heritable hyperlipidemic rabbits (Yokoyama et al, 1983).

d. Histamine

An enhanced contractile response to histamine in aorta from cholesterol-fed New

Zealand white rabbits (Heric & Tackett, 1985) and Watanabe heritable hyperlipidemic rabbits (Kolodgie et al, 1990) was observed by some groups, whereas Wines et al (1989) found a decreased contractile response in aorta from Watanabe heritable hyperlipidemic rabbits.

e. Angiotensin II

Either "increased" or "similar" maximum contraction to angiotensin II has been reported in aorta from hypercholesterolemic or atherosclerotic rabbits (Merkel et al, 1990; Kishi & Numano, 1984).

f. β-Adrenoceptor agonists

Vaso-relaxation responses to the β-adrenoceptor agonist, isoproterenol, were shown to be similar in coronary arteries from atherosclerotic patients and non-atherosclerotic patients by one group (Forstermann et al, 1988), but another group (Berkenboom et al, 1987) reported a significantly reduced response in atherosclerotic coronary arteries. Merkel et al (1990) also found that in modestly hypercholesterolemic rabbits (plasma cholesterol level 223 mg/dl), vaso-relaxation to isoproterenol was impaired in pre-constricted aorta.

g. Acetylcholine

The impairment of endothelium-dependent vaso-relaxation to acetylcholine or other agonists in isolated arteries from hypercholesterolemic or atherosclerotic humans and animals has been reported by many researchers. The impairment has been shown in (A) human atherosclerotic coronary arteries (Berkenboom et al, 1987; Bossaller et al, 1987b; Forstermann et al, 1988), (B) hypercholesterolemic or atherosclerotic rabbit aorta (Bossaller et al, 1987b; Guerra et al, 1989; Jayakody et al, 1987; Ibengwe & Suzuki, 1986; Verbeuren et al, 1986; Simonsen et al, 1991),

coronary arteries (Osborne et al, 1989; Vrints et al, 1990), and intracranial arteries (Kanamaru et al, 1989), (C) aorta from Watanabe heritable hyperlipidemic rabbits (Kolodgie et al, 1990), (D) hypercholesterolemic or atherosclerotic pig coronary arteries (Cohen et al, 1988; Shimokawa & Vanhoutte, 1989), and cerebral arteries (Shimokawa et al, 1988), (E) coronary arteries (Sellke et al, 1990) and iliac arteries (Friedman et al, 1986) from cholesterol-fed atherosclerotic monkeys. Most of these reports showed that the endothelium-independent vascular relaxation to nitroprusside or isoproterenol or nitroglycerin was preserved. As stressed by the Vanhoutte group, the impairment of endothelium-dependent vascular relaxation to aggregating platelets and related vasoactive substances in coronary and cerebral arteries may predispose these hypercholesterolemic or atherosclerotic animals to coronary or cerebral vasospasm, thus having significant pathophysiological implications associated with hypercholesterolemia or atherosclerosis (Shimokawa et al, 1988; Shimokawa & Vanhoutte, 1989).

In summary, there are many reports suggesting that hypercholesterolemia or atherosclerosis may alter the in vitro vascular responses of isolated arteries. The picture of change differs with regard to duration or severity of hypercholesterolemia, species of animal, vascular tissues examined, as well as agonists employed for study. To further complicate the issue, in vitro results are not always consistent with in vivo results.

4.1.6. Vascular reactivity and the duration of hypercholesterolemia and atherosclerosis

As mentioned briefly in the previous section, duration of hypercholesterolemia plays an important part in the vascular reactivity to agonists. Heric & Tackett (1985) reported that in rabbits fed on 0.6% cholesterol diet, the augmented contractile responses to noradrenaline, histamine, and serotonin were most prominent at 4

weeks of cholesterol feeding; the augmented contractile responses were attenuated with longer duration of cholesterol feeding (6 and 8 weeks), as compared to age-matched normal-diet rabbits. Lopez et al (1989a) compared the in vivo vascular responses of monkeys after 4,9, and 19 months of atherogenic diet with age-matched control animals, and found that with a longer duration of atherogenic diet, the vasoconstrictor response to serotonin was progressively potentiated, while the vasodilator response to adenosine 5'-diphosphate was progressively attenuated. Thus vascular responses to serotonin, adenosine 5'-diphosphate, and thromboxane A_2 analogue, all of which are products released after platelet aggregation, were altered in a direction that would favour greater vasoconstriction with longer duration of atherogenesis.

A progressive impairment of endothelium-dependent relaxation with longer duration of atherogenic diet has also been observed in rabbits (Verbeuren et al, 1986; Kanamaru et al, 1989) and monkeys (Lopez et al, 1989a), while the endothelium-independent relaxation was largely preserved. Kolodgie et al (1990) also found that the endothelium-dependent relaxation in aorta from Watanabe heritable hyperlipidemic rabbits was progressively impaired as the severity of intimal lesions increased with age.

Thus with the exception of the study of Heric & Tackett (1985), responses were more severely modified the longer the duration of atherogenesis.

In summary, both hypertension and hypercholesterolemia result in alterations in response to a range of agonists. The degree and direction of changes appear to depend on the agonist, the model studied, and the duration of disease. Impaired responses to acetylcholine and serotonin have received prominence in atherosclerosis, while in hypertension, increased responses to a wide range of contractile agonists including the endogeneous vasoconstrictors noradrenaline and

angiotensin II have been extensively reported.

In man, the two disease states are frequently found in conjunction with one another and could interact resulting in increased contraction and decreased relaxation and an enhanced risk of coronary vasospasm. Against this background, responses to a range of agonists have been examined during the development of hypertension and hypercholesterolemia, alone or in combination, in the rabbit.

4.2 Materials and methods

For a detailed description of the four groups of rabbits, see the "Overall Study Design" Chapter.

Groups of rabbits were studied at 14-21 days, 42-49 days, and 91-112 days postoperatively. An arterial line was inserted under local anaesthesia in one ear, for blood pressure and heart rate measurement, and a venous line for drug administration in the other. Basal mean arterial pressure and heart rate were obtained when the rabbit had been left for 60 minutes to recover after the insertion of the catheters. Each animal was conscious and was kept in an individual cage in a quiet warm laboratory throughout the study. Pressor and depressor responses to a range of agonists were measured in each rabbit. At most two drugs were given in one day to the same animal, with a 60 minute interval in between. The protocol for drug administration was as detailed below:

14-21 Days (2-3 Weeks) Study:

(A) Noradrenaline: 0.05, 0.10, 0.50, 3.00, & 10.0 μg/kg.

(B) Angiotensin II: 0.0005, 0.0010, 0.0050, 0.0200, 0.100, & 0.300 μg/kg.

(C) Acetylcholine: 0.05, 0.10, 0.30, 1.00, 3.00, & 10.0 μg/kg.

(D) Nitroprusside: 2.5, 5.0, 10.0, & 20.0 μg/kg/min, each dosage given for 10 minutes via a Braun infusion pump and the rate increased stepwise.

(E) Isoproterenol: 0.05, 0.10, & 0.30 μg/kg.

42-49 Days (6-7 Weeks) Study:

(A) Noradrenaline: 0.05, 0.10, 0.50, 1.00, 3.00, & 5.00 μg/kg.

(B) Angiotensin II: 0.0005, 0.0010, 0.0050, 0.0200, 0.100, & 0.300 μg/kg.

(C) Endothelin-1: 0.03, 0.05, 0.10, & 0.20 nmol/kg.

(D) Acetylcholine: 0.05, 0.10, 0.30, 1.00, 3.00, & 10.0 μg/kg.

(E) Nitroprusside: 2.5, 5.0, 10.0, & 20.0 μg/kg/min, each dosage given for 10 minutes via a Braun infusion pump and the rate increased stepwise.

(F) Isproterenol: 0.05, 0.10, & 0.30 μg/kg.

91-112 Days (13-16 Weeks) Study:

(A) Noradrenaline: 0.05, 0.10, 0.30, 1.00, 3.00, & 10.0 μg/kg.

(B) Angiotensin II: 0.0005, 0.0010, 0.0050, 0.0200, 0.100, & 0.300 μg/kg.

(C) Endothelin-1: 0.10 nmol/kg.

(D) Acetylcholine: 0.05, 0.10, 0.30, 1.00, 3.00, & 10.0 μg/kg.

The drugs given each day to rabbits were randomized and, except for nitroprusside, the doses of each agonist were given in random order. After the injection of endothelin-1, blood pressure was monitored continuously for the first 10 minutes and thereafter every 5 minutes for at least 30 minutes.

Most animals were studied at 2 out of the 3 time points. The animals could only be used a limited number of times, and it was not humanely possible to study all drugs in all rabbits at all times. In addition, some animals died during the course of the 16-week experimental period. It had been hoped to study all drugs at all time points; however, this was not possible within the time constraints of this thesis.

Noradrenaline, isoproterenol, acetylcholine, and angiotensin II were purchased from Sigma Chemical Co. (Poole, U.K.). Sodium nitroprusside was from Roche

Products (Welwyn Garden City, U.K.). Endothelin-1 was from Scientific Marketing (Barnet, U.K.). Noradrenaline and isoproterenol were prepared in 0.1% ascorbic acid. Acetylcholine and angiotensin II were dissolved in 0.9% saline. Sodium nitroprusside was prepared in 5% dextrose and protected from light. Endothelin-1 was prepared as a 10^{-4} M stock solution and 50 μl aliquots stored at -20 °C. All drug solutions were prepared fresh on the study day.

Pressor or depressor responses were examined in terms of changes in mean arterial pressure (△MAP). Each experimental group was compared to the control group using repeated measures analysis of variance for each agonist. When the hypertensive and the hypertensive-hypercholesterolemic groups were compared to the control group, repeated measures analysis of covariance with basal mean arterial pressure as covariate were also carried out to examine whether the starting blood pressure could account for any differences. In the case of isoproterenol, changes in heart rate (△ HR) were also compared. All results were expressed as Mean ± S.D. $p < 0.05$ was considered statistically significant.

4.3. Results

4.3.1. 2-3 Weeks Study:

(A) Noradrenaline

There was a greater pressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in blood pressure changes (△ MAP) reached significance at all doses except the highest for the hypertensive group, and at all doses for the hypertensive-hypercholesterolemic group versus the control group, but when examined in relation to the starting mean arterial pressure (analysis of covariance), this difference did not remain significant. (Tables 4.1 & 4.2)

There was no difference in the pressor response between the hypercholesterolemic and control groups. (Figure 4.1)

(B) Angiotensin II

There was a greater pressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in △MAP reached significance at 0.0005, 0.001, 0.005, 0.020, & 0.100 µg/kg for the hypertensive group, and at 0.0005, 0.001, 0.005, & 0.020 µg/kg for the hypertensive-hypercholesterolemic group versus the control group. When examined in relation to the starting mean arterial pressure, this difference did not remain significant. (Tables 4.1 & 4.2)

There was no difference in the pressor response between the hypercholesterolemic and control groups. (Figure 4.2)

(C) Acetylcholine

There was a greater depressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in △MAP reached significance at all dose points for the hypertensive group and at all dose points except the highest (10.0 µg/kg) for the hypertensive-hypercholesterolemic group versus the control group. Moreover, when examined in relation to the starting mean arterial pressure, these significant differences persisted. (Tables 4.3 & 4.4)

There was no difference in the depressor response between the hypercholesterolemic and control groups. (Figure 4.3)

(D) Nitroprusside

There was a greater depressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in △MAP reached significance at all infusion doses for both groups

versus the control group. When examined in relation to the starting mean arterial pressure, this difference remained significant only at the lowest dose (2.5 μg/kg/min for 10 minutes). (Tables 4.3 & 4.4)

There was no difference in the depressor response between the hypercholesterolemic and control groups. (Figure 4.4)

(E) Isoproterenol

There was a greater depressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in ΔMAP reached significance at all three dose points versus the control group. Moreover, when examined in relation to the starting mean arterial pressure, these significant differences persisted. (Tables 4.3 & 4.4)

There was no difference in the depressor response between the hypercholesterolemic and control groups. (Figure 4.5)

The chronotropic response (ΔHR) was also analyzed. There was no difference in chronotropic response in the hypertensive, hypercholesterolemic, or hypertensive-hypercholesterolemic groups as compared to the control group (Figure 4.6).

4.3.2. 6-7 Weeks Study:

(A) Noradrenaline

There was a greater pressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in ΔMAP reached significance at 0.05, 0.10, 0.50, & 1.00 μg/kg for the hypertensive group, and at 0.10 & 0.50 μg/kg for the hypertensive-hypercholesterolemic group versus the control group. When examined in relation to the starting mean arterial pressure, the significant difference persisted for the hypertensive-hypercholesterolemic group, but not for the hypertensive group. (Tables 4.1 & 4.2)

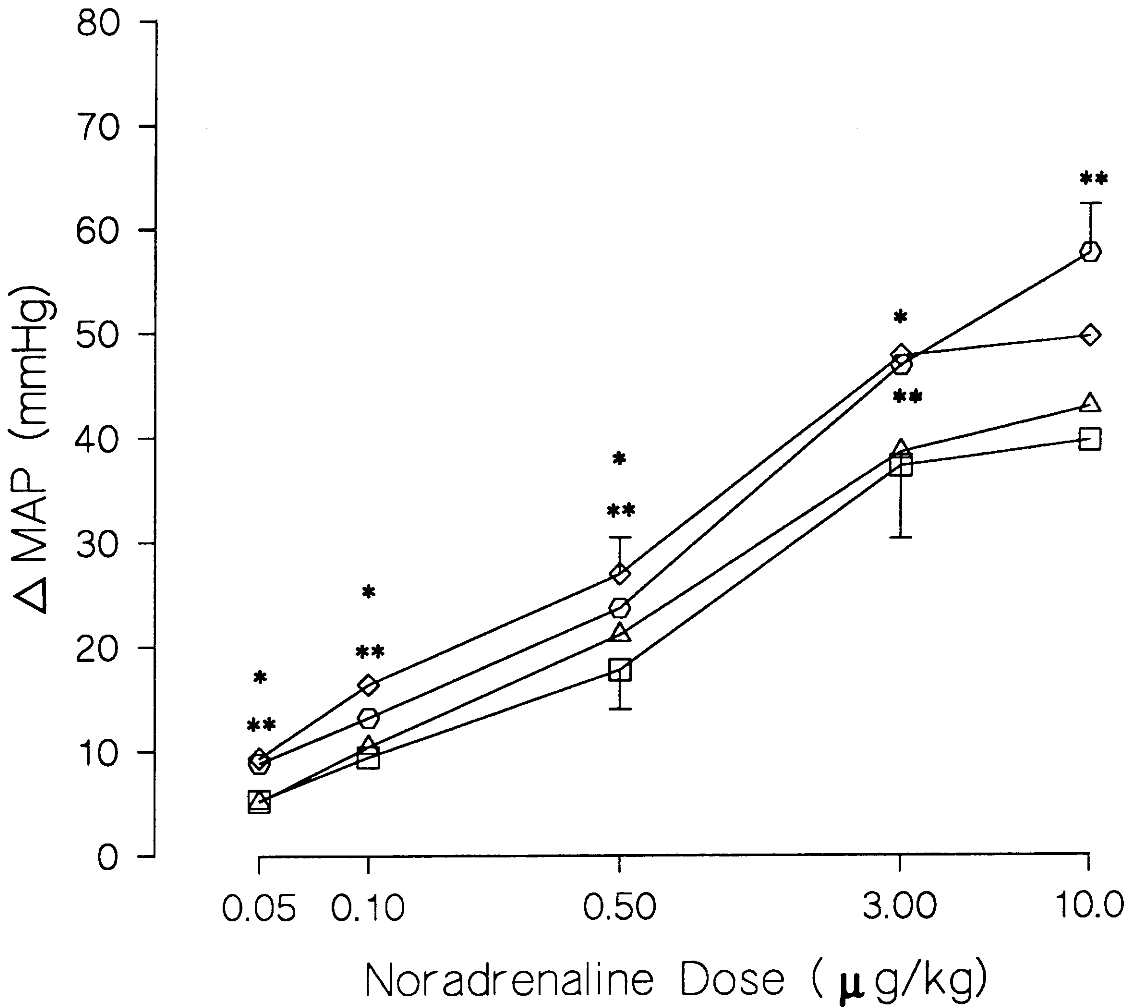


Figure 4.1: Changes in blood pressure (Δ MAP) in response to noradrenaline in groups of rabbits 2-3 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 6 - 9. * & **, p < 0.05 for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively.)

- □ **Control group.**
- △ **Hypercholesterolemic group.**
- ◇ **Hypertensive group.**
- ○ **Hypertensive-hypercholesterolemic group.**

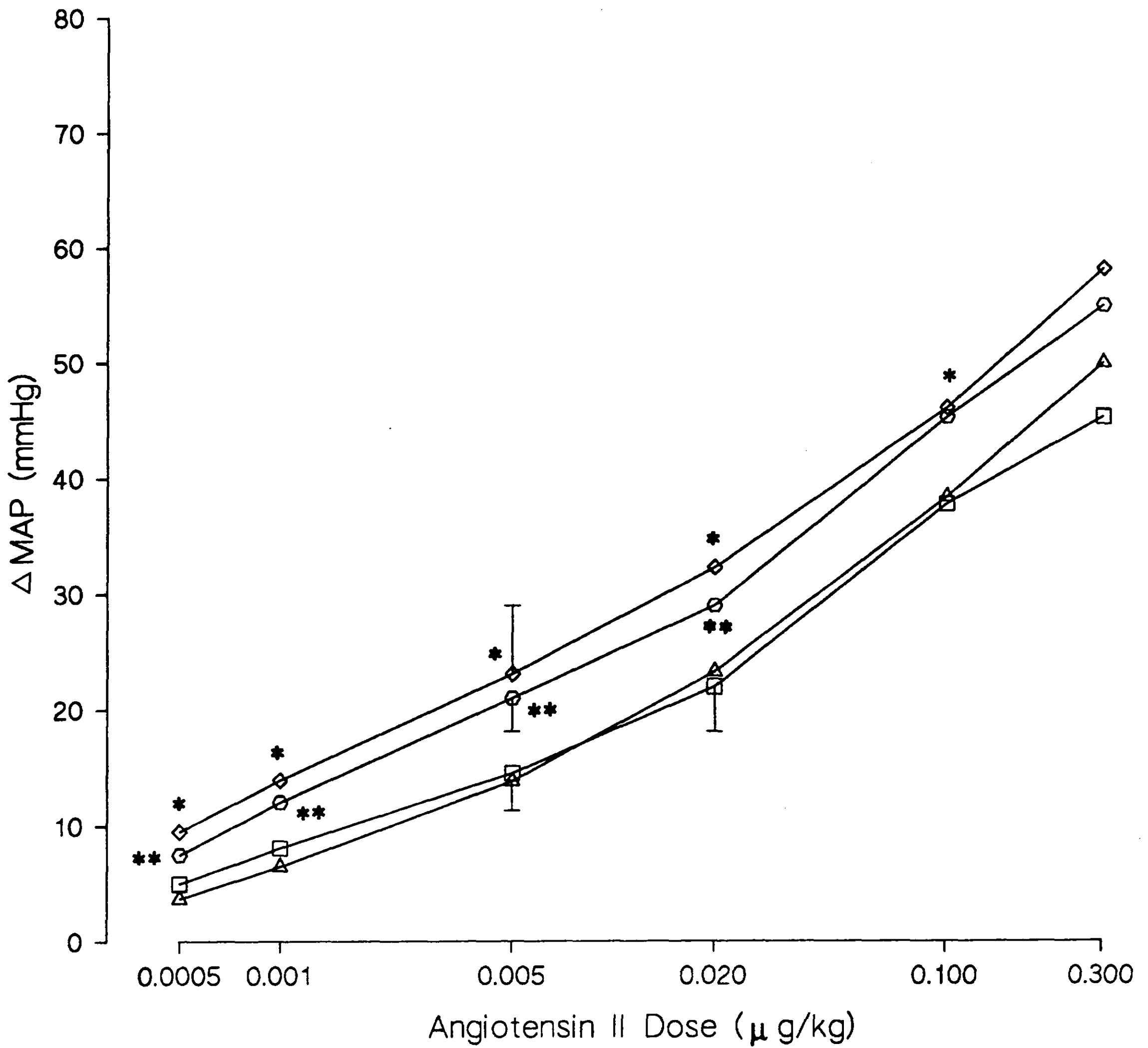


Figure 4.2: Changes in blood pressure (ΔMAP) in response to angiotensin II in groups of rabbits 2-3 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 8 - 9. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

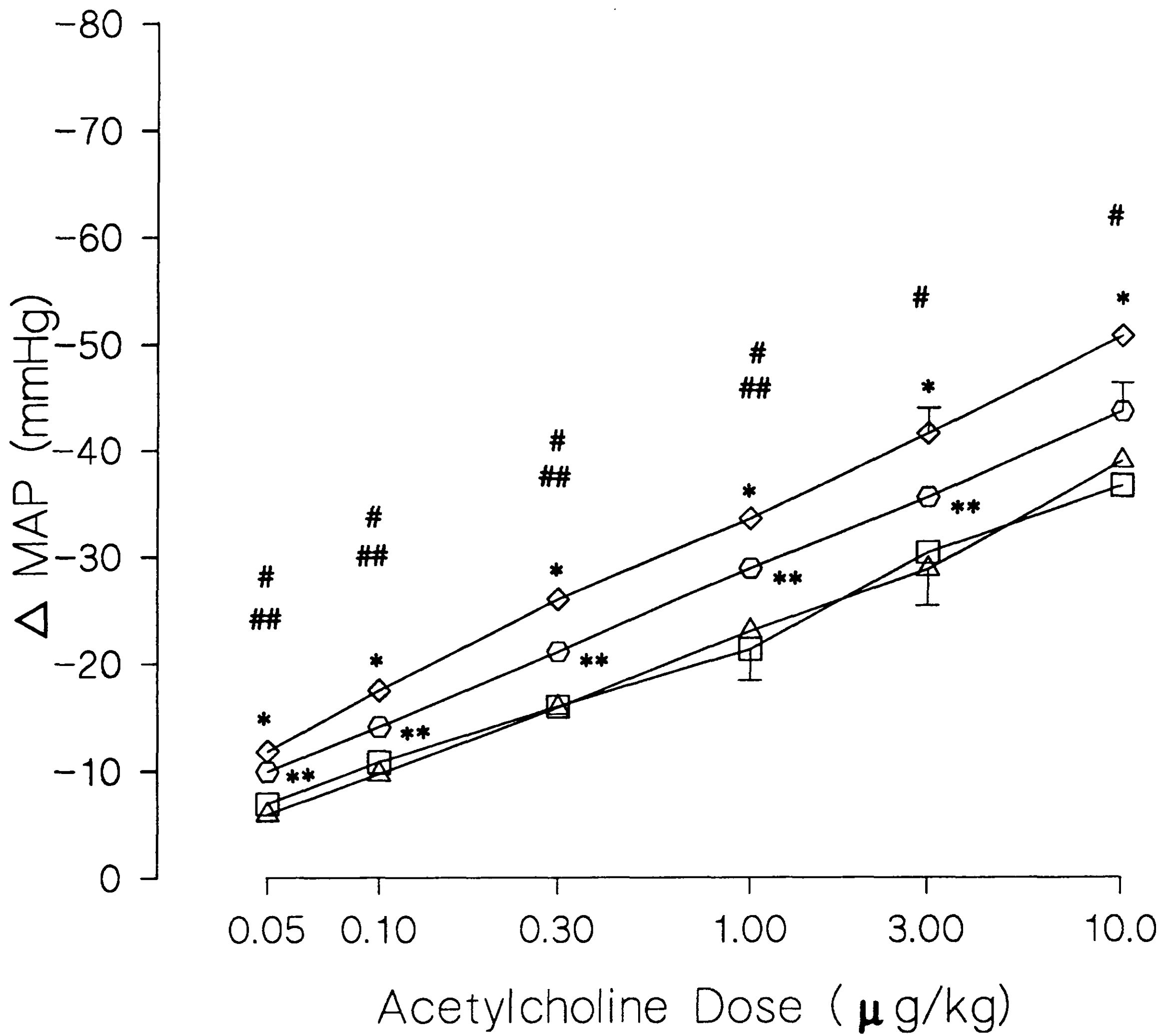


Figure 4.3: Changes in blood pressure (ΔMAP) in response to acetylcholine in groups of rabbits 2-3 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 7 - 9. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

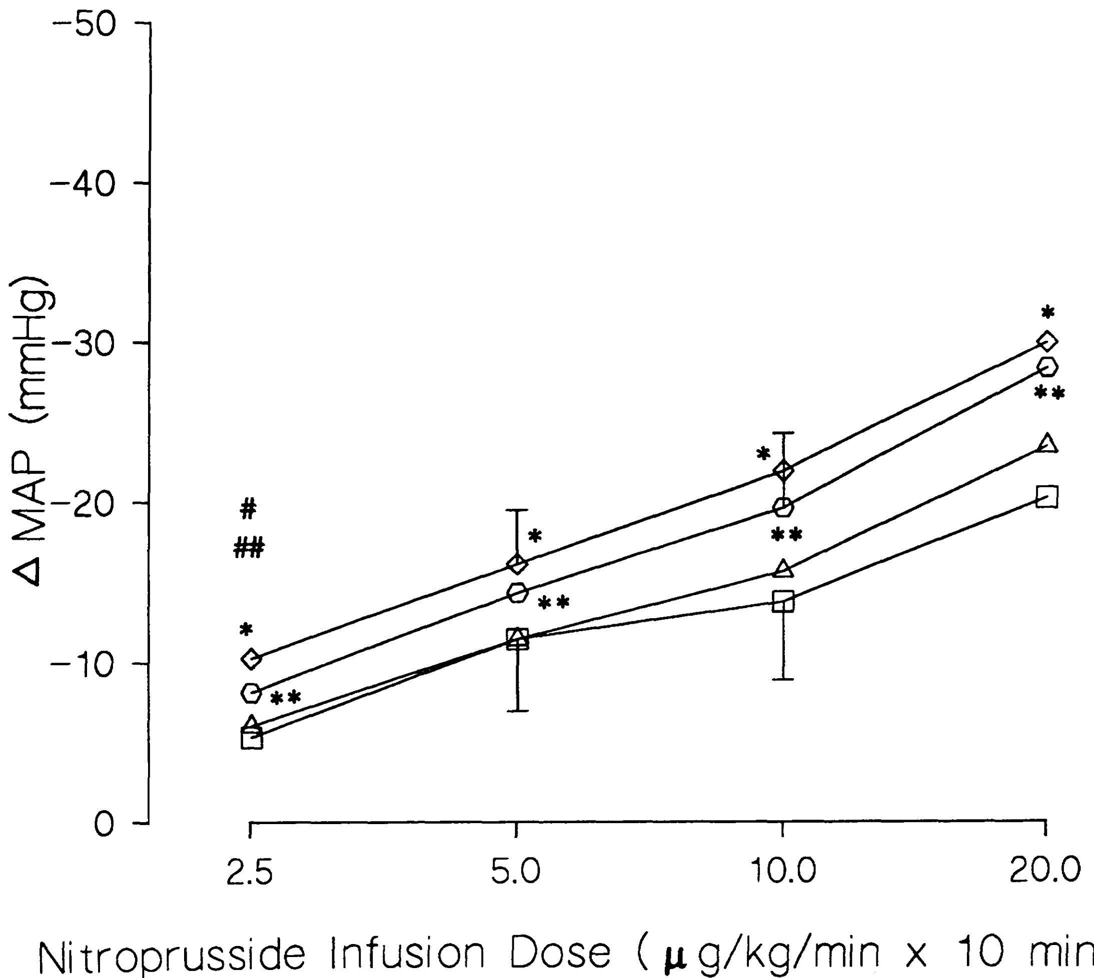


Figure 4.4: Changes in blood pressure (Δ MAP) in response to nitroprusside infusion in groups of rabbits 2-3 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 6 - 10. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

Δ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

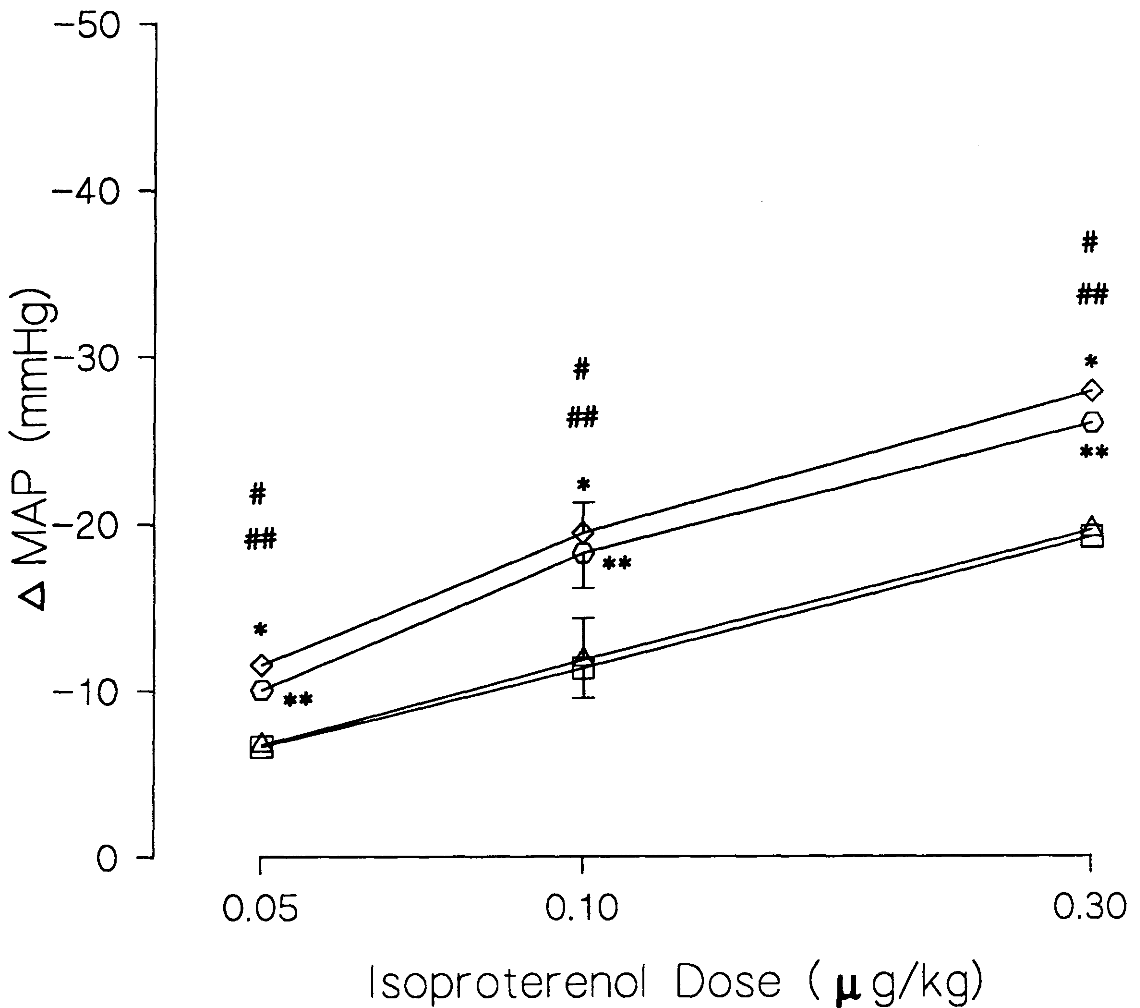


Figure 4.5: Changes in blood pressure (ΔMAP) in response to isoproterenol in groups of rabbits 2-3 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 6 - 9. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

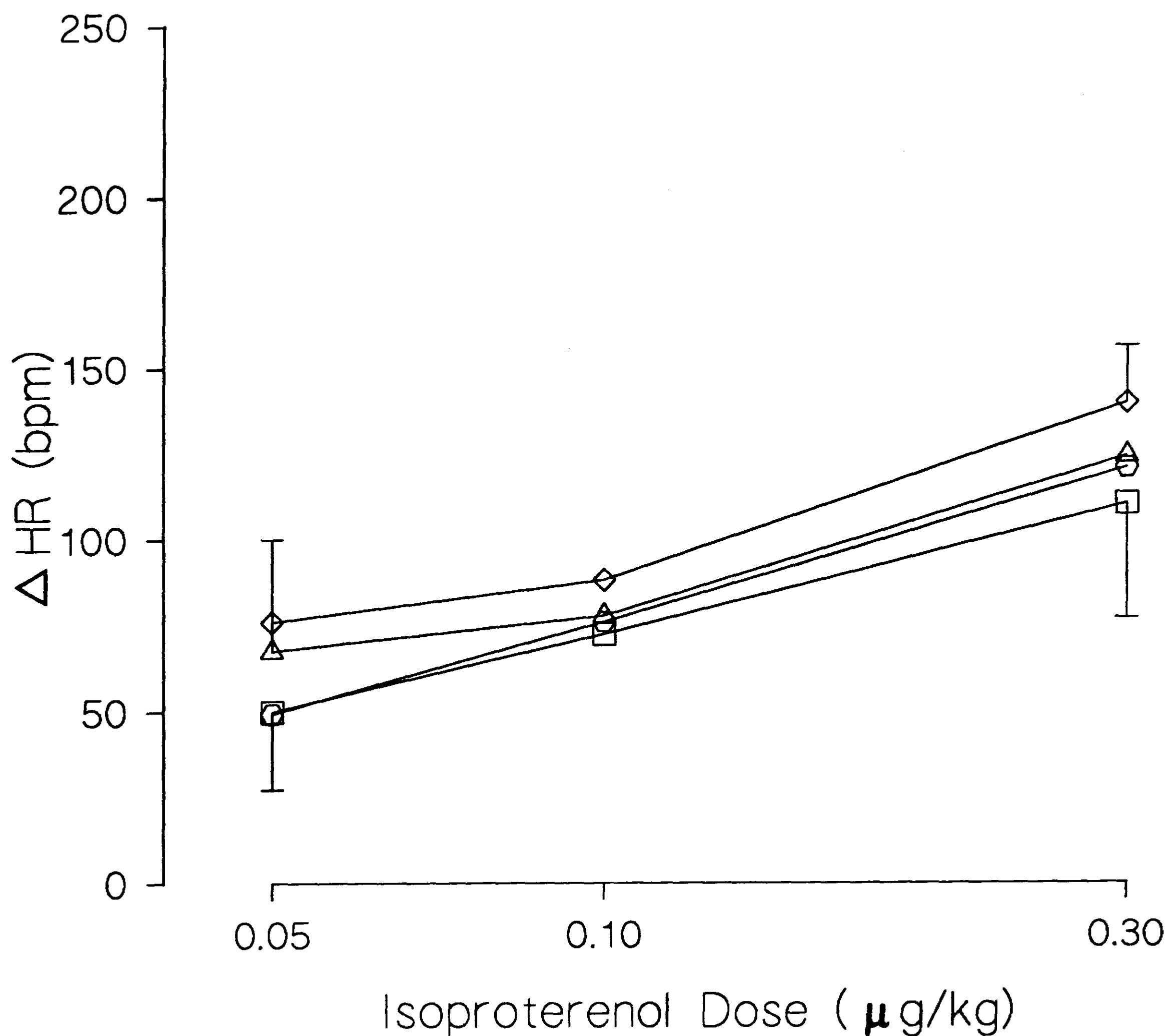


Figure 4.6: Changes in heart rate (Δ HR) in response to isoproterenol in groups of rabbits 2-3 weeks after surgery and dietary manipulation.

There is no difference in the chronotropic responsiveness (ΔHR) to isoproterenol between the control group and any of the experimental groups. (Mean ± S.D., n = 6 - 9.)

□ Control group.

Δ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

Table 4.1: 95% confidence intervals for the differences between the hypertensive vs the control groups and the hypertensive-hypercholesterolemic vs the control groups, respectively, in the pressor responses using analysis of variance.

	2 weeks		6 weeks		13-16 weeks	
	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C
NA (µg/kg):						
0.05	(0.3, 0.8)*	(0.2, 0.7)*	(0.005, 1.0)*	(-0.02, 0.9)	(0.4, 0.7)*	(0.4, 0.7)*
0.10	(0.3, 0.8)*	(0.1, 0.5)*	(0.02, 0.7)*	(0.2, 0.9)*	(0.4, 0.7)*	(0.3, 0.6)*
0.30	---	---	---	---	(0.01, 0.3)*	(0.5, 0.8)*
0.50	(0.2, 0.7)*	(0.01, 0.5)*	(0.08, 0.8)*	(0.2, 0.9)*	---	---
1.00	---	---	(0.005, 0.8)*	(-0.01, 0.8)	(0.4, 0.7)*	(0.4, 0.7)*
3.00	(0.03, 0.5)*	(0.06, 0.5)*	(-0.5, 0.7)	(-0.3, 0.5)	(0.3, 0.7)*	(0.3, 0.6)*
5.00	---	---	(-0.8, 1.1)	(-0.4, 0.8)	---	---
10.0	(-0.3, 0.8)	(0.1, 0.7)*	---	---	(0.2, 0.9)*	---
AII (µg/kg):						
0.0005	(0.3, 0.8)*	(0.03, 0.6)*	(0.4, 0.8)*	(0.4, 0.8)*	(0.7, 0.9)*	(0.6, 0.9)*
0.0010	(0.3, 0.8)*	(0.2, 0.6)*	(0.4, 0.7)*	(0.4, 0.7)*	(0.5, 0.8)*	(0.5, 0.8)*
0.0050	(0.3, 0.7)*	(0.2, 0.6)*	(0.4, 0.7)*	(0.3, 0.6)*	(0.5, 0.8)*	(0.4, 0.7)*
0.0200	(0.2, 0.6)*	(0.02, 0.5)*	(0.3, 0.6)*	(0.2, 0.5)*	(0.4, 0.6)*	(0.3, 0.6)*
0.100	(0.01, 0.5)*	(-0.02, 0.4)	(0.2, 0.5)*	(0.2, 0.5)*	(0.2, 0.5)*	(0.2, 0.5)*
0.300	(-0.08, 0.5)	(-0.2, 0.4)	(0.07, 0.6)*	(0.001, 0.7)*	(0.03, 0.5)*	(0.01, 0.5)*
ET-1 (nmol/kg):						
0.03			(2.1, 11.5)*	(0.9, 10.6)*	---	---
0.05			(-0.2, 12.1)	(-1.5, 10.3)	---	---
0.10			(-0.7, 15.1)	(-1.8, 13.6)	(0.7, 20.1)*	(2.2, 22.7)*
0.20			(-0.8, 20.8)	(-4.7, 16.3)	---	---

Note: *, significantly different between the groups ($p < 0.05$). If the interval includes zero, the groups are not statistically significantly different. However, if zero tends towards one end of the interval, then it is interpreted as a trend toward a difference (possibly Type II error). For endothelin-1 comparison, only the maximum MAP point for each dose was chosen. Abbreviations: NA, noradrenaline doses; AII, angiotensin II doses; ET-1, endothelin-1 doses; Ht, hypertensive group; C, control group; Ht-Chol, hypertensive-hypercholesterolemic group.

Table 4.2: 95% confidence intervals for the differences between the hypertensive vs the control groups and the hypertensive-hypercholesterolemic vs the control groups, respectively, in the pressor responses, using analysis of covariance with starting mean arterial pressure as covariate.

	2 weeks		6 weeks		13-16 weeks	
	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C
NA (µg/kg):						
0.05	(-0.4, 1.8)	(-0.4, 1.5)	(-0.1, 1.6)	(-0.1, 1.3)	(1.2, 4.2)*	(1.0, 3.3)*
0.10	(-0.5, 1.4)	(-0.5, 0.9)	(-0.2, 1.4)	(0.02, 1.4)*	(1.2, 4.1)*	(1.0, 3.2)*
0.30	---	---	---	---	(1.3, 4.2)*	(1.1, 3.4)*
0.50	(-0.5, 1.7)	(-0.5, 1.4)	(-0.2, 1.4)	(0.07, 1.4)*	---	---
1.00	---	---	(-0.2, 1.4)	(-0.1, 1.2)	(1.2, 4.1)*	(1.0, 3.3)*
3.00	(-0.7, 1.5)	(-0.5, 1.3)	(-0.6, 1.2)	(-0.4, 1.0)	(1.2, 4.1)*	(0.9, 3.2)*
5.00	---	---	(-0.8, 1.6)	(-0.4, 1.2)	---	---
10.0	(-0.8, 1.6)	(-0.4, 1.5)	---	---	(1.1, 4.2)*	---
AII (µg/kg):						
0.0005	(-5.1, 2.4)	(-3.7, 1.6)	(0.5, 2.7)*	(0.5, 2.3)*	(0.4, 1.6)*	(0.4, 1.5)*
0.0010	(-5.1, 2.4)	(-3.6, 1.7)	(0.5, 2.6)*	(0.4, 2.2)*	(0.3, 1.4)*	(0.2, 1.4)*
0.0050	(-5.2, 2.3)	(-3.6, 1.7)	(0.4, 2.5)*	(0.3, 2.1)*	(0.2, 1.4)*	(0.2, 1.3)*
0.0200	(-5.3, 2.2)	(-3.7, 1.6)	(0.3, 2.5)*	(0.2, 2.0)*	(0.1, 1.3)*	(-0.01, 1.1)
0.100	(-5.4, 2.1)	(-3.8, 1.5)	(0.2, 2.4)*	(0.3, 2.1)*	(-0.05, 1.1)	(-0.1, 1.1)
0.300	(-5.4, 2.1)	(-3.9, 1.5)	(0.2, 2.4)*	(0.2, 2.1)*	(-0.2, 1.1)	(-0.1, 1.0)
ET-1 (nmol/kg):						
0.03			(-48.5, 2.0)	(-47.8, 1.1)	---	---
0.05			(-17.1, 20.9)	(-16.5, 18.0)	---	---
0.10			(0.3, 39.0)*	(-0.4, 33.0)	(3.7, 37.6)*	(5.5, 35.3)*
0.20			(-38.2, 6.3)	(-33.2, 3.9)	---	---

Note: *, significantly different between the groups ($p < 0.05$). If the interval includes zero, the groups are not statistically significantly different. However, if zero tends towards one end of the interval, then it is interpreted as a trend toward a difference (possibly Type II error). For endothelin-1 comparison, only the maximum MAP point for each dose was chosen. Abbreviations: NA, noradrenaline doses; AII, angiotensin II doses; ET-1, endothelin-1 doses; Ht, hypertensive group; C, control group; Ht-Chol, hypertensive-hypercholesterolemic group.

Table 4.3: 95% confidence intervals for the differences between the hypertensive vs the control groups and the hypertensive-hypercholesterolemic vs the control groups, respectively, in the depressor responses using analysis of variance.

	2 weeks		6 weeks		13-16 weeks	
	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C
ACH (µg/kg):						
0.05	(0.4, 0.7)*	(0.2, 0.5)*	(0.2, 0.8)*	(-0.1, 0.5)	(0.4, 0.7)*	(0.3, 0.6)*
0.10	(0.3, 0.6)*	(0.1, 0.4)*	(0.06, 0.6)*	(-0.1, 0.4)	(0.3, 0.6)*	(0.2, 0.5)*
0.30	(0.3, 0.6)*	(0.1, 0.4)*	(-0.03, 0.8)	(-0.2, 0.7)	(0.3, 0.6)*	(0.2, 0.5)*
1.00	(0.3, 0.6)*	(0.1, 0.5)*	(0.006, 0.6)*	(-0.07, 0.5)	(0.3, 0.6)*	(0.1, 0.5)*
3.00	(0.2, 0.5)*	(0.002, 0.3)*	(-0.1, 0.7)	(-0.2, 0.7)	(0.3, 0.6)*	(0.2, 0.5)*
10.0	(0.02, 0.5)*	(-0.1, 0.3)	(-0.4, 1.1)	(-0.5, 1.2)	(0.3, 0.7)*	(0.2, 0.6)*
NIP (µg/kg/min X 10):						
2.5	(0.4, 1.0)*	(0.2, 0.7)*	(-0.07, 0.8)	(-0.2, 0.6)	---	---
5.0	(0.2, 0.7)*	(0.06, 0.6)*	(0.1, 0.9)*	(-0.3, 0.6)	---	---
10.0	(0.1, 0.7)*	(0.1, 0.6)*	(0.09, 0.9)*	(-0.2, 0.7)	---	---
20.0	(0.1, 0.7)*	(0.1, 0.6)*	(0.02, 0.8)*	(-0.1, 0.8)	---	---
ISP (µg/kg):						
0.05	(0.4, 0.7)*	(0.3, 0.6)*	(0.4, 0.7)*	(0.2, 0.6)*	---	---
0.10	(0.4, 0.7)*	(0.3, 0.6)*	(0.3, 0.6)*	(0.2, 0.6)*	---	---
0.30	(0.2, 0.5)*	(0.1, 0.5)*	(0.2, 0.6)*	(0.2, 0.5)*	---	---

Note: *, significantly different between the groups ($p < 0.05$). If the interval includes zero, the groups are not statistically significantly different. However, if zero tends toward one end of the interval, then it is interpreted as a trend toward a difference (possibly Type II error).
Abbreviations: ACH, acetylcholine doses; NIP, nitroprusside doses; ISP, isoproterenol doses; Ht, hypertensive group; C, control group; Ht-Chol, hypertensive-hypercholesterolemic group.

Table 4.4: 95% confidence intervals for the differences between the hypertensive vs the control groups and the hypertensive-hypercholesterolemic vs the control groups, respectively, in the depressor responses, using analysis of covariance with starting mean arterial pressure as covariate.

	2 weeks		6 weeks		13-16 weeks	
	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C	Ht vs C	Ht-Chol vs C
ACH (μg/kg):						
0.05	(0.3, 1.5)*	(0.2, 1.1)*	(0.2, 2.5)*	(-0.5, 1.3)	(0.2, 0.8)*	(0.1, 0.6)*
0.10	(0.2, 1.4)*	(0.06, 1.0)*	(-0.5, 1.8)	(-0.4, 1.3)	(0.1, 0.6)*	(0.04, 0.5)*
0.30	(0.3, 1.4)*	(0.1, 1.0)*	(-0.4, 1.9)	(-0.4, 1.5)	(0.1, 0.6)*	(0.01, 0.5)*
1.00	(0.2, 1.4)*	(0.1, 1.0)*	(-0.5, 1.8)	(-0.4, 1.4)	(0.1, 0.6)*	(0.02, 0.5)*
3.00	(0.1, 1.3)*	(-0.02, 0.9)	(-0.5, 1.8)	(-0.5, 1.4)	(0.1, 0.6)*	(0.02, 0.5)*
10.0	(0.006, 1.2)*	(-0.1, 0.9)	(-0.6, 2.0)	(-0.6, 1.8)	(0.1, 0.7)*	(0.05, 0.6)*
NIP (μg/kg/min X 10):						
2.5	(0.007, 1.4)*	(0.02, 0.9)*	(-0.5, 1.6)	(-0.5, 1.1)	---	---
5.0	(-0.3, 1.1)	(-0.1, 0.8)	(-0.3, 1.8)	(-0.5, 1.1)	---	---
10.0	(-0.3, 1.1)	(-0.1, 0.8)	(-0.4, 1.7)	(-0.4, 1.2)	---	---
20.0	(-0.3, 1.1)	(-0.09, 0.8)	(-0.4, 1.6)	(-0.4, 1.2)	---	---
ISP (μg/kg):						
0.05	(0.4, 1.5)*	(0.2, 1.3)*	(-5.0, 1.9)	(-5.8, 1.9)	---	---
0.10	(0.3, 1.5)*	(0.3, 1.4)*	(-5.1, 1.8)	(-5.8, 1.9)	---	---
0.30	(0.2, 1.3)*	(0.1, 1.2)*	(-5.2, 1.8)	(-5.9, 1.9)	---	---

Note: *, significantly different between the groups ($p < 0.05$). If the interval includes zero, the groups are not statistally significantly different. However, if zero tends toward one end of the interval, then it is interpreted as a trend toward a difference (possibly Type II error).
Abbreviations: ACH, acetylcholine doses; NIP, nitroprusside doses; ISP, isoproterenol doses; Ht, hypertensive group; C, control group; Ht-Chol, hypertensive-hypercholesterolemic group.

There was no difference in the pressor response between the hypercholesterolemic and control groups. (Figure 4.7)

(B) Angiotensin II

There was a greater pressor resopnse in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in △MAP reached significance at all dose points for both groups versus the control group. Moreover, when examined in relation to the starting mean arterial pressure, these significant differences still persisted for both groups. (Tables 4.1 & 4.2)

There was no difference in the pressor response between the hypercholesterolemic and control groups. (Figure 4.8)

(C) Endothelin-1

Each group of rabbits responded to endothelin-1 injection in two phases: an initial depressor response (within the first 30 seconds) followed by a pressor response which reached a maximum at 1-4 minutes and lasted for more than 20 minutes. There was a dose-related increase in the pressor response and the depressor response also tended to be dose-related.

The depressor response was greater in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. This depressor response was significantly greater at the 0.03 & 0.05 nmol/kg dose points for the hypertensive group, and at the 0.03 dose point for the hypertensive-hypercholesterolemic group versus the control group. When examined in relation to the starting mean arterial pressure, these significant differences still persisted for both groups at the 0.03 nmol/kg dose point.

The pressor response during the first 5 minutes after endothelin-1 injection was

greater in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The maximum pressor response was significantly greater in both groups at the 0.03 nmol/kg dose point versus the control group. When examined in relation to the starting mean arterial pressure, these differences no longer reached significance.

Neither the depressor nor the pressor response was different between the hypercholesterolemic and control groups at any dose of endothelin-1. (Figure 4.9, 4.10, 4.11, & 4.12)

(D) Acetylcholine

There was a greater depressor response in the hypertensive group as compared to the control group, and the difference in ΔMAP was significant at 0.05, 0.10, & 1.00 μg/kg dose points. When examined in relation to the starting mean arterial pressure, the significant difference persisted only for the 0.05 μg/kg dose point (Tables 4.3 & 4.4). The depressor response tended to be greater in the hypertensive-hypercholesterolemic group than in the control group, but the difference did not reach significance.

There was no difference in the depressor response between the hypercholesterolemic and control groups. (Figure 4.13)

(E) Nitroprusside

There was a greater depressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in ΔMAP reached significance at the 5.0, 10.0, & 20.0 μg/kg/min infusion doses for the hypertensive versus the control group. When examined in relation to the starting mean arterial pressure, this difference did not remain significant. (Tables 4.3 & 4.4)

There was no difference in the depressor response between the hypercholesterolemic and control groups (Figure 4.14)

(F) Isoproterenol

There was a greater depressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups compared to the control group. The difference in ΔMAP reached significance at all three dose points for both the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group. When examined in relation to the starting mean arterial pressure, these differences did not remain significant. (Tables 4.3 & 4.4)

There was no difference in the depressor response between the hypercholesterolemic and control groups. (Figure 4.15)

The chronotropic response (Δ HR) was also analyzed. There was no difference in the chronotropic response in the hypertensive, the hypercholesterolemic, or the hypertensive-hypercholesterolemic groups as compared to the control group. (Figure 4.16)

4.3.3. 13-16 Weeks Study:

(A) Noradrenaline

There was a greater pressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in ΔMAP was significantly greater at all dose points for both groups versus the control group. Moreover, when examined in relation to the starting mean arterial pressure, these significant differences still persisted for both groups. (Tables 4.1 & 4.2)

There was no difference in the pressor response between the hypercholesterolemic and control groups. (Figure 4.17)

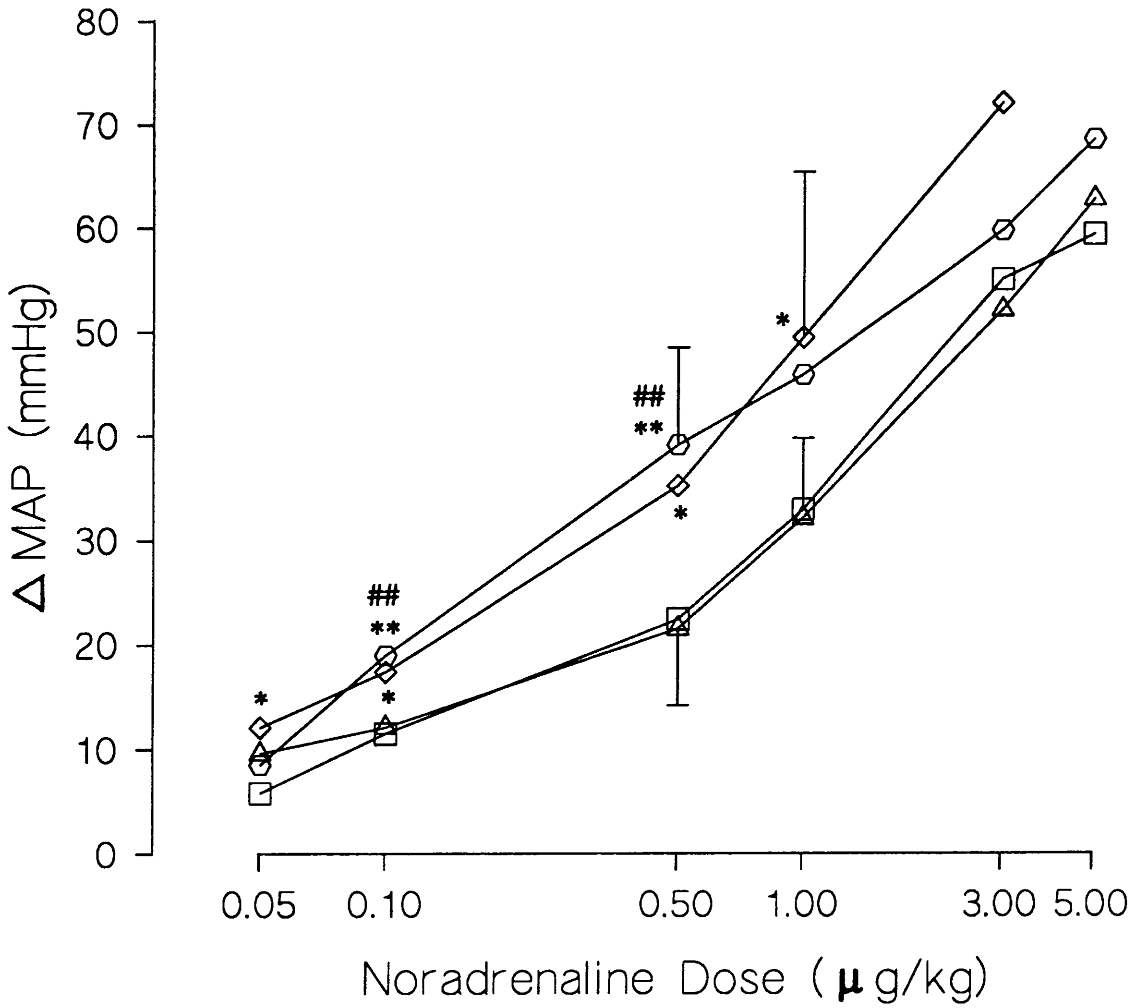


Figure 4.7: Changes in blood pressure (Δ MAP) in response to noradrenaline in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 6 - 9. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; ##, $p < 0.05$ for the hypertensive-hypercholesterolemic group versus the control group when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

Δ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

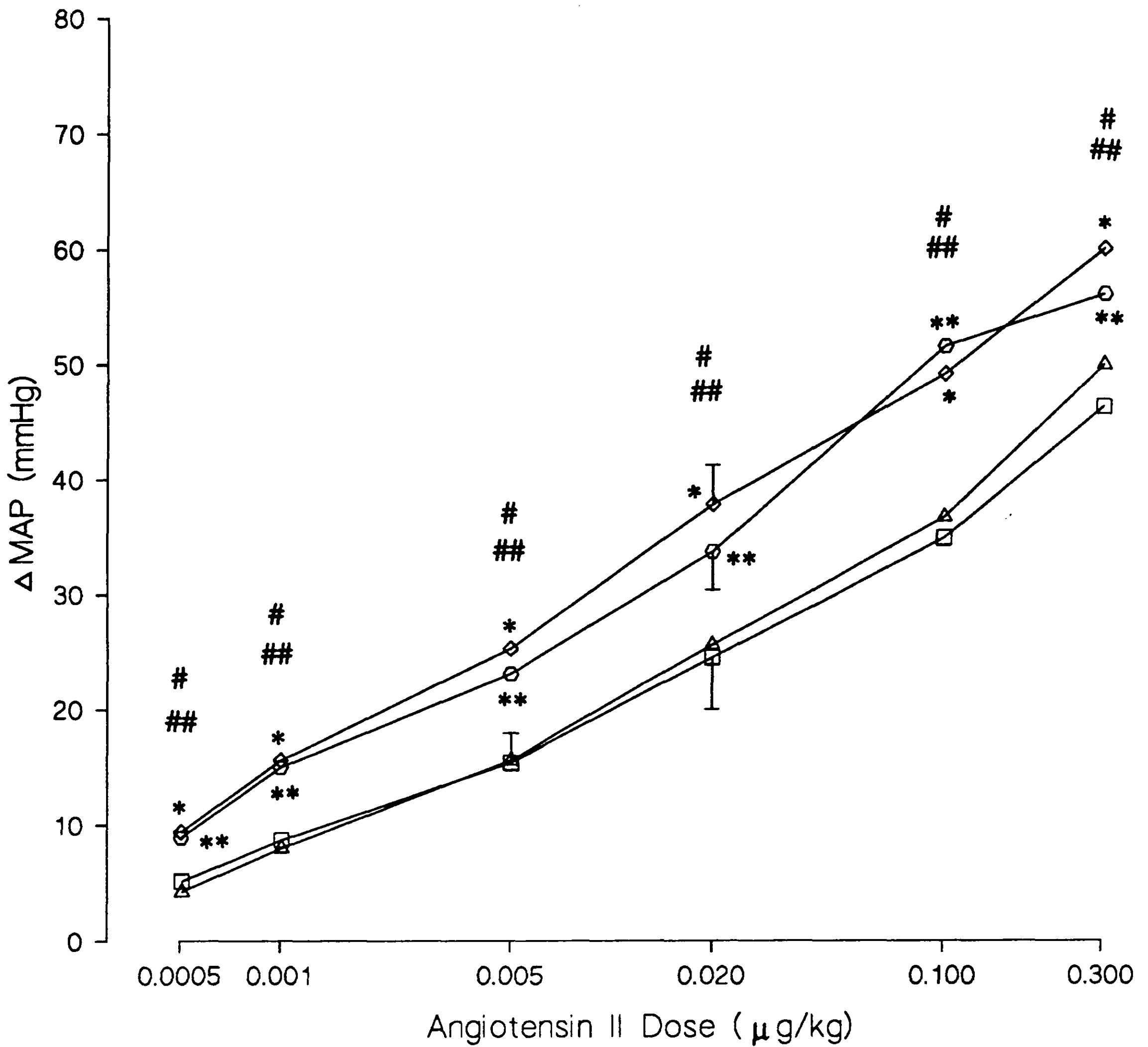


Figure 4.8: Changes in blood pressure (ΔMAP) in response to angiotensin II in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 7 - 8. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

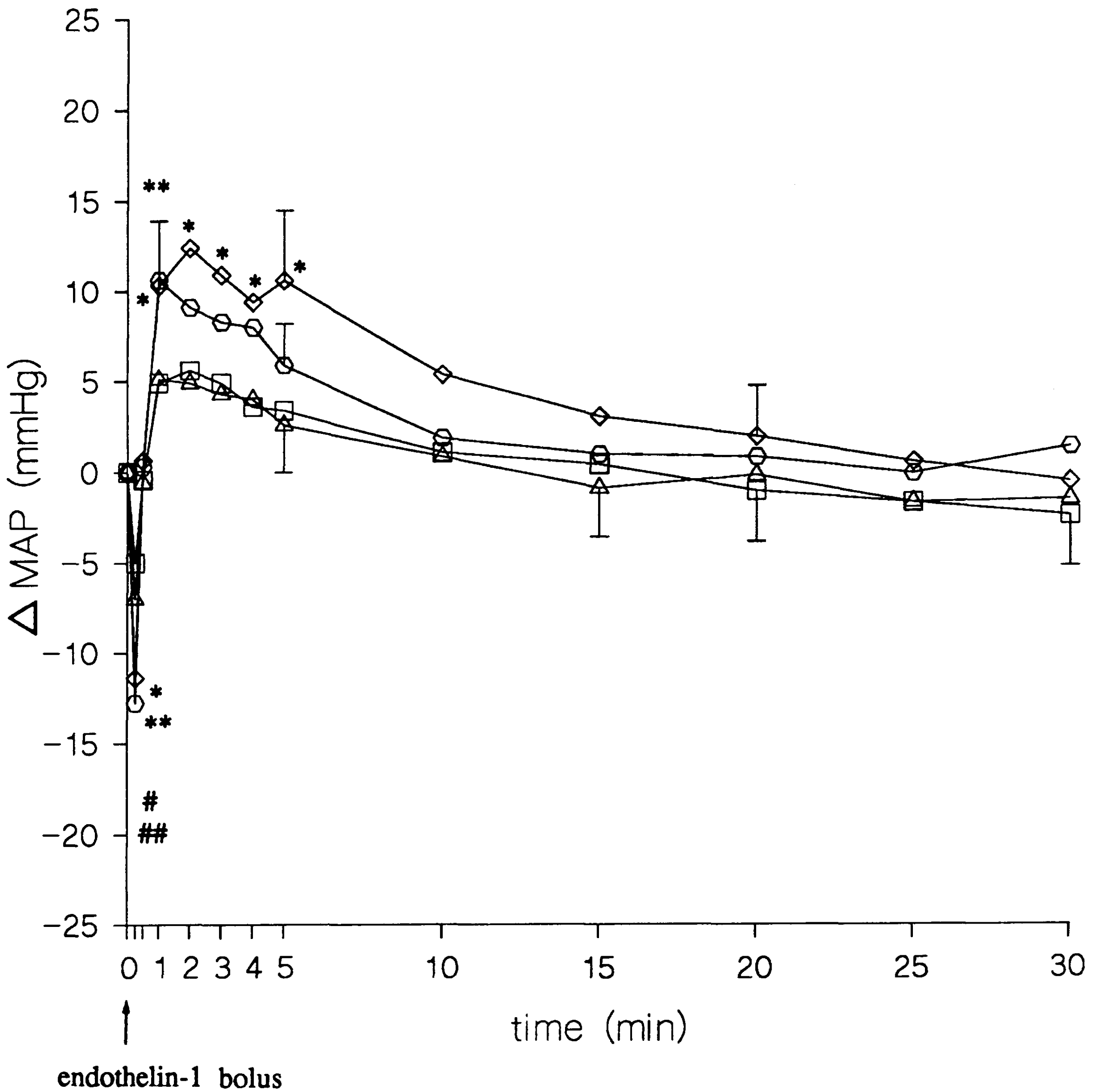


Figure 4.9: Changes in blood pressure (Δ MAP) in response to 0.03 nmol/kg endothelin-1 bolus in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

Note the biphasic profiles. (Mean ± S.D., n = 7 - 8. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

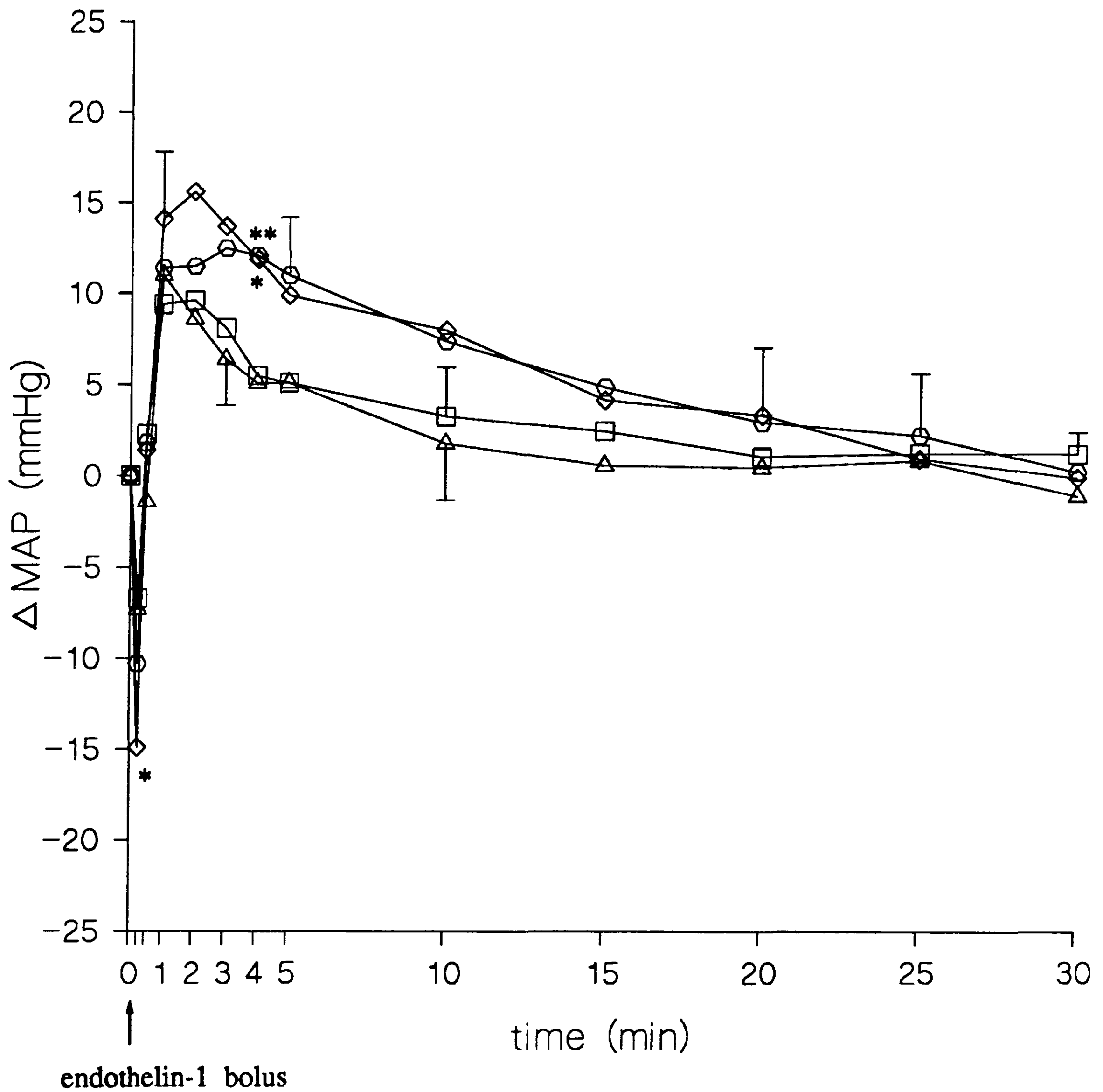


Figure 4.10: Changes in blood pressure (ΔMAP) in response to 0.05 nmol/kg endothelin-1 bolus in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

Note the biphasic profiles. (Mean ± S.D., n = 7 - 8. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively.)

□ Control group.

Δ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

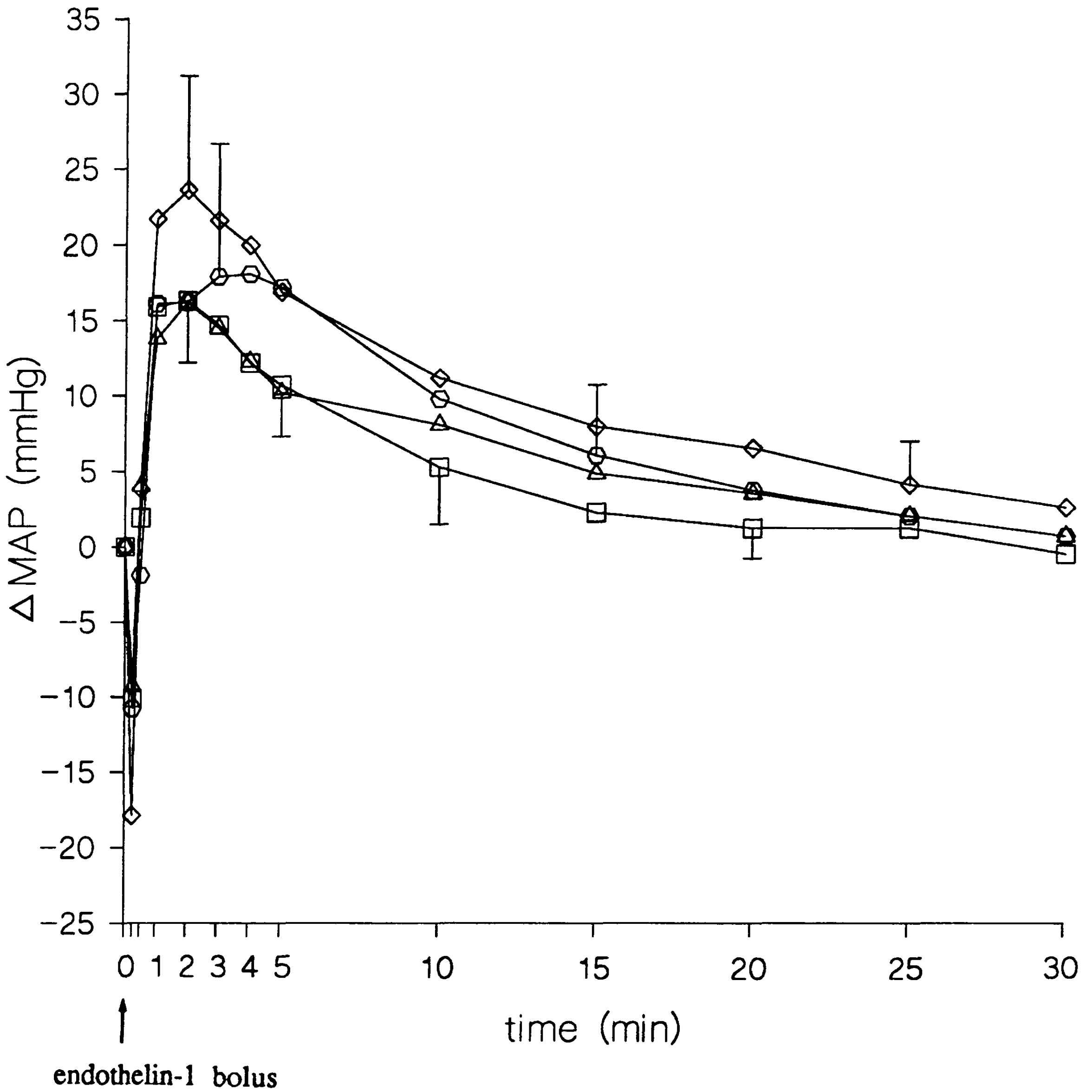


Figure 4.11: Changes in blood pressure (Δ MAP) in response to 0.10 nmol/kg endothelin-1 bolus in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

Note the biphasic profiles. (Mean ± S.D., n = 9 - 10.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

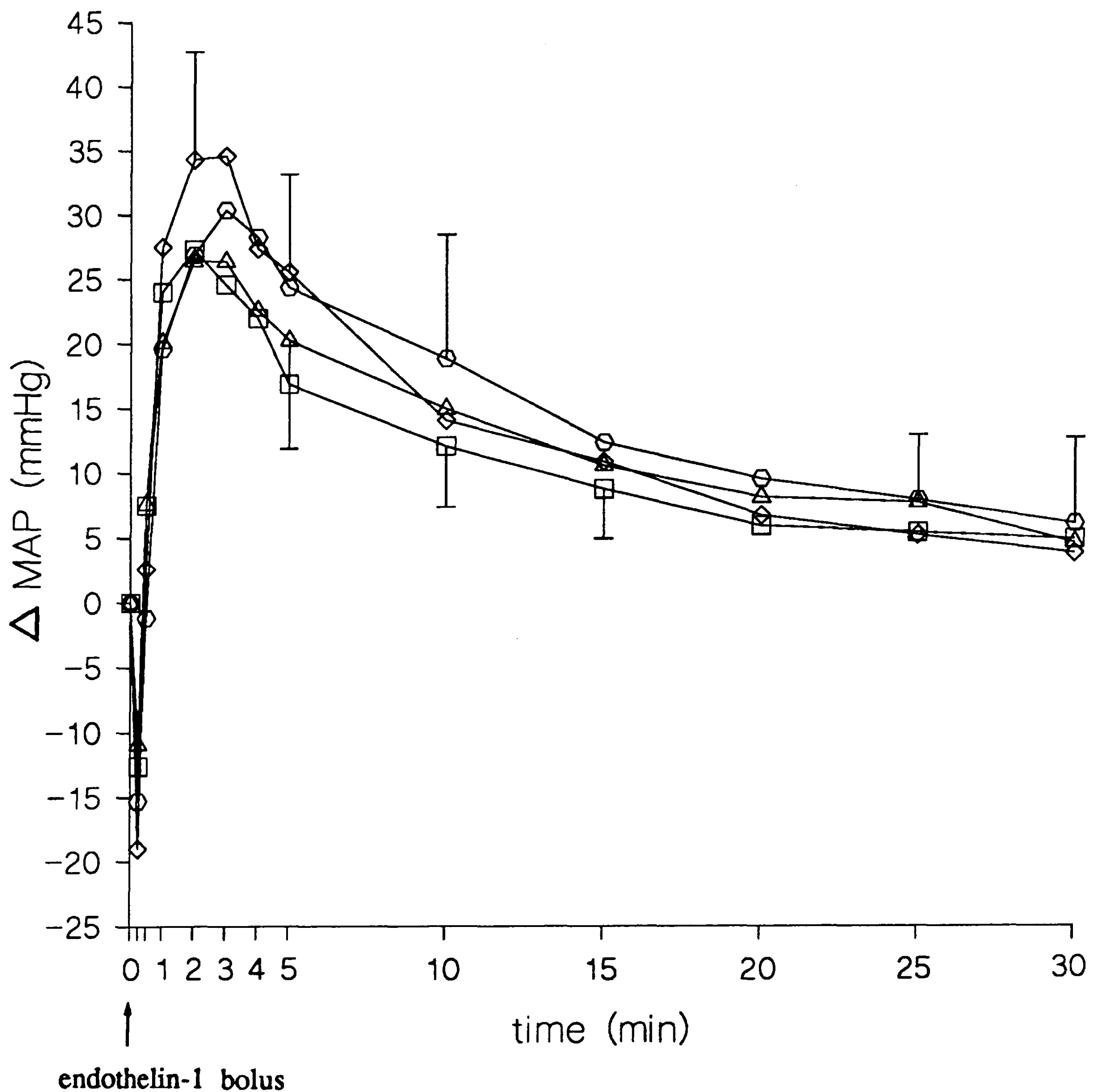


Figure 4.12: Changes in blood pressure (Δ MAP) in response to 0.20 nmol/kg endothelin-1 bolus in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

Note the biphasic profiles. (Mean ± S.D., n = 8 - 9.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

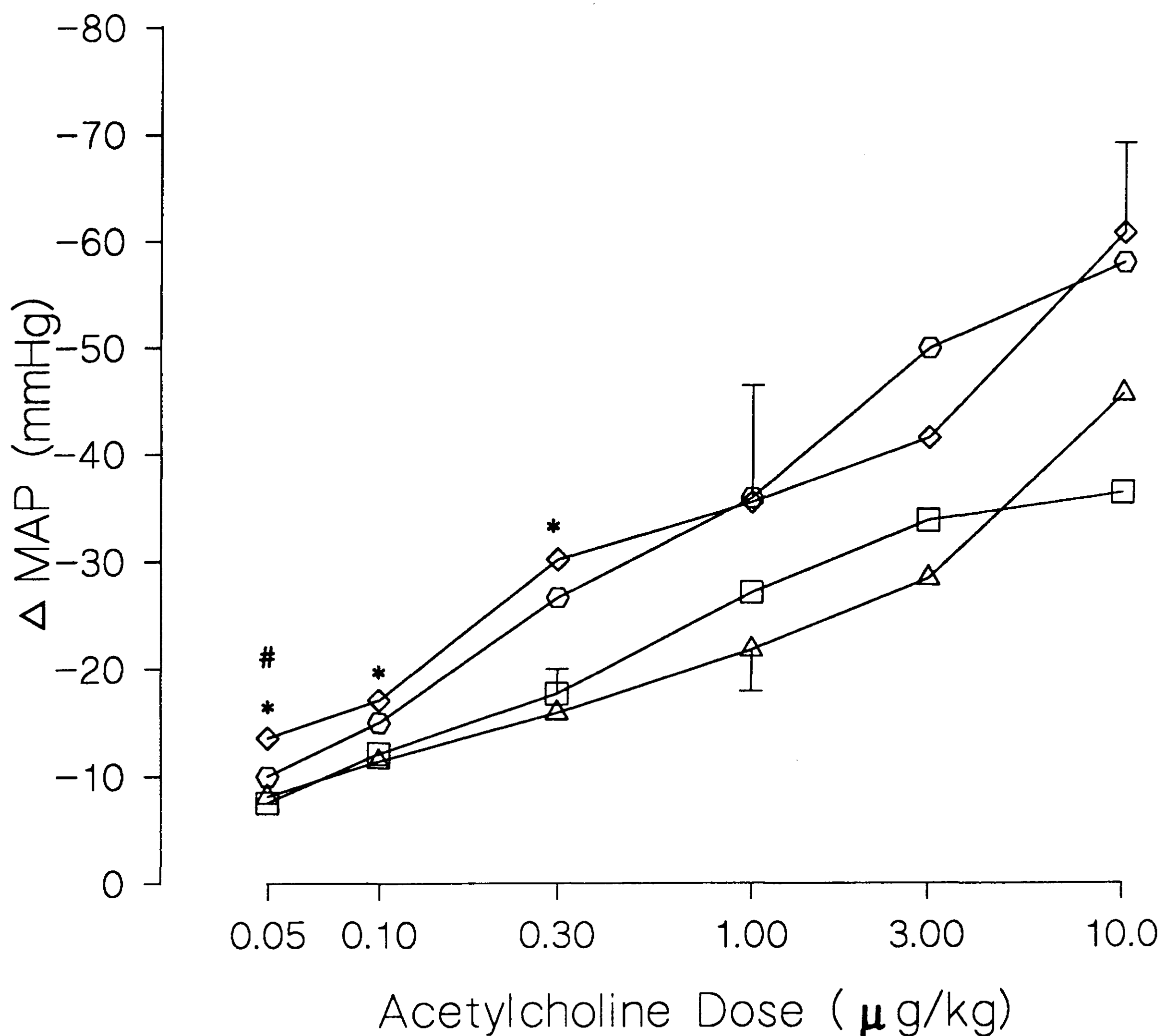


Figure 4.13: Changes in blood pressure (Δ MAP) in response to acetylcholine in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 9 - 11. *, $p < 0.05$ for the hypertensive group versus the control group; #, $p < 0.05$ for the hypertensive group versus the control group when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

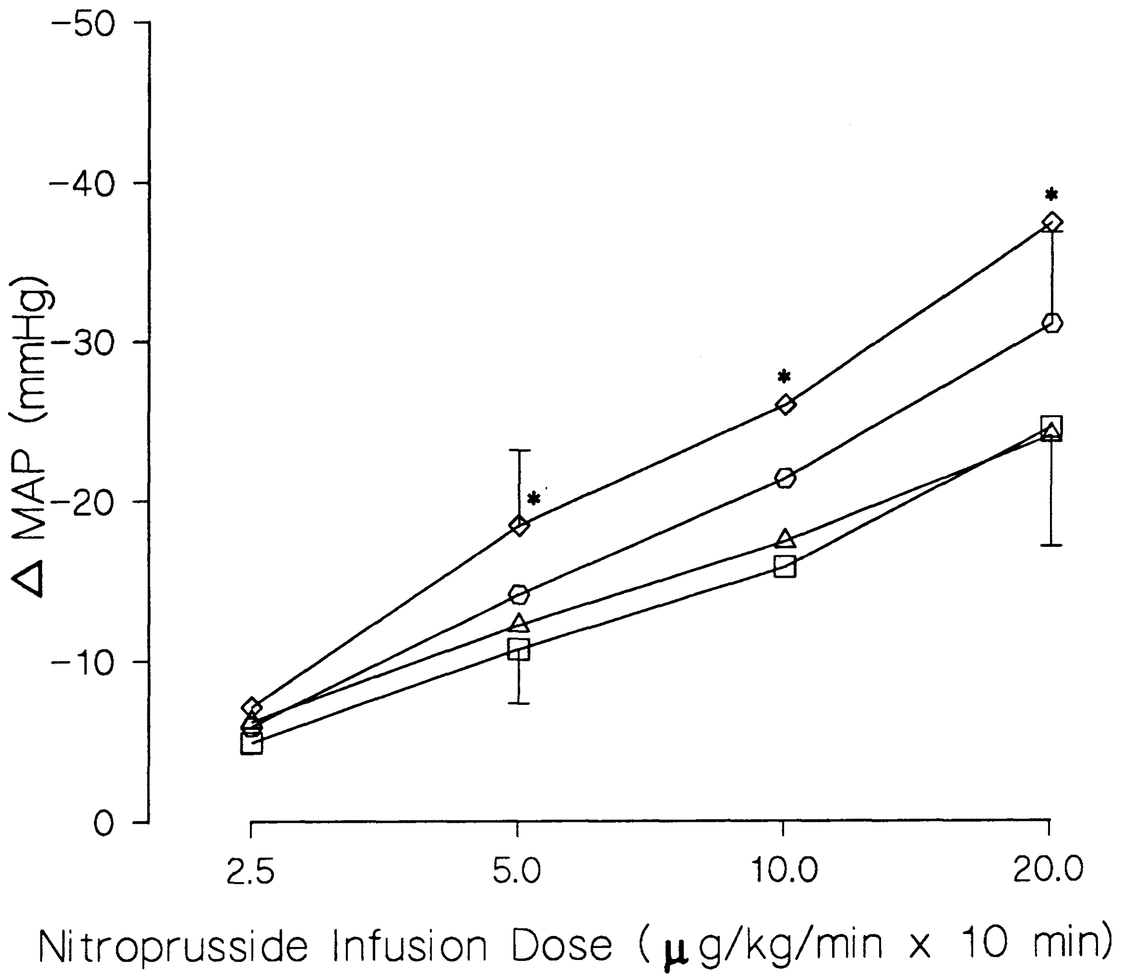


Figure 4.14: Changes in blood pressure (ΔMAP) in response to nitroprusside infusion in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 9 - 10. *, $p < 0.05$ for the hypertensive group versus the control group.)

- □ **Control group.**
- △ **Hypercholesterolemic group.**
- ◇ **Hypertensive group.**
- ○ **Hypertensive-hypercholesterolemic group.**

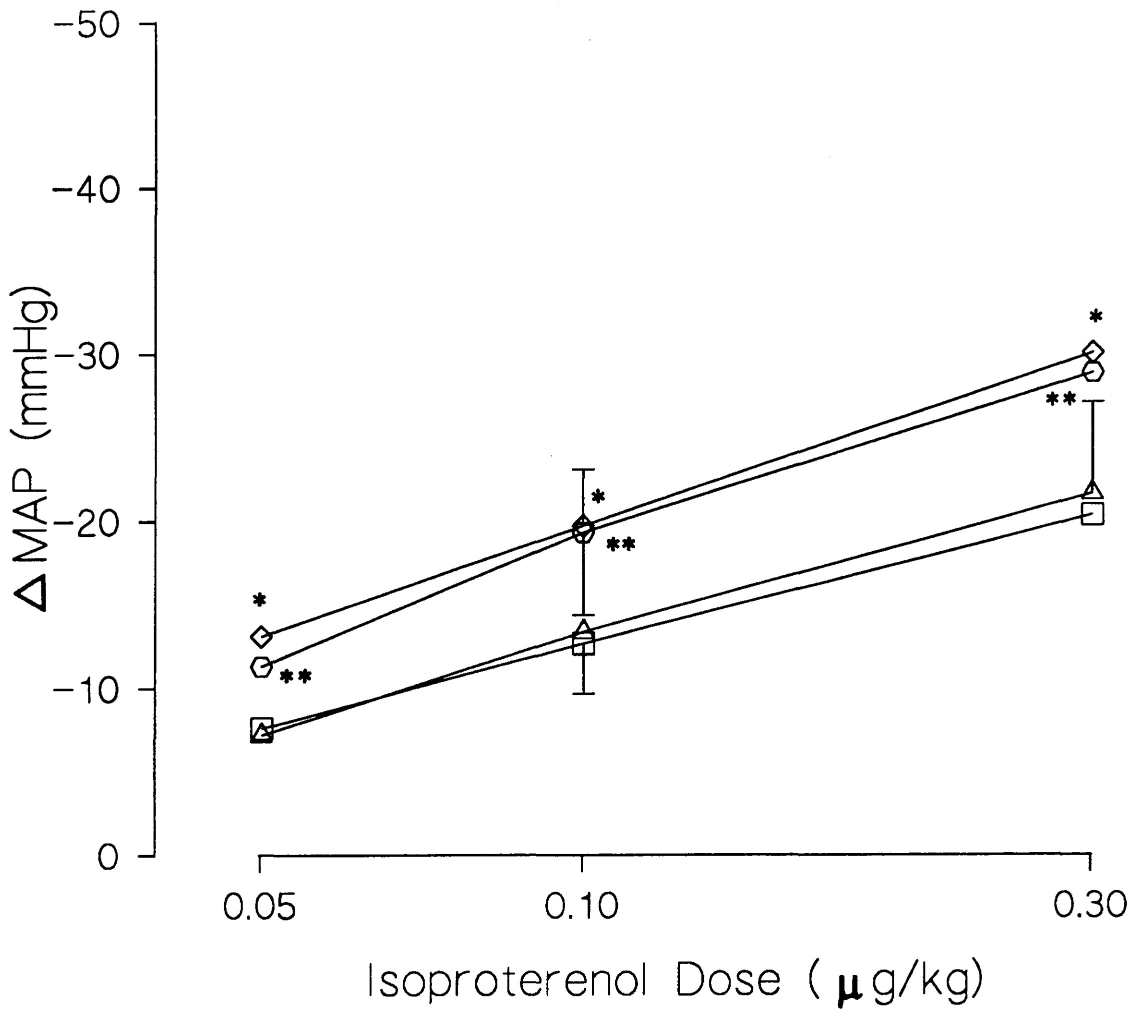


Figure 4.15: Changes in blood pressure (ΔMAP) in response to isoproterenol in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 6 - 8. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

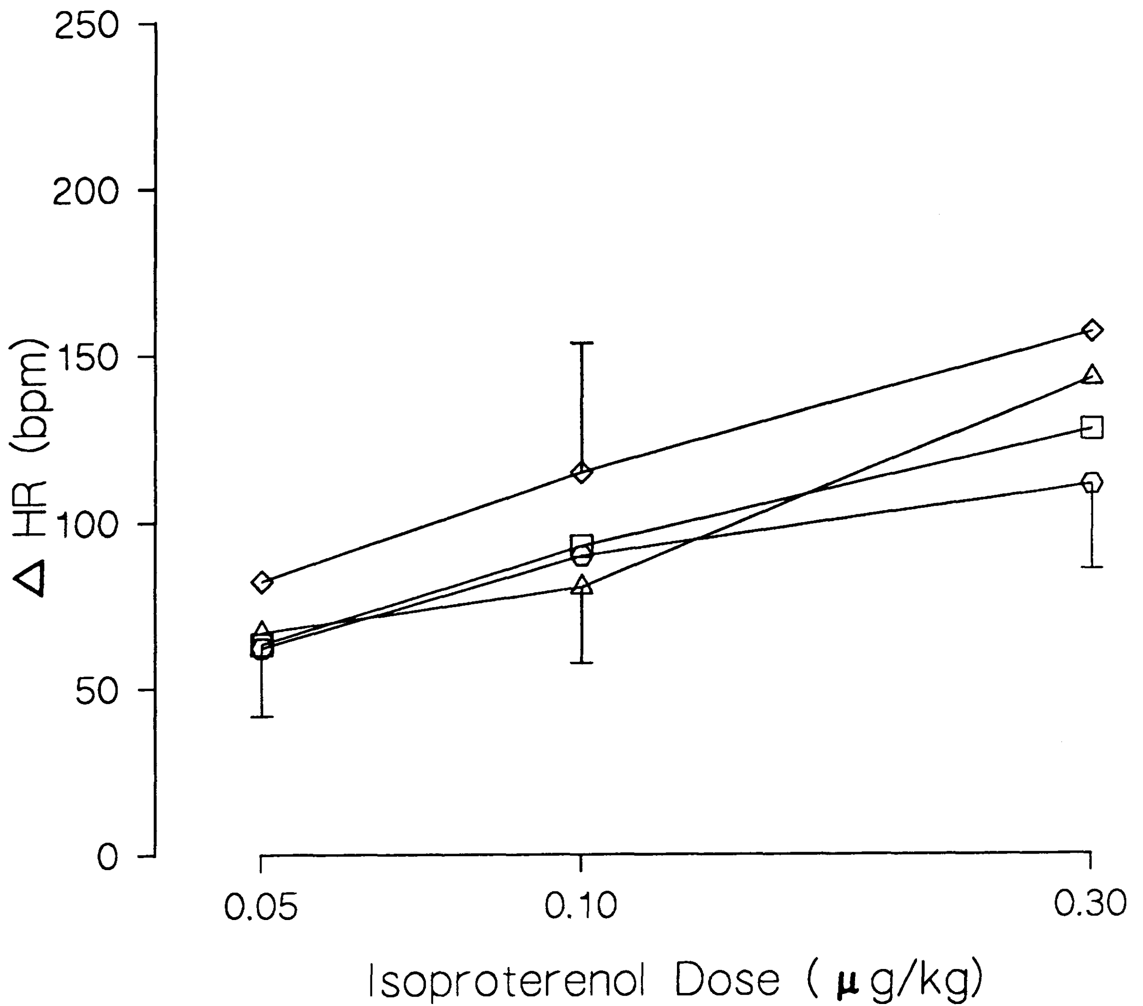


Figure 4.16: Changes in heart rate (ΔHR) in response to isoproterenol in groups of rabbits 6-7 weeks after surgery and dietary manipulation.

There is no difference in the chronotropic responsiveness (ΔHR) to isoproterenol between the control group and any of the experimental groups. (Mean ± S.D., n = 6 - 8.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

(B) Angiotensin II

There was a greater pressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in △MAP reached significance at all dose points for both groups versus the control group. When examined in relation to the starting mean arterial pressure, the differences remained significant at the 0.0005, 0.0010, 0.005, & 0.020 μg/kg for the hypertensive group, and at the 0.0005, 0.0010, & 0.005 μg/kg dose points for the hypertensive-hypercholesterolemic group versus the control group. (Tables 4.1 & 4.2)

There was no difference in the pressor response between the hypercholesterolemic and control groups. (Figure 4.18)

(C) Endothelin-1

Only one dose (0.10 nmol/kg) of endothelin-1 was given to each group. The depressor as well as the pressor responses were greater in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The maximum pressor response was significantly greater for both groups versus the control group. When examined in relation to the starting mean arterial pressure, these significant differences still persisted for both groups.

Neither the depressor nor the pressor response was different between the hypercholesterolemic and control groups. (Figure 4.19)

(D) Acetylcholine

There was a greater depressor response in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. The difference in △MAP reached significance at all dose points for both groups versus the control group. When examined in relation to the starting mean arterial pressure, these significant differences still persisted for both groups. (Tables 4.3 & 4.4)

There was no difference in the depressor response between the hypercholesterolemic and control groups. (Figure 4.20)

4.4. Discussion

(1) At 2-3 weeks after renal wrapping operation, when the rabbits were at the developing stage of hypertension, there were greater pressor responses to noradrenaline and angiotensin II, as well as greater depressor responses to acetylcholine, isoproterenol, and nitroprusside in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. When the starting mean arterial pressure was taken into account in analysis of covariance, only the depressor responses to isoproterenol and acetylcholine remained significantly greater in both groups versus the control group. Using the same model of hypertensive rabbits, Nixon et al (1990) reported that phospholipase C activity in response to noradrenaline, but not endothelin-1, was increased in the aorta and femoral artery of rabbits at 2 weeks during the development of hypertension. It is unclear how that finding relates to this study as there is no indication of changes in phospholipase C activity related to the development of hypertension in this study. However, Nixon et al did not examine inositol phosphates formation in the small arterioles, which are the major contributors to blood pressure changes in the conscious rabbits. It is possible that the changes observed in the aorta and femoral artery were not reflected in smaller vessels, or that changes occurred later or earlier in arterioles. Using the same model of hypertensive rabbits, Hamilton & Reid (1983) also found that the greater pressor response to noradrenaline was not apparent when the results were expressed as a percentage change. Berecek & Bohr (1978) also reported greater pressor responses to noradrenaline and angiotensin II at the developing stage of hypertension in deoxycorticosterone-acetate-salt hypertensive pigs, but no account was taken of baseline arterial pressure. An

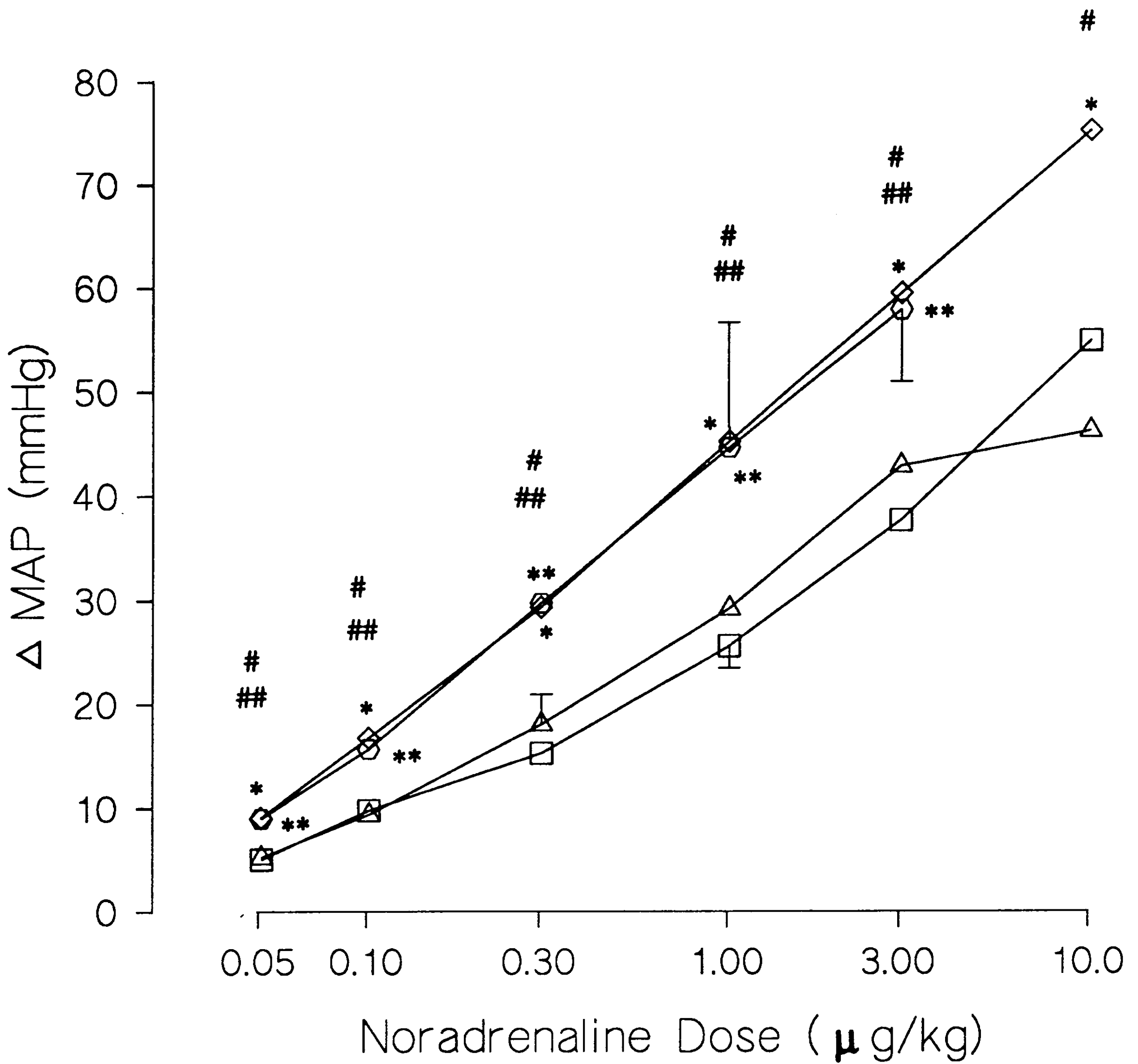


Figure 4.17: Changes in blood pressure (ΔMAP) in response to noradrenaline in groups of rabbits 13-16 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 7 - 9. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

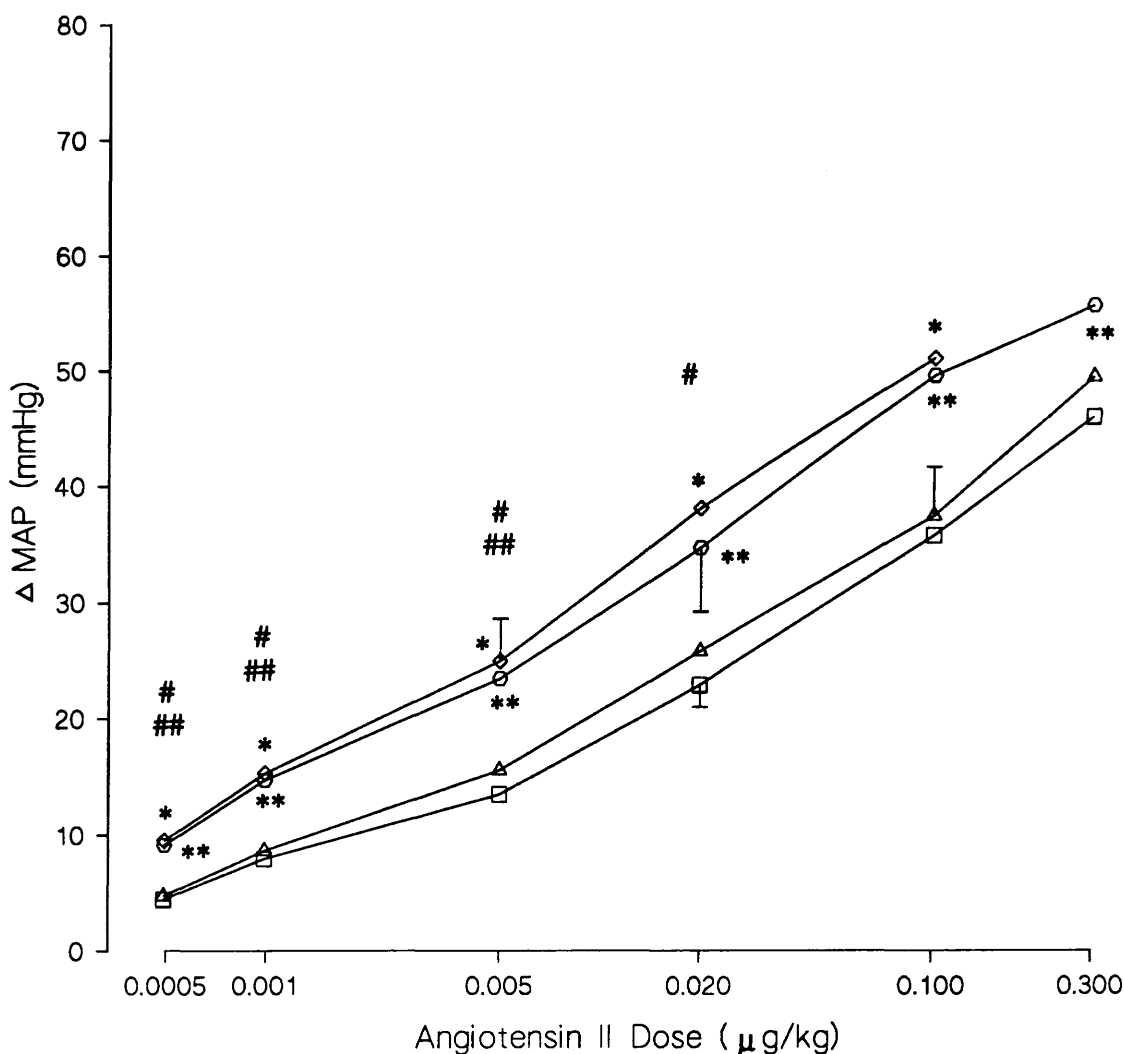


Figure 4.18: Changes in blood pressure (ΔMAP) in response to angiotensin II in groups of rabbits 13-16 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 7 - 8. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carrried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

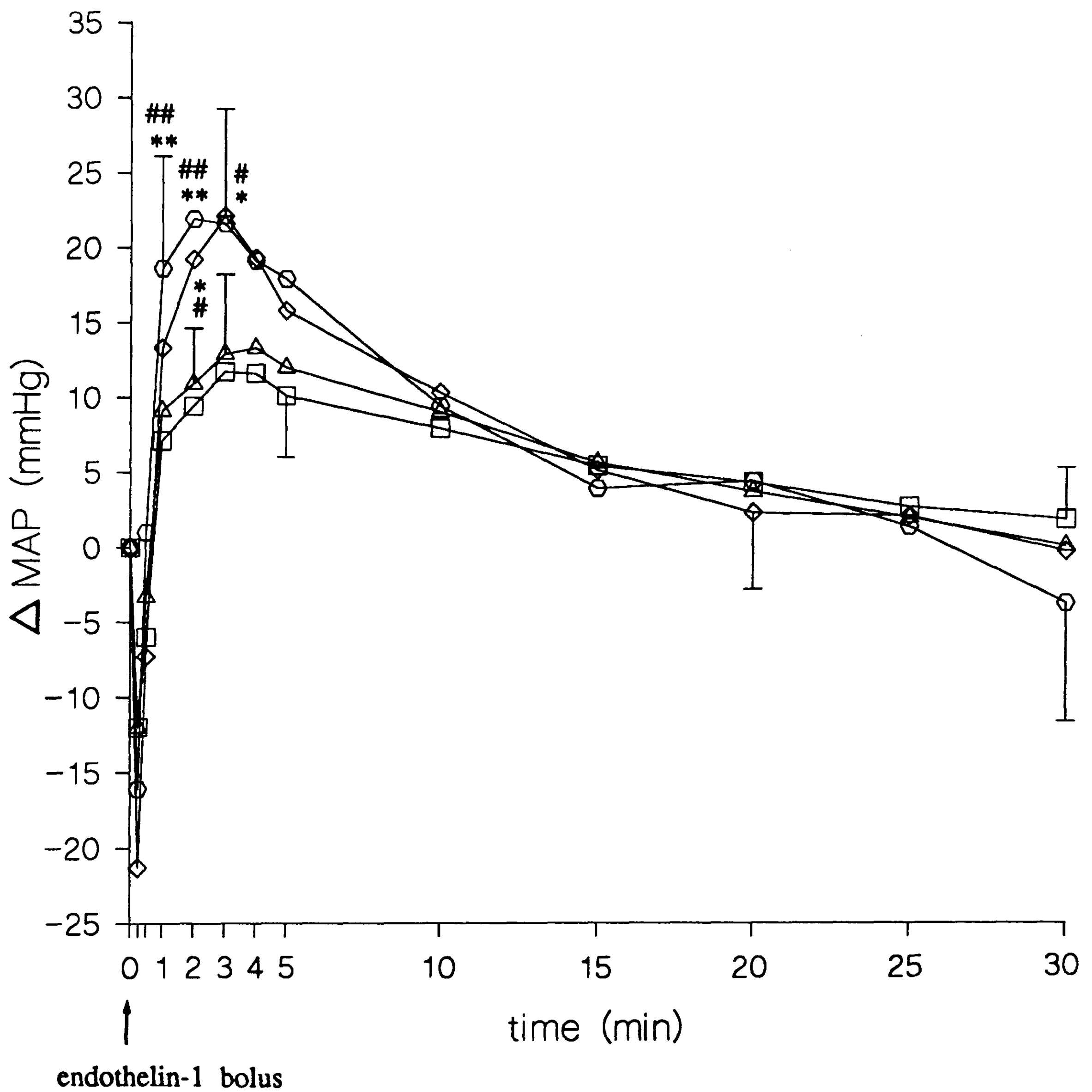


Figure 4.19: Changes in blood pressure (ΔMAP) in response to 0.10 nmol/kg endothelin-1 bolus in groups of rabbits 13-16 weeks after surgery and dietary manipulation.

Note the biphasic profiles. (Mean ± S.D., n = 7 - 9. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carried out with basal mean arterial pressure as covariate.)

□ Control group.

△ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

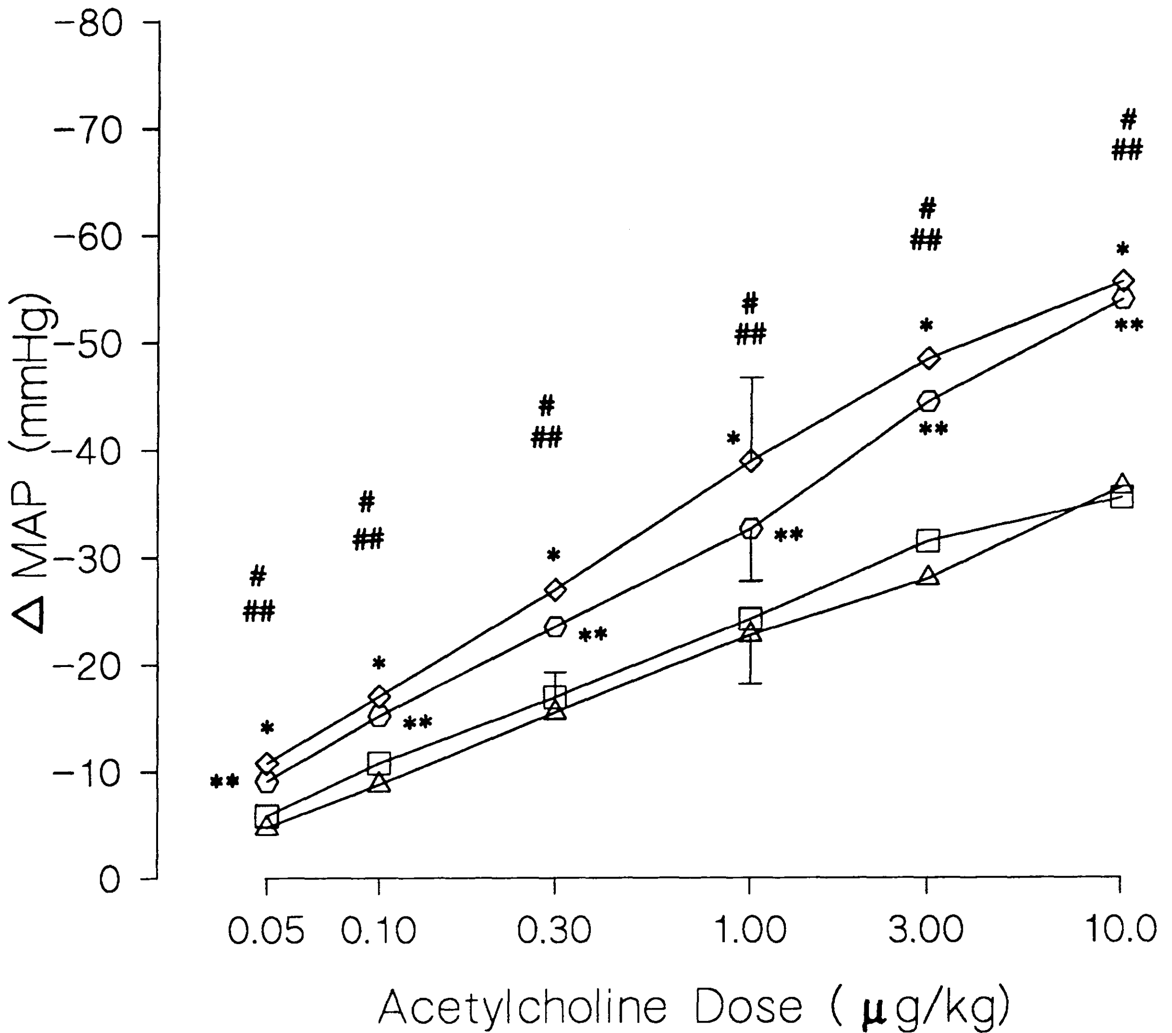


Figure 4.20: Changes in blood pressure (Δ MAP) in response to acetylcholine in groups of rabbits 13-16 weeks after surgery and dietary manipulation.

(Mean ± S.D., n = 7 - 10. * & **, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively; # & ##, $p < 0.05$ for the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, respectively, when analysis of covariance was carried out with basal mean arterial pressure as covariate.)

□ Control group.

Δ Hypercholesterolemic group.

◇ Hypertensive group.

○ Hypertensive-hypercholesterolemic group.

increased constrictor dose-response curve to noradrenaline and angiotensin II at the early, developing stage of hypertension was also observed in perfused renal vascular beds from spontaneously hypertensive rats (Berecek et al, 1980a) and in perfused mesenteric vasculature from Goldblatt renal hypertensive rats (Collis & Alps, 1975).

Our results also showed that there were greater depressor responses to acetylcholine, isoproterenol, and nitroprusside in both the hypertensive and the hypertensive-hypercholesterolemic groups at 2-3 weeks after operation, but only the receptor-mediated responses, i.e., acetylcholine and isoproterenol vasodilation, remained significantly greater after analysis of covariance with starting mean arterial pressure as covariate. Depressor responses to isoproterenol are mediated via activation of cAMP, and those to acetylcholine via cGMP activation. Unfortunately within the constraints of this thesis it was not possible to examine the activity of these second messenger systems. However, as nitroprusside acts directly on the cGMP system and the chronotropic response to isoproterenol involves cAMP, our results suggest that there is no generalized change in the activity of cAMP or cGMP during the development of perinephritis hypertension. However, changes in the cAMP system in relation to the development of genetic hypertension in rats have been reported (Hamet & Tremblay, 1990). Messerli et al (1976) reported that there was a greater depressor response to isoproterenol infusion in borderline hypertensive patients compared to normotensive controls. When the results were analyzed in terms of chronotropic responsiveness (Δ HR), there was no difference between either the hypertensive or the hypertensive-hypercholesterolemic group and the control group. Thus there were discordant patterns of vasodilator (β_2-adrenoceptor-mediated) and chronotropic (β_1-adrenoceptor-mediated) responsiveness in our hypertensive rabbits. No change in the chronotropic response has also been reported in borderline hypertensive patients (London et al, 1976; Henquet et al, 1982) and in early-stage aorta-coarctation hypertensive rats (Cervoni et al, 1981)

Taken as a whole, there were greater pressor and depressor responses to the agonists examined in both the hypertensive and the hypertensive-hypercholesterolemic rabbits at 2-3 weeks after renal operation; moreover a selective increase in responsiveness to receptor-mediated agonists was shown when comparing depressor responses to acetylcholine and isoproterenol with those to nitroprusside using analysis of covariance with starting mean arterial pressure as covariate.

On the other hand, 2-3 weeks of cholesterol feeding, which caused the plasma cholesterol level to rise from 1.3 ± 0.5 to 10.6 ± 5.9 mmol/L in the hypercholesterolemic group, had no effect on either the pressor or the depressor responses in the cholesterol-fed rabbits as compared to the control rabbits. Merkel et al (1990) reported that in moderately hypercholesterolemic rabbits, the in vitro aortic relaxation responses to isoproterenol and carbamylcholine chloride were decreased, while aortic constrictor responses to noradrenaline and angiotensin II were unchanged. One point of note is that the aorta is very susceptible to hypercholesterolemia, whereas resistance arterioles (the main determinant of peripheral resistance and therefore blood pressure) are little affected (Simonsen et al, 1991). Differences in experimental conditions (in vivo and in vitro) as well as vascular beds (systemic resistance arterioles and aorta) may also contribute to the different conclusions derived from our and Merkel et al's studies. Creager et al (1990) showed that in modestly hypercholesterolemic humans, the forearm resistance arterioles displayed impaired vasodilative responses to methacholine and nitroprusside, but similar vasoconstrictor responses to phenylephrine. Differences in species (human and rabbits) and investigation methods (local and systemic resistance arterioles) exist between ours and Creager et al's study.

The picture of generally similar pressor as well as depressor responses between the hypertensive and the hypertensive-hypercholesterolemic groups also suggested

that hypercholesterolemia had little effect on the vascular reactivity in these rabbits.

Thus at 2-3 weeks only the imposition of perinephritis hypertension altered the vascular responses of the rabbits, while hypercholesterolemia had little effect on responses.

(2) At 6-7 weeks after renal wrapping operation, when the rabbits were at the established stage of hypertension, there were greater pressor responses to noradrenaline and angiotensin II, as well as greater depressor responses to acetylcholine, isoproterenol, endothelin-1 and nitroprusside in the hypertensive group as compared to the control group. When starting mean arterial pressure was taken into account in the analysis of covariance, only the pressor response to angiotensin II and the initial depressor response to 0.03 nmol/kg endothelin-1 remained significantly greater in the hypertensive group. Hamilton & Reid (1983) also found that there was a greater pressor response to noradrenaline in 6-to-8-week perinephritis hypertensive rabbits; when expressed as a percentage change, the difference in the pressor response was not significant. In contrast, no difference in the pressor response to angiotensin II between hypertensive and control rabbits was reported in their study. The dose range of angiotensin II in their study (0.5--5.0 μg/kg) was higher than that of this study (0.0005--0.300 μg/kg), which was similar to that used in Hof & Hof (1988)'s study. Enhanced in vivo pressor responses to noradrenaline and angiotensin II, and the depressor response to acetylcholine, were also observed in the hindlimb vessels from 5-week bilateral-renal-wrapped hypertensive rabbits (Wright et al, 1987; Wright & Angus, 1986). Angell-James et al (1980) also found there were greater in vivo pressor responses to angiotensin II and phenylephrine in perinephritis hypertensive rabbits, although only one or two doses were given in that study. Enhanced in vivo pressor responses to noradrenaline and angiotensin II at the established phase of hypertension were also reported in other animal models of secondary hypertension, such as deoxycorticosterone-salt

hypertensive rats (Finch, 1971), and pigs (Berecek & Bohr, 1978), and Goldblatt renal hypertensive rats (Collis & Alps, 1975).

In line with our previous findings (Huang et al, 1990), endothelin-1 (0.03--0.20 nmol/kg) caused a dose-related increase in the pressor responses in the hypertensive as well as normotensive rabbits. The depressor as well as pressor responses tended to be higher in the hypertensive group compared to the control group. Miyauchi et al (1989) reported that in 12-week spontaneously hypertensive and Wistar-Kyoto rats, the pressor response was only significantly increased at the highest dose (2 nmol/kg). Yokokawa et al (1990) also observed a greater pressor response to endothelin-1 in 6-week deoxycorticosterone-acetate-salt hypertensive rats.

In contrast to the earlier study at 2-3 weeks, the depressor responses to acetylcholine and isoproterenol were no longer significantly increased when analysis of covariance was used. It is possible that the early increase in depressor responses was related to changes occurring during the development of hypertension which were not apparent in the established phase of the disease.

Our studies showed that although there was a greater depressor response to isoproterenol in the hypertensive group compared to the control group, there was no difference in terms of chronotropic responsiveness between these two groups, again suggesting a discordant picture of vasodilative and chronotropic responsiveness in the hypertensive rabbits. No difference in chronotropic responsiveness was observed in the aorta-coarctation hypertensive rats (Cervoni et al, 1981), and Goldblatt renal hypertensive rats (Ayobe & Tarazi, 1984) as compared to their normotensive controls. There are few data on the in vivo depressor responsiveness to isoproterenol in secondary hypertension to compare with our findings of increased responsiveness in perinephritis hypertensive rabbits, although some in vitro strip or perfused vessel studies also showed an enhanced vasorelaxing response to isoproterenol in the developing and established phase of animal hypertension

(Spector et al, 1969; Deragon et al, 1978; Dadkar et al, 1980).

In contrast, 6-7 weeks of cholesterol feeding, which caused plasma cholesterol levels to rise from 1.3 ± 0.5 to 17.6 ± 8.1 mmol/L in the hypercholesterolemic group, had no effect on either the pressor or depressor responses in the cholesterol-fed rabbits as compared to the controls. Wright & Angus (1986) found that the sensitivity (ED_{50})of the hindlimb vascular beds from 1%-cholesterol-diet-for-4-week rabbits to acetylcholine was not changed. Verbeuren et al (1986) observed that in vitro aortic contractile responsiveness to serotonin was not changed in 0.3%-cholesterol-for-8-week rabbits. (We also used 0.3% cholesterol diet.) In their study, the relaxant response to acetylcholine was attenuated in aorta from cholesterol-fed rabbits, but the response in pulmonary artery was not affected. No results were available in Verbeuren et al's study with regard to vascular resistance vessels, which may be more relevant to in vivo studies, as there is good evidence that vascular reactivity may be altered in large arteries from cholesterol-fed rabbits, but not in small arteries (Simonsen et al, 1991)

In the hypertensive-hypercholesterolemic group, there was a greater pressor response to angiotensin II and a greater depressor response to isoproterenol, as compared to the control group. The pressor responses to noradrenaline and endothelin-1 and the depressor responses to acetylcholine and nitroprusside, tended to be higher in the hypertensive-hypercholesterolemic group than those in the control group. The generally similar picture of the pressor as well as the depressor responses in the hypertensive-hypercholesterolemic group in comparison with the hypertensive group also strengthens our claim that only the imposition of perinephritis hypertension altered the vascular reactivity of the rabbits, while cholesterol-feeding did not have any effect.

(3) At 13-16 weeks after renal wrapping operation, when the rabbits had well established hypertension, there were greater pressor responses to noradrenaline,

angiotensin II, and endothelin-1 (0.1 nmol/kg), as well as a depressor response to acetylcholine in both the hypertensive and the hypertensive-hypercholesterolemic groups as compared to the control group. In addition, when starting mean arterial pressure was taken into account using analysis of covariance, these differences remained significant for both groups versus the control group. Thus as the rabbits progressed from the developing stage (2-3 weeks), through the established stage (6-7 weeks), and into the long established stage (13-16 weeks), the pressor responses to noradrenaline, angiotensin II, and endothelin-1 in the hypertensive rabbits progressively diverged from those in the normotensive rabbits. As shown in Tables 4.1 & 4.2, the degree of difference in the pressor responses increased with the duration of hypertension. This may, at least in part, be due to altered geometry of the arterial wall. It was hoped that analysis of covariance would allow for this. However, in Poiseuille's law, the pressure (P) and flow (Q) are related to fourth power of the radius (r^4), while analysis of covariance assumes a linear relationship between the covariate (basal mean arterial pressure) and response. It is possible that a linear relationship between basal mean arterial pressure and the responsiveness to agonists is not the most appropriate; the relationship may be more complex, although this was not apparent from inspection of plots of blood pressure versus response.

A progressive increase in the pressor response to noradrenaline and angiotensin II with increased duration of hypertension has also been observed in spontaneously hypertensive rats (Berecek et al, 1980a), Goldblatt renal hypertensive rats (Collis & Alps, 1975), and deoxycorticosterone-salt hypertensive rats (Berecek et al, 1980b). The presence of increased pressor responsiveness to agonists in the developing stage of hypertension, together with further increases as the disease progresses, may indicate that both "functional" and "structural" factors are important in the pathophysiological changes of hypertension.

In contrast, 13-16 weeks of cholesterol feeding, which caused the plasma

cholesterol level to rise from 1.3 ± 0.5 to 33.1 ± 10 mmol/L in the hypercholesterolemic group, had no effect on either the pressor or the depressor responses in the cholesterol-fed rabbits as compared to the controls. This was also echoed in the similar picture of the pressor and depressor responses in the hypertensive and the hypertensive-hypercholesterolemic groups. Hof & Hof (1988) reported that pressor responses to noradrenaline and angiotensin II were greater in the cholesterol-fed atherosclerotic rabbits, while the depressor response to acetylcholine was similar between atherosclerotic and control rabbits. On the other hand, Armstrong et al (1985) observed that the in vivo pressor responses to noradrenaline and angiotensin II in diet-induced atherosclerotic monkeys were not changed as compared to control animals. Chilian et al (1990) also showed that the in vivo pressor response to phenylephrine in the coronary resistance arteries was not altered in atherosclerotic monkeys. Simonsen et al (1991) reported that the vascular reactivity to noradrenaline, serotonin, acetylcholine, or nitroprusside was not changed in the resistance arteries from 12-week-1%-cholesterol-diet rabbits. Thus there are several reports supporting our observations that cholesterol feeding does not alter the vascular reactivity of the systemic or resistance vessels, despite induction of impressive hypercholesterolemia in the animals.

(4) The parallel increase in the pressor responsiveness (to noradrenaline, angiotensin II, and endothelin-1) in the hypertensive rabbits suggested that this is a generalised response. This finding agrees with Berecek et al (1980b)'s results in deoxycorticosterone-salt hypertensive rats, and Collis & Alps (1975)s' in Goldblatt renal hypertensive rats. One possible common denominator for this could be the enhanced responsiveness of the receptor-mediated phosphoinositide pathway, but our results (see "Chapter 6. Phosphoinositide Hydrolysis in Aorta from Hypertensive and Hypercholesterolemic Rabbits") and those of Nixon et al (1990), using the aorta in the same model of perinephritis hypertension, do not support this hypothesis.

However these studies were carried out in aorta, and it remains to be investigated whether the resistance vessels from the hypertensive animals have generalised perturbations of phosphoinositide metabolism which could explain the results of the pressor responses. One other possible explanation for the increased pressor responses is that there is altered sensitivity of actin-myosin fibrils to Ca^{2+} occurring with developing hypertension, resulting in a generalised increase in the pressor responses.

In contrast to the pressor responses, the depressor responses did not show any increase with the duration of hypertension. When examining the depressor responses to acetylcholine the greatest increases were observed early (at 2-3 weeks) and late (at 13-16 weeks) and it is possible that different mechanisms are involved at the different stages of hypertension. The discordant patterns of changes in the pressor responses as compared to the depressor responses with the duration of hypertension suggest that the structural factor is not the only mechanism for the altered vascular reactivity in the hypertensive rabbits, and the imposition of perinephritis hypertension could have different impacts on the contractile (phosphoinositide-related) processes and the relaxant (cAMP- or cGMP-related) processes.

In summary, our results showed that during the developing as well as the established stages of perinephritis hypertension, there were greater pressor responses to noradrenaline, angiotensin II, and endothelin-1, and greater depressor responses to acetylcholine, isoproterenol and nitroprusside. On the other hand, hypercholesterolemia and atherosclerosis induced by 0.3% cholesterol diet had no effect on either the pressor or the depressor responses in the rabbits throughout the study. Both functional and structural changes contributed to the altered vascular reactivity in the hypertensive rabbits.

CHAPTER 5

PLATELET INTRACELLULAR CALCIUM LEVELS IN HYPERTENSIVE AND HYPERCHOLESTEROLEMIC RABBITS

Chapter 5. Platelet Intracellular Calcium Levels in Hypertensive and Hypercholesterolemic Rabbits

5.1. Introdution

5.1.1. Platelets as a model for studying intracellular calcium levels ($[Ca^{2+}]_i$) in hypertension

Changes in intra-cellular calcium concentrations ($[Ca^{2+}]_i$) are important signals for contraction and tension development in vascular smooth muscle cells (Karaki,1989). Blaustein (1984) has proposed that the elevation of peripheral vascular resistance, which is the hallmark of primary hypertension, may be due to an elevation of $[Ca^{2+}]_i$; this enhances the contractile state of the smooth muscle cells. This hypothesis has engendered efforts to measure $[Ca^{2+}]_i$ of vascular smooth muscle cells in hypertension (Sada et al, 1990). However, work on vascular smooth muscle cells is technically more demanding and in human essential hypertension, almost impracticable for ethical reasons. Hence, there has been an endeavour to find a substitute which is easily accessible, available for multiple sampling, and reliable as an assay specimen. Platelets are a potential candidate because: (1) they can be isolated as a homogeneous specimen. (2) they are readily accessible and acceptable for multiple sampling. (3) they have some intracellular apparatus in common with vascular smooth muscle cells, e.g., an alpha-2-adrenoceptor adenyl cyclase system and a calcium-dependent contraction-coupling mechanism (Erne et al, 1984). Nevertheless, some major differences exist between platelets and vascular smooth muscle cells. For example, voltage-dependent calcium channels, which play an important role in the regulation of calcium content of vascular smooth muscle cells, have been reported to be absent in platelets (Doyle & Ruegg, 1985). Secondly,

platelets do not have the capacity to synthesise receptor proteins, thus may not be a suitable model for studying receptor regulation in response to drug challenges in vascular tissues (Hamilton & Reid, 1986).

5.1.2. Signalling systems for platelet activation or inhibition

There are three main second messenger systems that are involved in the activation or inhibition of platelets: (1) the inositol trisphosphate & diacylglycerol pathway, (2) the cAMP pathway, and (3) the cGMP pathway (Rink & Sage, 1990).

(1) Agonists that increase the production of inositol trisphosphate & diacylglycerol by way of phosphatidylinositol 4,5-bisphosphate hydrolysis, increase $[Ca^{2+}]_i$ and activate platelets. Exogenous diacylglycerol, and the tumour promoter, phorbol ester, activate protein kinase C and stimulate a slow secretion and aggregation without elevating $[Ca^{2+}]_i$ (Rink, 1983).

(2) Agonists that elevate cAMP levels suppress platelet function in response to stimuli (Rink & Sage, 1990), whereas alpha-2-adrenoceptor agonists that inhibit adenyl cyclase and decrease cAMP levels, can activate platelets (Grant & Scrutton, 1979).

(3) Agonists such as nitroprusside that elevate cGMP cause inhibition of platelet responses to stimuli (Rink & Sage, 1990). Endothelium-dependent relaxing factor and nitric oxide have been shown to inhibit platelet aggregation and adhesion, with stimulation of guanylate cyclase and elevation of cGMP levels (Radomski et al, 1987).

5.1.3. Platelet $[Ca^{2+}]_i$ in hypertension and atherosclerotic diseases

Erne et al in 1984 reported that platelet $[Ca^{2+}]_i$ was significantly higher in patients with essential hypertension than in normotensive controls, was significantly correlated with blood pressure levels, and was reduced from the elevated levels in hypertensive patients after treatment with anti-hypertensive drugs, correlating with the fall in blood pressure (Erne et al, 1984). Ever since there have been reports on this subject with conflicting findings. Although several groups also showed a

significantly higher platelet $[Ca^{2+}]_i$ in hypertensive patients than in controls (Astarie et al, 1989; Bruschi et al, 1985; Haller et al, 1987; Pritchard et al, 1989), no significant difference in platelet $[Ca^{2+}]_i$ between hypertensives and controls has also been reported (Barr & Reid, 1989; Taylor et al, 1989). Moreover, in contrast to the strong correlation between platelet $[Ca^{2+}]_i$ and blood pressure as reported by Erne et al (1984), recent studies have suggested either a weak correlation (Haller et al, 1987; Lechi et al, 1988; Pritchard et al, 1989), or no correlation (Le Quan-Sang et al, 1987; Cooper et al, 1987; Hvarfner et al, 1988) between $[Ca^{2+}]_i$ and blood pressure.

Studies in animal models of hypertension also gave inconsistent findings. Increased basal platelet $[Ca^{2+}]_i$ in spontaneously hypertensive rats as compared to Wistar-Kyoto rats has been reported by some groups (Bruschi et al, 1985; Vasdev et al, 1988; Baba et al, 1987; Oshima et al, 1990). In contrast, Murakawa et al (1986) and Zimlichman et al (1986) found no differences in platelet $[Ca^{2+}]_i$ between spontaneously hypertensive rats and Wistar-Kyoto rats. In secondary, experimentally-induced hypertension, such as deoxycorticosterone-acetate-salt hypertensive rats (Murakawa et al, 1986; Baba et al, 1987), two-kidney-one-clip hypertensive rats (Baba et al, 1987), and perinephritis hypertensive rabbits (Barr et al, 1989), no increase in platelet $[Ca^{2+}]_i$ has been reported.

So far there are little data on platelet $[Ca^{2+}]_i$ in hypercholesterolemic or atherosclerotic conditions. Nevertheless, platelet activity has been reported to be elevated in patients with hypercholesterolemia, hyperlipidemia, and atherosclerosis (For review, see Aviram & Brook, 1987).

We therefore undertook to investigate basal platelet $[Ca^{2+}]_i$ in hypertensive or hypercholesterolemic rabbits, in conjunction with studies on phosphoinositide hydrolysis and in vivo vascular dose response curves in these animals.

5.2. Materials and methods

For a detailed description of the four groups of rabbits, see the "Overall Study Design" Chapter. At the 17th week after renal operation, an arterial line was inserted in the rabbit ear to collect blood for platelet $[Ca^{2+}]_i$ measurement.

9 ml of blood from each rabbit was collected into 1 ml 3.8% sodium citrate. Platelet rich plasma was prepared from the blood by centrifugation at 800 rpm, at 22 °C for 15 minutes. The platelet rich plasma was removed, and prostacyclin (PGI_2, $5x10^{-7}$ M) was added and incubated for 5 minutes at 37 °C to prevent activation of platelets during centrifugation. A platelet pellet was obtained by centrifugation at 2000 rpm, at 22 °C for 15 minutes. The supernatant was discarded and the platelet pellet resuspended in a Ca^{2+} free buffer (136.9 mM NaCl, 2.7 mM KCL, 2mM $MgCl_2$, 11.9 mM $NaHCO_3$, 5.5 mM glucose, 0.32 mM NaH_2PO_4, 0.35% bovine serum albumin; pH 6.5). Quin 2-AM (20 μM) was added and the platelet suspension was incubated for 30 minutes at 37 °C. Five minutes before the completion of incubation, PGI_2 ($5x10^{-7}$ M) was added. The platelet suspension was then separated from any extraneous dye by centrifugation at 2000 rpm, at 22 °C for 15 minutes. The platelet pellet was resuspended in a Ca^{2+} containing buffer (136.9 mM NaCl, 2.7 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 5.5 mM glucose, 0.32 mM NaH_2PO_4, 0.35% bovine serum albumin; pH 7.4) and incubated at 37 °C before use. Fluorescence was measured on a Perkin-Elmer LS-3 luminescence spectrometer with standard monochromator settings of 339 nm excitation and 492 nm emission. Three separate aliquots of each sample was used for platelet $[Ca^{2+}]_i$ measurement and the mean of these was calculated.

Basal platelet $[Ca^{2+}]_i$ was calculated from the fluorescence levels using the equation :

$$[Ca^{2+}]_i = 115 \times [(F-F_{min}) \div (F_{max}-F)],$$

where 115 is the dissociation constant for Quin 2-Ca^{2+} complex,

F is the reading from the resting platelets,

F_{max} is obtained by lysing the platelets with digitonin (50 μM),

F_{min} is obtained (after F_{max}) by chelating all the free Ca^{2+} with EGTA (40 mM).

Quin-2-AM ester was purchased from Lancaster Synthesis (Morecambe, UK), and prepared by dissolving 50mg in 7.43 ml dimethylsulphoxide (DMSO) and making up to 10 ml solution. Aliquots were stored at -20°C. All other laboratory reagents were from Sigma Chemical Co. (Poole, UK).

Analysis of variance was used to compare the platelet $[Ca^{2+}]_i$ in the four groups of rabbits. All results were expressed as mean ± S.D. $P < 0.05$ was considered statistically significant.

5.3. Results

As shown in Figure 5.1, there were no significant differences in basal platelet $[Ca^{2+}]_i$ between the four groups. Mean values of platelet $[Ca^{2+}]_i$ in the control, hypercholesterolemic, hypertensive, and hypertensive-hypercholesterolemic groups were 205.0 ± 37.7, 215.3 ± 50.5, 212.2 ± 42.9, and 211.9 ± 53.1 nM respectively. (n= 7-11)

The basal mean arterial pressure, heart rate, body weight, and plasma cholesterol levels at this time for each group of rabbits are shown in Table 5.1.

5.4. Discussion

Our results show that neither high blood pressure (30-40 mmHg above normal) nor hypercholesterolemia (> 25 mmol/L) was reflected as changes in the basal platelet $[Ca^{2+}]_i$ in rabbits four months after the cellophane wrapping or 0.3% cholesterol diet. Taking together our results (16-17 weeks after operation) and earlier results from our department (6-8 weeks after operation) (Barr et al, 1989), we conclude that in the established phase of perinephritis hypertension in the rabbits, there is no change in basal platelet $[Ca^{2+}]_i$. Other forms of secondary hypertension

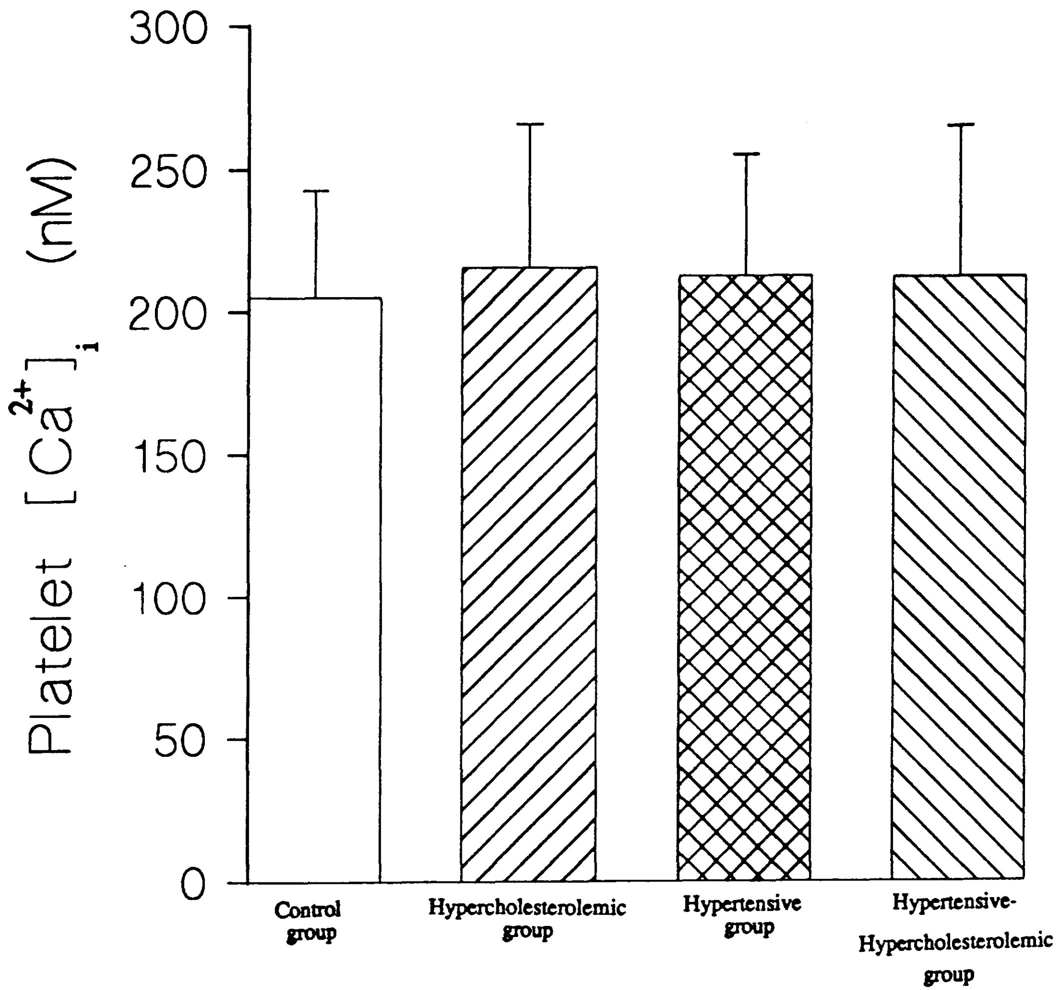


Figure 5.1: Basal platelet $[Ca^{2+}]_i$ in each group of rabbits 17 weeks after surgery and dietary manipulation. (Mean ± S.D., n= 7-11)

Table 5.1. Mean arterial pressure, heart rate, plasma cholesterol, and body weight at the 17th week when platelet $[Ca^{2+}]_i$ was measured in each group of rabbits. (Mean ± S.D., n=13-17)

Category	Control Gr.	Chol. Gr.	Ht. Gr.	Ht-Chol. Gr.
Mean arterial pressure (mmHg)	78±5	78±5	116±13*	108±7*
Heart rate (bpm)	211±21	179±25*	199±28	180±25*
Plasma cholesterol (mmol/L)	0.7±0.3	33.1±9.6*	0.7±0.3	29.5±11.9*
Body weight (kg)	4.1±0.5	3.5±0.4*	3.5±0.5*	3.5±0.3*

* $p < 0.05$ vs Control group.

Abbreviations for groups: Chol. ------- Hypercholesterolemic; Ht. --- Hypertensive;
Ht-Chol. --- Hypertensive-Hypercholesterolemic

in animals (deoxycorticosterone-acetate-salt hypertension, and 2-kidney-1-clip hypertension in rats) (Baba et al, 1987; Murakawa et al, 1986) also did not cause an increase in platelet $[Ca^{2+}]_i$. However, it would be interesting to see whether platelet $[Ca^{2+}]_i$ is increased in the developing stage of secondary hypertension, for example, during the 2nd and 3rd week after cellophane operation in our model. Or whether increased platelet $[Ca^{2+}]_i$ is only to be found in primary hypertension----in spontaneously hypertensive rats or essentially hypertensive patients, as suggested in some reports (Bruschi et al, 1985; Baba et al, 1987; Vasdev et al, 1988; Erne et al, 1984; Haller et al, 1987; Pritchard et al, 1989). It is also possible that platelet $[Ca^{2+}]_i$ does not in fact reflect hypertensive states, either primary or secondary (Murakawa et al, 1986; Zimlichman et al, 1986; Barr & Reid, 1989; Barr et al, 1989).

Our results also indicate that basal platelet $[Ca^{2+}]_i$ was not increased in severe hypercholesterolemia (> 25 mmol/L) with concurrent atherosclerotic lesions.

Although platelet activity in terms of secretion or aggregation has been reported to be different in hypercholesterolemic animals, there was no mention of basal platelet $[Ca^{2+}]_i$ levels or relevant Ca^{2+} kinetics in platelets (Aviram & Brook, 1987). However, Strickberger et al (1988) reported that there is an increased influx of Ca^{2+}, namely plasma membrane calcium permeability, in aortic tissue in cholesterol-fed rabbits.

Two questions arise from these reports:

Firstly, if there is any perturbation in the Ca^{2+} dynamics as well as kinetics in the vascular smooth cells in hypertensive and/or hypercholesterolemic states, is it always mirrored in platelets ?

Secondly, Ca^{2+} perturbation---dynamics or kinetics---may not be confined to one single parameter, i.e., other counter or compensatory mechanisms may also come into play so that the overall picture is not different under various hypertensive and/or

hypercholesterolemic conditions. In other words, the gross normality can conceal a host of abnormalities offsetting each other. Thus, whether platelet $[Ca^{2+}]_i$ measurement is a good approach or not to unravel any changes or abnormalities in cellular Ca^{2+} dynamics or kinetics in hypertension or hypercholesterolemia is open to question.

CHAPTER 6

PHOSPHOINOSITIDE HYDROLYSIS IN AORTA FROM HYPERTENSIVE AND HYPERCHOLESTEROLEMIC RABBITS

Chapter 6. Phosphoinositide Hydrolysis in Aorta from Hypertensive and Hypercholesterolemic Rabbits

6.1. Introduction

6.1.1. Intracellular Ca^{2+} concentrations and vascular smooth muscle contraction

Contraction-relaxation cycles in vascular smooth muscle cells are largely dependent on changes in the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Karaki, 1989). Activation of receptors by agonists may lead to release of intracellular second messengers, which in turn modulate intracellular $[Ca^{2+}]_i$ levels and vascular smooth muscle contraction. There are three main "second messenger systems" involved in the regulation of $[Ca^{2+}]_i$ and thereby the contraction-relaxation of vascular smooth muscle cells. They are:

(1) The phosphoinositide hydrolysis system (i.e., inositol 1,4,5-trisphosphate and 1,2-diacylglycerol system).

(2) The cAMP system.

(3) The cGMP system. (Figure 6.1) (Karaki, 1989)

Agonists such as noradrenaline can stimulate the production of inositol trisphosphate and diacylglycerol by way of phosphatidylinositol 4,5-bisphosphate hydrolysis, increase $[Ca^{2+}]_i$ and cause the contraction of vascular smooth muscle cells.

Agonists such as isoproterenol can elevate cAMP levels in vascular smooth muscle cells, decrease $[Ca^{2+}]_i$ as well as the Ca^{2+} sensitivity of contractile elements, and thus relax the smooth muscle (Karaki, 1989).

Agonists such as nitroprusside can elevate cGMP levels in vascular smooth muscle cells, decrease $[Ca^{2+}]_i$ as well as Ca^{2+} sensitivity of contractile elements,

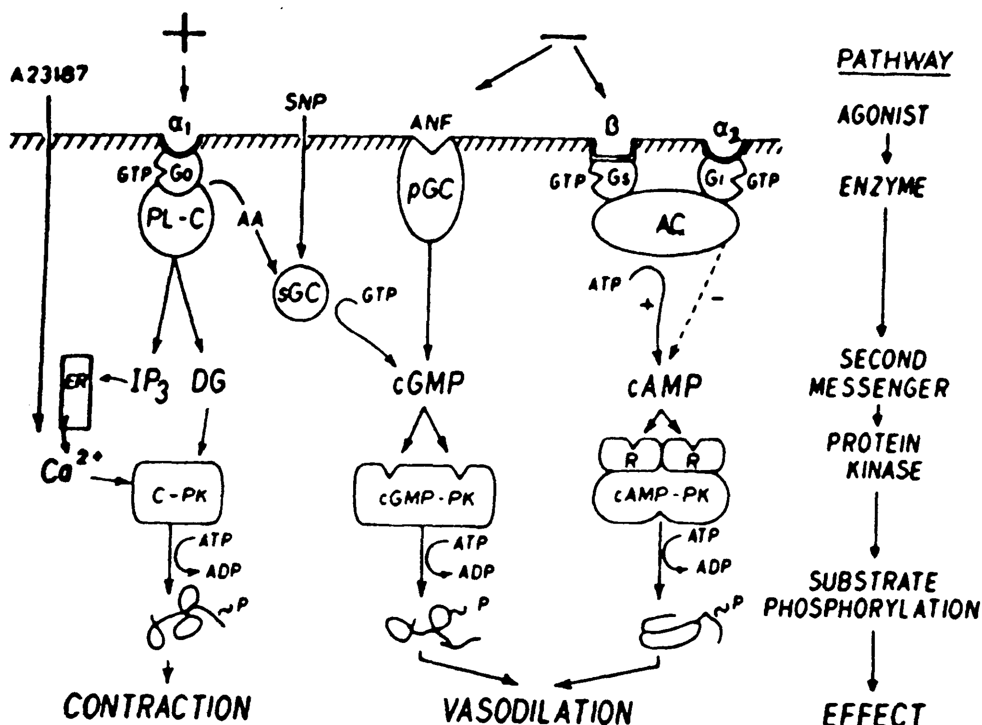


Figure 6.1: Second messenger systems involved in the contraction or relaxation of vascular smooth muscle cells.

(Abbreviations: AC, adenyl cyclase; ANF, atrial natriuretic factor; ER, endoplasmic reticulum; Gs& Gi, stimulatory and inhibitory G-proteins for adenyl cyclase, respectively; pGC & sGC, particulate and soluble guanylate cyclase, respectively; PK, protein kinase; SNP, sodium nitroprusside.) For more detailed description, see the text.

(Source of figure: Hamet P & Tremblay J. Cyclic nucleotides in the pathogenesis of hypertension. In Laragh JH, Brenner BM, eds. Hypertension: Pathophysiology, Diagnosis, and Management. New York: Raven Press, 1990: 617-635.)

and cause relaxation of the smooth muscle (Karaki, 1989).

6.1.2. The phosphoinositide second messenger system in vascular smooth muscle contraction

Phosphoinositide hydrolysis is the most important pathway in receptor-activated vascular smooth muscle contraction.

In the following section, an outline of the general pattern of the phosphoinositide messenger system in vascular smooth cells is presented.

As shown in Figures 6.1 and 6.2, on the binding of agonists to receptors, an enzyme phospholipase C on the cell membrane is activated. It stimulates the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate into two products: inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Agonists acting via phospholipase C include noradrenaline, angiotensin II, vasopressin, serotonin, endothelin, and platelet-derived growth factor (Hashimoto et al, 1986; Rapoport, 1987; Nabika et al, 1985; Griendling et al, 1986; Roth et al, 1986; Resink et al, 1988; Block et al, 1990). Although G-proteins are believed to be involved as transducers of the external signals to the membrane phosphoinositide second messenger system, the identity of the G proteins remains speculative (LaBelle & Murray, 1990; Cheung et al, 1990; Takuwa et al, 1990; Reynolds et al, 1989). It is possible that more than one G protein is involved. Some research groups have reported that in α-adrenergic mediated phosphoinositide hydrolysis or vasoconstriction, pretreatment with pertussis toxin, which is a widely used G_i-protein ribosylating agent, only partially reduced the response (Berta et al, 1988; Liebau et al,1989; Boonen & De May, 1990), while other groups have suggested a crucial role of one or more "non G_i-proteins" in mediating the phosphoinositide hydrolysis response to noradrenaline or endothelin in vascular smooth muscle cells (Cheung et al, 1990; Takuwa et al, 1990; LaBelle & Murray, 1990).

The inositol trisphophate formed from phosphatidylinositol 4,5-bisphosphate

hydrolysis then diffuses through the cytosol to bind to a receptor site on the endoplasmic reticulum, thereby releasing Ca^{2+} stored intracellularly in the latter (Kowarski et al, 1985; Somlyo et al, 1985; Somlyo et al, 1988). Inositol trisphosphate induces a rapid, short-lived increase in $[Ca^{2+}]_i$ and thereby a phasic contraction of vascular smooth muscle cells (Somlyo et al, 1985; Somlyo et al, 1988). Inositol trisphosphate is rapidly metabolized and the increase in $[Ca^{2+}]_i$ is not maintained (Hashimoto et al, 1986; Grieldling et al, 1986; Alexander et al, 1985; Takuwa et al, 1990; Sasaguri et al, 1985). The inositol trisphosphate binding site on the endoplasmic reticulum has been shown to exhibit enantiomer specificity as well as structural-activity relationships. D-1,4,5-trisphosphate is the most potent stimulant for Ca^{2+} release as compared to L-1,4,5-trisphosphate or other inositol trisphosphate analogues or inositol 1,3,4,5-tetrakisphosphate (Polokoff et al, 1988; Ehrlich & Watras, 1988). Some reports suggest that inositol trisphosphate-induced Ca^{2+} release from the endoplasmic reticulum could be specifically inhibited by heparin (Ehrlich & Watras, 1988; Kobayashi et al, 1988a; Yamamoto et al, 1990) and might involve some guanine nucleotide-binding protein (G protein) on the endoplasmic reticulum for the Ca^{2+} release process (Kobayashi et al, 1988a; Kobayashi et al, 1988b; Somlyo et al, 1988).

Thus the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) rises from approximately 0.1 μM in the unstimulated state to 1-10 μM in the agonist-stimulated state (Abdel-Latif, 1986; Sato et al, 1988; Hirano et al, 1991). Ca^{2+} ions bind to calmodulin. The Ca^{2+}-calmodulin complex then activates myosin light chain kinase, which in turn phosphorylates the myosin light chain, thus facilitating the "cross-bridging " with actin, and consequently initiating smooth muscle contraction (Kamm & Stull, 1989).

Inositol 1,3,4,5-tetrakisphosphate is one of the metabolic products of inositol 1,4,5-trisphosphate (Downes & MacPhee, 1990). It has been detected in the arterial tissues after noradrenaline stimulation (Ollerenshaw, et al, 1988a; Pijuan & Litosch,

1988). However, the exact role of inositol 1,3,4,5-tetrakisphosphate in the sequence of events of vascular contraction remains to be defined, despite some speculations about its role in $[Ca^{2+}]_i$ dynamics and kinetics in other cell types (Berridge & Irvine, 1989).

1,2-Diacylglycerol, the other product of phosphatidylinositol 4,5-bisphosphate hydrolysis, remains within the cell membrane, and, in conjuction with the elevated $[Ca^{2+}]_i$, translocates the cytosolic protein kinase C to the cell membrane and thereby activates protein kinase C (Nishizuka, 1986; Haller et al, 1990).

It has been proposed that the activated protein kinase C performs a variety of functions:

(1) Activated protein kinase C has been shown to cause a slow, but sustained contraction of vascular smooth muscle cells (Haller et al, 1990; Jiang & Morgan, 1987; Forder et al, 1985), in line with a sustained increase in diacylglycerol production after agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis (Griendling et al, 1986; Griendling et al,1989; Takuwa et al, 1990). It has been suggested that protein kinase C causes tonic contraction by phosphorylating the late-phase contractile apparatus called filamin-actin-desmin (Rassmussen et al, 1987; Haller et al, 1990). Activated protein kinase C has also been reported to enhance the Ca^{2+} sensitivity of vascular contractile proteins (Itoh et al, 1988), and vascular contraction can be induced by activated protein kinase C without raising $[Ca^{2+}]_i$ (Jiang & Morgan, 1987; Itoh & Lederis, 1987).

(2) Activated protein kinase C has been shown to exert an inhibitive effect on the subsequent phosphatidylinositol 4,5-bisphosphate hydrolysis (Roth et al, 1986; Brock et al, 1985; Araki et al, 1989) and vascular contraction (Danthuluri & Deth, 1984; Khalil & Van Breemen, 1988).

(3) Activated protein kinase C was found to induce an increase in Ca^{2+} influx from the extracellular space into vascular smooth muscle cells (Khalil & Van Breemen,

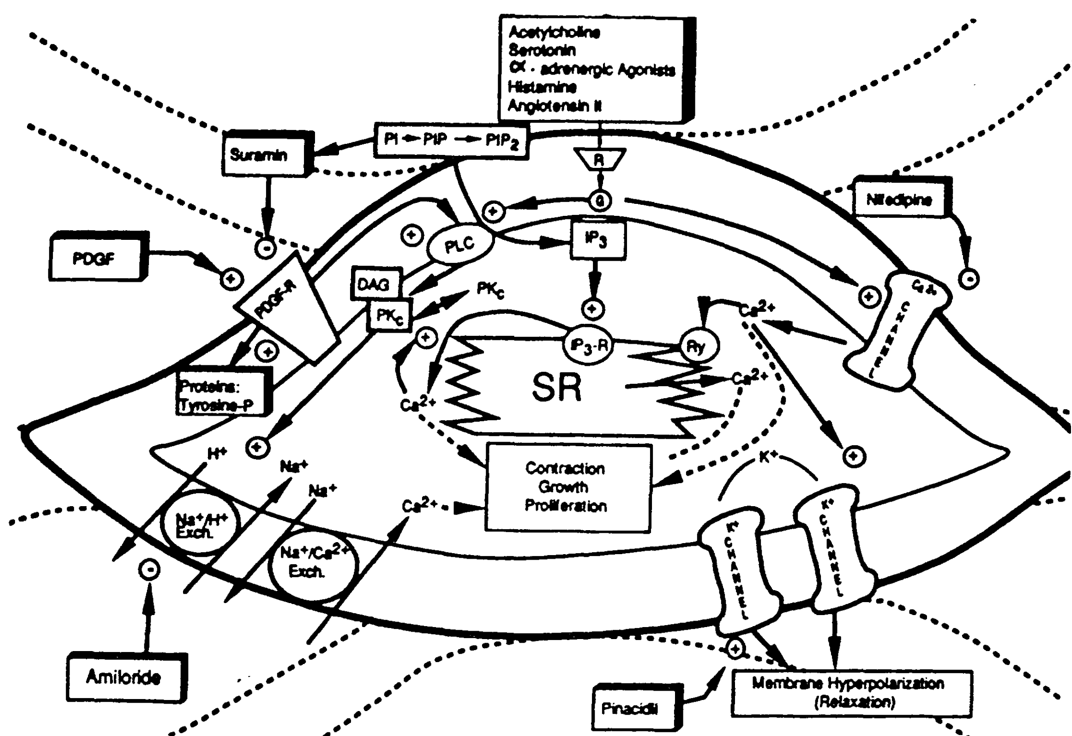


Figure 6.2: Shematic picture of the phosphoinositide messenger system in vascular smooth muscle cells.

An agonist, e.g., noradrenaline, binds to the receptor (R) in the cell membrane and activates phospholipase C (PLC) via a transducer G-protein (G) and thereby stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). The hydrolysis products, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), are keys to specific subsequent cellular processes. IP_3 stimulates the release of Ca^{2+} from sarcoplasmic (endoplasmic) reticulum (SR) via a specific IP_3-receptor protein, and eventually triggers muscle contraction. DAG translocates cytoplasmic protein kinase C (PK_c) to the cell membrane and the activated PK_c may phosphorylates a range of substrates including Na^+/H^+ exchange, and contractile proteins. Mitogens, e.g., platelet-derived growth factor (PDGF) possibly induce cell growth or proliferation in part via phosphoinositide second messenger pathway.

(Source of figure: Hathaway DR, March KL, Lash JA, Adam LP, & Wilensky RL. Vascular smooth muscle: A review of the molecular basis of contractility. Circulation 1991; 83: 382-390).

1988).

(4) Activated protein kinase C was reported to be involved in the activation of membrane Na^+/H^+ antiport and thereby cellular alkalisation (Berk et al, 1987; Hatori et al, 1987).

6.1.3. The role of the phosphoinositide messenger system in mitogenecity and the proliferative process of vascular smooth muscle cells

Proliferation of vascular smooth muscle is well recognized as central to the pathology of major vascular diseases such as hypertension and atherosclerosis (Schwartz et al, 1986; Mulvany & Simonsen, 1989). Studies on cellular and molecular responses to growth factors suggest an involvement of phosphoinositide hydrolysis in cell proliferation. At earlier stages of cell proliferation the following changes have been observed (For review, see Taylor, 1986; Heagerty & Ollerenshaw, 1990; Berridge, 1986):

(1) An increase in phosphoinositide turnover reflecting increased phospholipase C activity.

(2) An increase in cytosolic Ca^{2+} concentrations.

(3) Stimulation of a Na^+/H^+ antiport leading to an increase in cytosolic pH (namely, cellular alkalisation).

(4) An increase in tyrosine-specific protein kinase activity.

In cultured vascular smooth muscle cells, an enhanced cell proliferation or gene expression, associated with increased phosphoinositide metabolism (increase in $[Ca^{2+}]_i$, inositol trisphosphate, phospholipase C activity, or protein kinase C activity) could be induced with the addition of angiotensin II (Paquet et al, 1990), serotonin (Paquet et al, 1989), bradykinin (Paquet et al, 1989), endothelin (Muldoon et al, 1989), platelet-derived growth factor (Block et al, 1990), epidermal growth factor (Scott-Burden et al, 1989a) and low-density lipoprotein (Block et al, 1990; Scott-Burden et al, 1989b)

In cultured vascular smooth muscle cells, both phosphoinositide metabolism and cell proliferation rate were increased in spontaneously hypertensive rats as compared to Wistar-Kyoto rats, in non-stimulated (Resink et al, 1987) and more distinctively, in agonist-stimulated conditions (Paquet et al, 1989; Scott-Burden et al, 1989a; Paquet et al, 1990).

However, the phosphoinositide messenger system is not the only trigger for cell growth in the regulation of proliferation of vascular smooth muscle cells; the cAMP messenger system has also been implicated (Hamet & Tremblay, 1990).

6.1.4. Phosphoinositide metabolism in hypertension and atherosclerotic diseases

Since the phosphoinositide pathway is a common signalling system for both smooth muscle contraction and proliferation (See Sections 6.1.2. and 6.1.3.), much effort has been gone into investigating whether phosphoinositide metabolism is perturbed in hypertension and atherosclerotic diseases. In this regard, both vascular smooth muscle cells and other non-muscle cells have been employed in investigating the possible perturbations of phosphoinositide metabolism in hypertension or atherosclerotic diseases. The latter includes blood elements such as platelets, erythrocytes, and leukocytes, mainly because these are more easily accessible than vascular smooth muscle cells and they also have the phosphoinositide system present in the cell membrane. In the following section, the literature reports on phosphoinositide metabolism in hypertension and atherosclerotic diseases are reviewed and discussed.

6.1.4.1. Phosphoinositide metabolism in hypertension

Studies on phosphoinositide metabolism can be broadly categorized into three types:

(1) On the content of the phosphoinositides in the cell membrane, namely the amount of phosphotidylinositol, phosphatidylinositol 4-phosphate, and

phosphatidylinositol 4,5-bisphosphate, in the "non-stimulated" cells, measured after [^{3}H] or [^{32}P] radiolabelling.

(2) On the activity of membrane phospholipase C under basal, non-stimulated conditions, measured as radioactivity of various inositol phosphates or diacylglycerol after radiolabelling the phosphoinositides with [^{3}H] or [^{32}P]; this is the basal rate of phosphoinositide hydrolysis.

(3) On the response of phosphoinositide hydrolysis to the challenge of agonists, namely the relative yield of inositol 1-monophosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate or diacylglycerol after stimulation as compared to non-stimulated, basal states. This gives a measure of the activity of membrane phospholipase C in response to agonists.

(1) The content of the phosphoinositides in the membrane

A higher content of, or a higher [^{3}H] or [^{32}P] incorporation into, phosphatidylinositol 4,5-bisphosphate, has been reported in (A) resistance arteries from 5-week spontaneously hypertensive rats (Durkin et al, 1990), (B) cultured vascular smooth muscle cells from 20-week spontaneously hypertensive rats (Resink et al, 1987), (C) erythrocytes from 12-week spontaneously hypertensive rats (Boriskina et al, 1978), as compared to their controls from age-matched Wistar-Kyoto rats. Similarly, a higher [^{32}P] incorporation into phosphatidylinositol 4,5-bisphosphate was found in platelets (Dimitrov et al, 1986) and erythrocytes (Marche et al, 1985) from patients with essential hypertension as compared to their controls from age-matched normotensive subjects.

In contrast, no difference in [^{32}P] incorporation into phosphatidylinositol 4,5-bisphosphate was observed in (A) aorta from 3-to-4-month spontaneously hypertensive rats (Ek et al, 1989), (B) platelets from 12-to-15-week spontaneously hypertensive rats (Koutouzov et al, 1987), (C) platelets from patients with essential hypertension (Remmal et al, 1988), (D) erythrocytes from patients with essential hypertension (Riozzi et al, 1987), compared to their proper controls.

To further complicate the issue, a decreased [^{32}P] incorporation into phosphatidylinositol 4,5-bisphosphate in primary hypertension was reported to be manifest at the pre-hypertensive stage, and tended to disappear at the established stage of hypertension (Durkin et al, 1990; Riozzi et al, 1987). Kiselev et al (1981) also observed that the higher incorporation of [^{32}P] into phosphatidylinositol 4,5-bisphosphate in erythrocytes from 1-month spontaneously hypertensive rats was reversed to the opposite at 4-month of age.

In secondary, experimental hypertension, Boriskina et al (1978) found a significantly higher content of phosphatidylinositol 4,5-bisphosphate in erythrocytes from Goldblatt renal hypertensive rats, 8 weeks after operation, compared to their controls from Wistar-Kyoto rats.

(2) Basal, non-stimulated rate of phosphoinositide hydrolysis (or phospholipase C activity)

A significantly higher rate of basal phosphoinositide hydrolysis has been reported in (A) aorta from 5-week spontaneously hypertensive rats (Heagerty et al, 1986), (B) aorta from 4-week as well as 14-week spontaneously hypertensive rats (Uehara et al, 1988), (C) renal cortex and medulla from 10-week and 40-week stroke-prone spontaneously hypertensive rats (Kawaguchi et al, 1987), (D) erythrocytes from 16-week spontaneously hypertensive rats (Tremblay et al, 1990), and (E) cultured aortic smooth muscle cells from 20-week spontaneously hypertensive rats (Resink et al, 1987), compared to their proper controls from Wistar-Kyoto rats.

In contrast, no difference in basal phosphoinositide hydrolysis was observed in (A) aorta from 19-week (Heagerty et al,1986) and 3-to-4-month (Ek et al, 1989) spontaneously hypertensive rats, and from 4-to-8-month stroke-prone spontaneously hypertensive rats (Turla & Webb, 1990), (B) resistance arteries from 5-week and 12-week spontaneously hypertensive rats (Durkin et al, 1990), (C) cultured aortic smooth muscle cells from 11-week spontaneously hypertensive rats (Paquet et al,

1989), (D) renal cortex from 13-week spontaneously hypertensive rats (Jeffries et al, 1988), (E) platelets from 12-to-15-week spontaneously hypertensive rats (Koutouzov et al, 1988), (F) cultured neurons from 1-day spontaneously hypertensive rats (Feldstein et al, 1986), (G) cultured fibroblasts from 10-week spontaneously hypertensive rats (Zhu et al, 1990), as compared to their proper controls from Wistar-Kyoto rats.

In secondary, experimental hypertension, the results were also divergent. A higher rate of basal phosphoinositide hydrolysis was observed in (A) aorta from aldosterone-salt hypertensive rats (3-to-4-week administration) (Jones et al, 1988), (B) aorta of the "pressure-loaded" segment from coarctation hypertensive rats (9-to-20-day after operation) (Ollerenshaw et al, 1988b), (C) cardiac atria and ventricles (but not femoral arteries) from deoxycorticosterone-acetate-salt hypertensive rats (4-week administration) (Eid & de Champlain, 1988), compared to their proper controls from normotensive rats.

In contrast, no difference in basal phosphoinositide hydrolysis was reported in (A) aorta from perinephritis hypertensive rabbits (1-, 2-, & 6-week after operation) (Nixon et al, 1990), (B) mesenteric (Takata et al, 1989) and femoral (Eid & de Champlain, 1988) arteries from deoxycorticosterone-acetate-salt hypertensive rats (4-week administration), compared to their proper controls from normotensive animals.

(3) Response of phosphoinositide hydrolysis or phospholipase C activity to agonist stimulation

An increased response of phosphoinositide hydrolysis to agonist stimulation has been observed in (A) aorta challenged by serotonin from 20-week spontaneously hypertensive rats (Huzoor-Akbar et al, 1989) and 4-to-8-month stroke-prone spontaneously hypertensive rats (Turla & Webb, 1990), (B) resistance arteries stimulated by noradrenaline from 5-week and 12-week spontaneously hypertensive rats (Durkin et al, 1990), (C) platelets stimulated by thrombin from both 12-to-15-

week spontaneously hypertensive rats (Koutouzov et al, 1987) and patients with essential hypertension (Marche et al, 1989), (D) cultured aortic smooth muscle cells from 11-week spontaneously hypertensive rats, stimulated by angiotensin II, serotonin, and bradykinin (Paquet et al, 1989; Paquet et al, 1990), and from 20-week spontaneously hypertensive rats, stimulated by angiotensin II, endothelin-1, and epidermal growth factor (Resink et al, 1989b; Resink et al, 1990; Scott-Burden et al, 1989a), (E) cultured fibroblasts from 10-week spontaneously hypertensive rats, stimulated by thrombin and arginine-vasopressin (Zhu et al, 1990), compared to their proper controls.

In contrast, a reduced response of phosphoinositide hydrolysis (or phospholipase C activity) was shown in (A) aorta stimulated by noradrenaline from 5-week and 19-week (Heagerty et al, 1986), as well as 3-to-4-month (Ek et al, 1989), spontaneously hypertensive rats, (B) renal cortex stimulated by noradrenaline from 4-week and 13-week spontaneously hypertensive rats (Jeffries et al, 1988), (C) cultured neurons stimulated by noradrenaline from 1-day spontaneously hypertensive rats (Feldstein et al, 1986), compared to their proper controls.

In secondary, experimental hypertension, an increased response of phosphoinositide hydrolysis was reported in (A) aorta and femoral arteries stimulated by noradrenaline from perinephritis hypertensive rabbits (2-week, but not 1-week or 6-week after operation) (Nixon et al, 1990), (B) aorta (Takata et al, 1989), or cardiac atria and ventricles, and femoral arteries (Eid & de Champlain, 1988), stimulated by noradrenaline from 4-week-treated deoxycorticosterone-acetate-salt hypertensive rats, or cardiac atria and mesenteric arteries stimulated by endothelin-1 or noradrenaline (de Champlain et al, 1989) from 3-week-treated deoxycorticosterone-acetate-salt hypertensive rats, (C) aorta stimulated by noradrenaline from 3-to-4-week-treated aldosterone-salt hypertensive rats (Jones et al, 1988), compared to their proper controls.

In contrast, no change in the response of phosphoinositide hydrolysis was observed in (A) aorta stimulated by endothelin-1 from 2-week-after-operation perinephritis hypertensive rabbits (in contrast to the results from noradrenaline stimulation) (Nixon et al, 1990), (B) platelets stimulated by thrombin from 6-to-7-week-treated deoxycorticosterone-acetate-salt hypertensive rats (Limon et al, 1990), as compared to their proper controls.

From the results surveyed above, some points are worthy of note:

(1) Changes in phosphoinositide metabolism may differ with regard to the stage of hypertension (Durkin et al, 1990; Heagerty et al, 1986; Nixon et al, 1990).

(2) Changes in phosphoinositide metabolism may differ with regard to different tissues examined (Eid & de Champlain, 1988) and even differ between aorta and resistance arteries (Heagerty et al, 1986; Durkin et al, 1990).

(3) Changes in phosphoinositide metabolism may differ with regard to the agonists used for stimulation (Nixon et al, 1990; Zhu et al, 1990).

(4) Changes in phosphoinositide metabolism may differ with regard to different models of hypertension (Limon et al, 1990).

(5) Changes in phosphoinositide metabolism may differ with regard to the presence or absence of agonist stimulation (Durkin et al, 1990; Nixon et al, 1990; Turla & Webb, 1990; Paquet et al, 1989).

6.1.4.2. Phosphoinositide metabolism in hypercholesterolemic or atherosclerotic states

In contrast to the published reports on phosphoinositide metabolism in hypertension, little is known about phosphoinositide metabolism in hypercholesterolemic or atherosclerotic states.

Winocour et al (1990) reported that there was a significantly higher phosphoinositide hydrolysis in thrombin-stimulated platelets from hypercholesterolemic rats than normocholesterolemic rats. Moreover, a similar

conclusion was drawn whether the hypecholesterolemia was "genetically-determined" or "diet-induced".

In summary, there is a considerable body of evidence suggesting that alterations in phosphoinositide metabolism may occur in cardiovascular diseases. However, controversy exists with different results reported depending on the experimental models, the parameters examined, the agonists used, and the duration or severity of the disease state. We have therefore included an investigation into phosphoinositide metabolism in our studies on hypertension and hypercholesterolemia in the rabbits. Phosphatidylinositol 4,5-bisphosphate hydrolysis in response to two very different agonists, noradrenaline and endothelin-1, was examined.

6.2. Materials and methods

For a detailed description of the four groups of rabbits, see the "Overall Study Design" chapter. At the 18th week after renal operation, the rabbits were killed by intravenous injection of sodium pentobarbitone (60 mg/kg). The thoracic and abdominal aorta were isolated immediately and cleared of adherent connective tissue and then washed for 10 minutes X 3 times in Krebs Ringer bicarbonate buffer (KRB) solution, at 37 °C with continuous bubbling of 95% O_2/5% CO_2. (KRB composition: NaCl 118.3 mM, KCl 4.7 mM, $CaCl_2$ 0.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, glucose 11.1 mM, myoinositol 0.1 mM, bovine serum albumin 1.5%; pH 7.4) The tissue was incubated for a further 10 minutes in another KRB solution containing 10 mM LiCl and 2 μM imipramine. Afterwards the aorta was put on a McIlwan tissue chopper and cut into 1 mm rings. 5 Thoracic and 4 abdominal rings were weighed and put into each tube (except blank tubes) for incubation. The incubation medium was 0.3 ml KRB solution containing 10 mM LiCl (inhibiting the breakdown of inositol-1-monophosphate) and 2 μM

imipramine (blocking the neuronal uptake of noradrenaline) and 0.5 μ Ci [^{3}H]-myoinositol (Amersham International, Amersham, U.K.). The tubes were incubated at 37 °C under an atmosphere of 95% O_2/ 5% CO_2 for 3.5 hours. Then agonists (noradrenaline 10^{-6}, 10^{-5}, & 10^{-4} M; endothelin-1 10^{-7}, 10^{-6}, & 10^{-5} M) were added as appropriate. Noradrenaline was prepared in 0.1% ascorbic acid solution. 0.1% Ascorbic acid solution was added into blank and control tubes, which gave a measure of radioactivity in blanks and basal inositol phosphates formation, respectively. Tubes were incubated for a further 30 minutes. At the end of incubation, 2 ml of methanol: chloroform: hydrochloric acid (40:20:1, v/v) was added into each tube. Tubes were sonicated for 45 minutes. More chloroform (0.63 ml) and distilled water (1.26 ml) was added to form two layers. The tubes were centrifuged at 2500 rpm for 10 minutes. 2 ml of aqueous (upper) phase was removed and the pH adjusted to between 6.8 and 7.2. This aqueous phase, which contained inositol mono-, bis-, and tris- phosphates, was then passed through a 1 ml Dowex anion-exchange resin column (Dowex 1-X8 resin, 100-200 mesh, formate form, pH 4.0-5.0). 15 ml of unlabelled myoinositol (5 mM) was run through the column to wash away extraneous [^{3}H]-myoinositol. Finally, 2 ml of 1 M ammonium formate in 0.1 M formic acid was added to elute all the [^{3}H]-inositol phosphates. The elute was collected in scintillation vials and 15 ml scintillant added. [^{3}H]-Inositol phosphates formation was counted by a liquid scintillation counter.

Basal [^{3}H]-inositol phosphates formation is calculated as counts/min per mg of tissue weight. Stimulated [^{3}H]-inositol phosphates formation was calculated as a percentage of basal values.

Endothelin-1 was purchased from Scientific Marketing (Barnet, U.K.). All other reagents unless specified were from Sigma Chemical Co. (Poole, U.K.).

Between-group comparisons were carried out using repeated measures analysis of variance for each agonist. All results were expressed as Mean ± S.D. $p< 0.05$ was considered statistically significant.

6.3 Results

(1) Tissue weights

As shown in Table 6.1, tissue weights (5 thoracic and 4 abdominal aortic rings, 1 mm width each) were significantly higher in the hypercholesterolemic as well as the hypertensive-hypercholesterolemic groups than in the control group. The mean tissue weights were also higher in the hypertensive group than in the control group, but the difference did not reach significance.

(2) Basal $[^3H]$-inositol phosphates formation

There was no difference in basal $[^3H]$-inositol phosphates formation per mg of tissue weight between the four groups. (Table 6.1)

(3) $[^3H]$-Inositol phosphates formation by noradrenaline

As shown in Figure 6.3, there was a dose-related increase in $[^3H]$-inositol phosphates formation in response to noradrenaline (10^{-6} - 10^{-4} M) in each group.

The stimulation tended to be higher in each renal operated group than its normotensive counterpart, but the difference only reached significance for the hypertensive group vs the control group at 10^{-4} M noradrenaline.

The stimulation tende to be lower in each cholesterol-fed group than its normal-diet counterpart, and this difference reached significance for both the control group vs the hypercholesterolemic group, and the hypertensive group vs the hypertensive-hypercholesterolemic group at 10^{-4} M noradrenaline.

(4) $[^3H]$-Inositol phosphates formation stimulated by endothelin-1

As shown in Figure 6.4, there was a dose-related increase in $[^3H]$-inositol phosphates formation in response to endothelin-1 (10^{-7} - 10^{-5} M) in each group.

There was no statistical difference in the $[^3H]$-inositol phosphates formation in response to endothelin-1 between each renal operated group and its normotensive counterpart.

Table 6.1. Tissue weights and basal $[^3H]$-inositol phosphates ($[^3H]$-IPs) formation in aortic (5 thoracic & 4 abdominal) rings in each group of rabbits. (Mean ± S.D., n=6-8)

Category	Control Gr.	Chol. Gr.	Ht. Gr.	Ht-Chol. Gr.
Tissue weight (mg)	31.2±3.6	52.6±12.9*	40.4±7.2	56.8±10.2*
Basal $[^3H]$-IPs/tissue weight (cpm/mg)	36.7±8.6	38.0±7.9	29.0±3.5	36.3±5.3

* $p < 0.05$ vs Control group.

Abbreviations for groups: Chol. ------- Hypercholesterolemic; Ht. --- Hypertensive; Ht-Chol. --- Hypertensive-Hypercholesterolemic

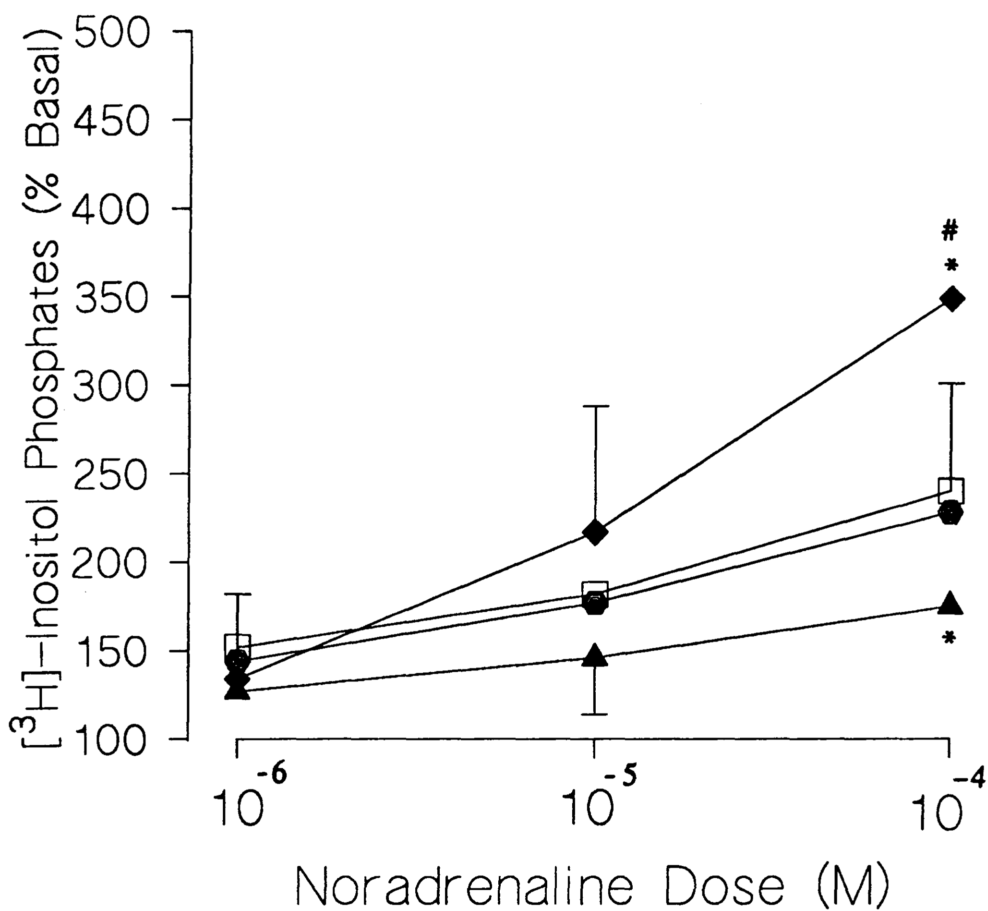


Figure 6.3: Noradrenaline-stimulated [^{3}H]-inositol phosphates formation in each group of rabbits 18 weeks after surgery and dietary manipulation.

(Mean ± S.D., n= 7-11. * $p < 0.05$ versus control group; # $p < 0.05$ Hypertensive group versus Hypertensive-Hypercholesterolemic group.)

□ Control group.

▲ Hypercholesterolemic group.

◆ Hypertensive group.

● Hypertensive-Hypercholesterolemic group.

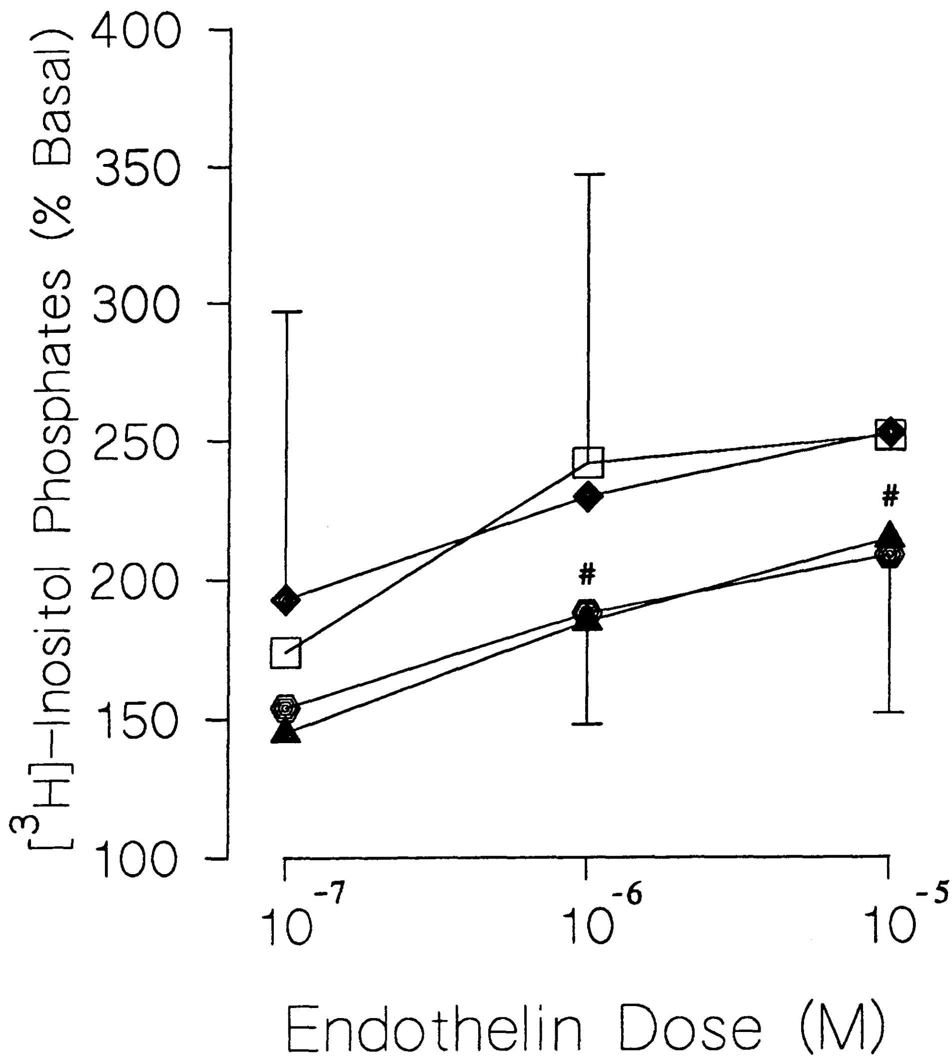


Figure 6.4: Endothelin-1-stimulated [^{3}H]-inositol phosphates formation in each group of rabbits 18 weeks after surgery and dietary manipulation.

(Mean ± S.D., n= 6-10. # $p < 0.05$ Hypertensive group versus Hypertensive-Hypercholesterolemic group.)

□ Control group.

▲ Hypercholesterolemic group.

◆ Hypertensive group.

● Hypertensive-Hypercholesterolemic group.

The stimulation tended to be lower in each cholesterol-fed group than its normal-diet counterpart, but the difference reached significance only for the hypertensive group vs the hypertensive-hypercholesterolemic group at 10^{-6} & 10^{-5} M endothelin-1.

6.4. Discussion

Our results show that there was no difference in the basal [^{3}H]-inositol phosphates formation in the aorta from any experimental group as compared to the control group, suggesting that neither perinephritis hypertension nor 0.3% cholesterol diet for 18 weeks had any effect on the basal metabolism of aortic membrane phosphoinositides. No difference in the basal phosphoinositide hydrolysis was also observed in aorta from 1-, 2-, or 6-week perinephritis hypertensive rabbits versus their proper controls (Nixon et al, 1990).

The greater tissue weights in the two cholesterol-fed groups as compared to the control group were echoed in the picture of severe fatty depositions in the aorta of these two groups visualized by Sudan Red staining. (See Colour Plates 1 to 8)

Our results also showed that there was a dose-related increase in [^{3}H]-inositol phosphates formation in response to noradrenaline and endothelin-1 in each group of rabbits. Severe hypercholesterolemia (> 25 mmol/L at 18th week of study) tended to attenuate the [^{3}H]-inositol phosphates formation in response to noradrenaline as well as endothelin-1. However, hypertension (30-40 mmHg above normal at the 18th week of study) tended to increase [^{3}H]-inositol phosphates formation by noradrenaline, but not endothelin-1. The divergent picture of [^{3}H]-inositol phosphates formation by noradrenaline and by endothelin-1 in perinephritis hypertension has also been shown by Nixon et al (1990) when earlier stages of hypertension were examined (1 to 6 weeks after renal operation as compared to 18 weeks in this study). Nixon et al observed no difference in endothelin-1-stimulated

[^{3}H]-inositol phosphates formation between hypertensive and normotensive rabbits, whereas noradrenaline-stimulated [^{3}H]-inositol phosphates formation was higher in hypertensive rabbits, especially at 2 weeks after renal operation. One explanation for this difference between endothelin-1 and noradrenaline is that, although both stimulate phosphoinositide hydrolysis, their receptor-coupling processes for the hydrolysis of phosphatidylinositol 4,5-bisphosphates may not be the same. In other words, noradrenaline and endothelin-1 may act via different isoenzymes of phospholipase C or transducer G proteins, and perinephritis hypertension might have differential effects on these phospholipase C isoenzymes or transducer G proteins. Ashkenazi et al (1989) reported that functionally distinct G proteins selectively coupled different receptors to phosphoinositide hydrolysis in the same cell. Different patterns of second messenger mobilization from the phosphoinositide messenger system by different agonists have also been shown in vascular smooth muscle cells (Kawahara et al,1988; Haller et al, 1990). Similarly, Zhu et al (1990) found that among agonists capable of stimulating phosphoinositide hydrolysis, some caused significantly greater phosphoinositide hydrolysis in fibroblasts from spontaneously hypertensive rats than in Wistar-Kyoto rats, while others caused a similar degree of stimulation in the two groups.

Enhanced noradrenaline-stimulated [^{3}H]-inositol phosphates formation in aorta or arteries from secondary hypertensive animals has also been reported by others, mostly using the model of deoxycorticosterone-acetate-salt hypertensive rats (Jones et al, 1988; Eid & de Champlain, 1988; Takata et al, 1989; de Champlain et al, 1989). However, while de Champlain et al (1989) found a significantly greater stimulation of phosphoinositide hydrolysis by both noradrenaline and endothelin-1 in mesenteric arteries from deoxycorticosterone-acetate-salt hypertensive rats, in this study and that of Nixon et al (1990) a greater stimulation by noradrenaline, but not endothelin-1, in aorta from perinephritis hypertensive rabbits was observed. Differences in species, mode of hypertension, and tissue location may contribute to

this difference, as reviewed in the Introduction Section of this chapter.

The attenuation of agonist-induced [^{3}H]-inositol phosphates formation in hypercholesterolemic rabbits was surprising. However there are a few possible explanations:

(1) Hypercholesterolemia induced atherosclerotic lesions in the rabbit aorta, such that there was an increased structural barrier to agonists approaching receptor sites in the cell membrane. Indeed, the tissue weights of aortic rings from cholesterol-fed groups were 40-70% higher than their normal-diet counterparts. However, the [^{3}H]-inositol uptake of the aortic muscle cells appeared to be un-impaired, since there was no decrease in basal [^{3}H]-inositol phosphates formation in the cholesterol-fed groups. It is not known how much the fatty depositions contributed to the 40-70% increase in aortic weights from cholesterol-fed groups. It would be interesting to measure the dry weight of the aortic tissue (we only measured wet weight) and also extract the lipid from the aortic tissue in order to calculate the "fatty component" and compare this with the "smooth muscle cells and connective tissue content" of the vessels. More detailed knowledge of the changes in tissue composition of vessels from the hypercholesterolemic animals would provide further insights into the mechanisms behind the biochemical abnormalities observed.

(2) Hypercholesterolemia may alter some part of the receptor-coupling apparatus of the phosphoinositide messenger system in the aortic muscle membrane, such that the phosphoinositide messenger system became less responsive to agonist challenge. Alteration of membrane calcium channels by cholesterol enrichment of the cell membrane has been reported in rabbit arterial smooth muscle (Bialecki & Tulenko, 1989). Changes in adrenergic receptor number in aorta or myocardium from "cholesterol-fed" rabbits has also been observed (Nanda & Henry, 1982; Tsuji et al, 1987). A decrease in receptor number could lead to decreased receptor activation and stimulation of phosphoinositide hydrolysis.

In our study, hypercholesterolemia and hypertension had opposing effects on the agonist-stimulated [^{3}H]-inositol phosphates formation, with hypercholesterolemia attenuating, and hypertension augmenting or not changing, the hydrolysis of phosphatidylinositol 4,5-bisphosphates. Although smooth muscle proliferation is a common essential feature of both hypertension and atherosclerosis, there are also important differences between these two pathologenetic processes:

(1) The main pathological changes inflicted by hypertension are in the "medial" layer of the artery, whereas those inflicted by hypercholesterolemia are in the "intimal" layer (Mulvany & Simonsen, 1989).

(2) Hypercholesterolemia can cause pervasive lipid deposition intracellularly or extracellularly in the artery, while hypertension alone rarely causes lipid deposition without the concurrence of hypercholesterolemia (Chobanian, 1990).

These differences in pathogenesis of hypercholesterolemia and hypertension may contribute to the discrepancies in effects on the agonist-stimulated [^{3}H]-inositol phosphates formation in our hypertensive and hypercholesterolemic rabbits.

Thus, although hypercholesterolemia and hypertension may have additive effects on the extent of atherosclerotic lesion development (McGill et al, 1985), their respective effects on the agonist-stimulated phosphoinositide hydrolysis were not additive in our study. The assumption of synergism between hypertension and hypercholesterolemia on the pathogenesis of vascular lesions deserves qualification and further investigation.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Chapter 7. General Discussion and Conclusion

Both hypertension and hypercholesterolemia are important risk factors for cardiovascular disease. The aim of this study was to investigate potential sites of interaction between these two pathogenetic processes.

In the course of the study, the following observations were made:

1. The imposition of perinephritis hypertension in New Zealand white rabbits caused an increase of about 40 mmHg in mean arterial pressure in the operated rabbits, which stabilized after 6-7 weeks. The feeding of 0.3% cholesterol diet induced a continual rise in plasma cholesterol in the rabbits, which reached 30 mmol/L after 4 months. Perinephritis hypertension did not change the plasma cholesterol profile in the rabbit, and 0.3% cholesterol feeding did not have any effect on mean arterial pressure.

2. After 18-weeks cholesterol feeding, heavy fatty deposition on the aortic intimal surfaces was observed in the treated rabbits by the Sudan Red staining technique. This was echoed in the significantly greater tissue weight in the aorta from both cholesterol-fed groups than the control group. In contrast, there was little or no Sudan Red staining in either the hypertensive or the control group. The fatty depositions appeared to have no effect on mortality.

3. In contrast to 0.3% cholesterol feeding, perinephritis hypertension was a significant risk factor for cardiovascular deaths in the course of a 4-month study, as there were significantly greater numbers of cardiovascular deaths in both the hypertensive and the hypertensive-hypercholesterolemic groups versus the control group, but no significant difference between the hypercholesterolemic and the control group, or between the hypertensive-hypercholesterolemic and the

hypertensive group. Nevertheless, when the hypertensive-hypercholesterolemic group was compared to the hypertensive group, 0.3% cholesterol feeding tended to enhance the cardiovascular deaths in these hypertensive animals, although this was not statistically significant with the sample size used.

4. The imposition of perinephritis hypertension enhanced the in vivo pressor responses to noradrenaline, angiotensin II, and endothelin-1, as well as the depressor responses to acetylcholine, isoproterenol, and nitroprusside at 2-3, 6-7, and 13-16 weeks of study. The differences tended to increase with the duration of hypertension for the pressor responses, but not for the depressor responses. The patterns of changes in the duration of hypertension differed from one pressor agonist to another, suggesting that the structural thickening in the vessel wall was not the sole explanation for enhanced vascular reactivity. Similar conclusions held for the depressor agonists. In contrast, the imposition of 0.3% cholesterol diet had no effect on the in vivo vascular reactivity at any time point of the study. The difference in vascular reactivity changes between hypertension and hypercholesterolemia could be partly due to the different predilection sites for the two disease states, as experimental hypertension induces pathological changes in the resistance as well as conduit vessels, and hence changes in pressor and depressor responses. Experimental hypercholesterolemia, on the other hand, induces pathological changes predominantly in the aorta, and thus may not affect the pressor and depressor responses which are determined mainly by the resistance vessels rather than the aorta.

5. Neither perinephritis hypertension nor 0.3% cholesterol feeding caused any changes in the basal platelet $[Ca^{2+}]_i$ at the 17th week of study. However, measurement of the basal platelet $[Ca^{2+}]_i$ may not be a good model for the vascular

smooth muscle cells in the disease states. Other parameters of platelet activity, such as aggregation, adhesion, or $[Ca^{2+}]_i$ changes in response to agonist stimulation, could be studied to clarify this matter in relation to the disease states.

6. Perinephritis hypertension tended to enhance the noradrenaline-stimulated, but not the endothelin-1-stimulated phosphoinositide hydrolysis in the rabbit aorta, while 0.3% cholesterol feeding tended to decrease both noradrenaline- and endothelin-1-stimulated, phosphoinositide hydrolysis, at the 18th week of study. Thus, hypertension and hypercholesterolemia tended to oppose, rather than augment, each other in terms of phosphoinositide hydrolysis stimulation. The discrepant picture of noradrenaline- vs endothelin- stimulated phosphoinositide hydrolysis in perinephritis hypertensive rabbits suggests that, although both agonists can stimulate phosphoinositide hydrolysis, their receptor-coupling processes may not be the same. It remains to be studied whether noradrenaline and endothelin may act via different isoenzymes of phospholipase C or transducer G proteins; perinephritis hypertension might have differential effects on these phospholipase C isoenzymes or transducer G proteins. Although only the aorta was investigated in our study, it would be interesting to investigate the phosphoinositide hydrolysis in other vessels, such as the resistance arterioles, to see whether either disease state causes more distinct changes in phosphoinositide metabolism in these vessels.

There was no difference in the basal $[^3H]$-inositol phosphates formation between any experimental group and the control group, suggesting that neither perinephritis hypertension nor 0.3% cholesterol feeding for 18 weeks altered the basal phosphoinositide metabolism in rabbit aorta.

7. Overall, perinephritis hypertension caused increases in cardiovascular deaths, in the pressor as well as depressor responses, and the noradrenaline-stimulated phosphoinositide hydrolysis. In contrast, 0.3% cholesterol feeding with consequent

hypercholesterolemia decreased the agonist-stimulated phosphoinositide hydrolysis, but had little effect on all other parameters.

Overall, no evidence of interactions was observed in any of the parameters examined between perinephritis hypertension and 0.3% cholesterol feeding. Questions remain whether sites of interaction, if any, could exist in other vascular beds such as coronary or cerebral vessels, which are the predilection sites for cardiovascular events. Moreover, we only studied one of the second messenger systems, i.e., the phosphoinositide messenger system. Further investigation in these animals into the other messenger systems such as the cAMP and cGMP systems in these disease states could be useful, as it has been reported that these second messenger systems can be perturbed in other models of hypertension or hypercholesterolemia.

8. There is much evidence that the endothelium-dependent relaxation in conduit and resistance arteries is impaired in disease states such as hypertension and atherosclerosis. However, we were unable to show any impairment in responses to acetylcholine in rabbits with perinephritis hypertension. Recently, it has been suggested that enhanced "endothelium-dependent contraction" may be involved in the functional impairment of the vascular endothelium in these disease states. Since the discovery of endothelin-1, one of the endothelium-derived contracting factors, and later on, reports of hypertensive patients with "endothelin-secreting tumours", and increased plasma concentrations of endothelin-1 in patients with essential hypertension and vasospastic diseases, it has been speculated that endothelin-1 might be one of the keys to these pathologically vasotonic states. Our work on endothelin-1 showed that intravenous injection of endothelin-1 caused a greater pressor response in vivo in the perinephritis hypertensive rabbits as compared to the control rabbits, in line with other reports of increased pressor responses in various models of

hypertension. The mechanisms and significance of these observations in the pathophysiology of hypertension remains to be clarified.

APPENDIX: Publications and presentations containing the work undertaken for this thesis.

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3. Huang YT, Hamilton CA, Howie CA, & Reid JL. Responses to endothelin in hypertensive rabbits. Br J Pharmacol 1990; 101 (Proc Suppl): 509P.

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