STUDIES IN ANIMAL MODELS OF ATHEROSCLEROSIS

by

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DECLARATION.

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been submitted previously for a higher degree.

This research was carried out in the Department of Medicine and Therapeutics, University of Glasgow, under the supervision of Dr C. A. Hamilton and Professor J. L. Reid.

Fiona J. Dowell.

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SUMMARY.

1. Alterations in vascular function have been investigated in two clinically relevant situations - atherosclerosis and exposure to free radicals / reactive oxygen species. Two animal models of atherosclerosis were studied - the cholesterol fed New Zealand White rabbit and the genetically hyperlipidaemic WHHL rabbit. The *in vitro* model for free radical / reactive oxygen species exposure was investigated in aortic tissue from New Zealand White rabbits. Contractile and relaxant properties of the vasculature were examined in these models.

2. In the cholesterol fed New Zealand White rabbits a high proportion displayed hypercholesterolaemia resistance. However, within the limitations of the data, it was shown that in this model phenylephrine induced contraction is unaffected by dietary elevation of serum cholesterol. However carbachol induced endothelium dependent relaxation is impaired following the twenty week period of cholesterol feeding (0.3%). A high dose of sodium nitroprusside (SNP), a directly acting nitrovasodilator, induced 100% relaxation regardless of the degree of impairment of the carbachol response. Subsequent normalisation of dietary cholesterol intake for a further twenty weeks initiated repair mechanisms, inducing the partial restoration of carbachol induced endothelium dependent relaxation.

3. Genetically hyperlipidaemic WHHL rabbits, an animal model of human familial hypercholesterolaemia, were also studied. Responses to phenylephrine and carbachol were studied in both homozygous and heterozygous WHHL rabbits at 3, 6, 9 and 12 months of age, in the thoracic aorta, aortic arch and carotid artery. Normocholesterolaemic New Zealand White rabbits were studied in age matched groups for comparison. At three months of age a slight hyper-reactivity to

phenylephrine was observed in homozygous WHHL rabbits compared to controls. However with increasing age a clear decline in contractile function of the thoracic aorta was observed in homozygous and to a lesser extent heterozygous WHHL rabbits when compared to New Zealand White rabbits.

4. Studies of carbachol induced endothelium dependent relaxation in the thoracic aorta from WHHL rabbits showed a progressive decline in the ability to relax. Even as early as three months of age endothelium dependent relaxation was impaired in heterozygous and to a greater extent homozygous WHHL rabbits. Relaxation of the thoracic aorta was unaltered by age in the New Zealand White rabbits.

5. Similar patterns of impairment of both contractile and relaxant function in ring segments of aortic arch from WHHL rabbits. However no alteration in responsiveness was observed in ring segments of carotid artery in the genetically hyperlipidaemic rabbits.

6. Parallel histological studies were carried out using both macro- and microscopic techniques to qualitatively assess the extent of atheroma in the thoracic aorta of the WHHL rabbit. This revealed the progressive development of atheroma in homozygous WHHL rabbits with increasing age. In heterozygous WHHL rabbits early stages of atheroma were visible. The atheroma was found to closely resemble classic human atheroma.

7. Elevation of serum cholesterol was found to be significantly related to both the decline in vasoactive function and the physical development of atheroma. The relationship between functional and structural parameters was also found to be significant. With regard to contractile function intimal thickening appeared to exert the greatest influence on the ability of the vessel to contract. Decline of endothelium

dependent relaxation was found to be significantly related to both the proportion of intimal involvement and the relative thickness of the developed plaque.

8. The effects of the HMG CoA reductase inhibitor simvastatin on the development and progression of atherosclerosis in both homozygous and heterozygous WHHL rabbits were also studied. Four groups of rabbits were treated with 10 mg / Kg / day :- young (3-6 month) heterozygous; older (9-12 month) heterozygous; young (3-6 month) homozygous and older (9-12 month) homozygous WHHL rabbits.

9. Simvastatin was found to significantly reduce serum cholesterol levels in the young heterozygous WHHL rabbits and a non-significant reduction was observed in the older heterozygous WHHL rabbits. In the homozygous WHHL rabbits no significant fall was observed.

10. It was observed that simvastatin was clearly able to retard the progressive loss of both contractile and relaxant function in the young heterozygous WHHL rabbit group, similar but less effective trends were observed in the young homozygous group and in the older heterozygous group. However no beneficial effect was observed in the older homozygous group. These effects were not parallelled by inhibition of structural development of atheroma.

11. Rings of thoracic aorta from New Zealand White rabbits were exposed to a xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system. This system generates the superoxide anion, hydrogen peroxide and the hydroxyl radical. Vasoactive properties of the thoracic aorta were examined before and after exposure to the system. It was observed that phenylephrine induced contraction, carbachol induced endothelium dependent relaxation and SNP induced non-endothelium dependent relaxation were impaired following exposure to this

system.

12. Subsequent experiments using a series of inhibitors / enzymes :- mannitol, N-(2-mercaptopropionyl)-glycine (MPG), captopril, catalase and superoxide dismutase (SOD) allowed the elucidation of the primary cytotoxic species causing impairment of carbachol induced relaxation. The primary cytotoxic agent was identified as hydrogen peroxide. It was also shown that hydrogen peroxide could directly mimic the effects of the xanthine oxidase / hypoxanthine system with all three agonists studied.

CHAPTER ONE.

GENERAL INTRODUCTION.

1.1 THE CARDIOVASCULAR SYSTEM.

The mammalian cardiovascular system consists of two interlinked circuits :- the systemic circulation, which carries the blood from the heart to the peripheral tissues to supply oxygen and nutrients and remove metabolites; and the pulmonary circulation, which conducts the blood through the lungs so that reoxygenation can occur. The blood vessels in the circulatory system have different structures adapted to their function: arteries and arterioles are responsible for the conduit of the blood to the periphery; the aorta and other large arteries are strong elastic vessels to allow distention and recoil in response to the pulsatile pressure generated by the pumping of the heart; further down the arterial tree the vessels become more muscular, the arterioles are the last small branches of the arterial tree, these vessels are highly muscular in nature and are able to constrict or dilate in response to various stimuli in order to regulate blood flow into the appropriate tissues; the capillaries are responsible for the supply of blood to the peripheral tissues, capillary walls are very thin and selectively permeable to allow exchange of water, oxygen, electrolytes and nutrients as required by the tissue. Capillaries also serve to remove the metabolites and waste products away from the site of production. After the blood has passed through the capillaries it enters the venous circulation - venules progressively merge to form larger veins which conduct the blood back towards the heart.

1.1.1 ARTERIAL STRUCTURE.

The structure of the arterial wall consists of three major layers:-

The outermost layer of the artery is the adventitia. The adventitia is primarily connective tissue such as collagen and functions to maintain the physical shape of the vessel. The adventitia also contains the nerves and blood vessels which supply

the arterial wall.

The next layer is the media. Under normal circumstances this is the thickest layer of the arterial wall. The main components of the media are smooth muscle, elastic tissue and collagen. Some arteries are very elastic in nature such as the aorta, whereas others are more muscular such as the resistance arteries. The supply of oxygen and nutrients for the media come partly from small blood vessels in the adventitia and partly direct from the lumen of the vessel.

The innermost layer of the artery is the intima. Under normal circumstances the intima is a very thin layer comprising of a single endothelial cell layer and a small amount of connective tissue between the endothelium and the media (subendothelial space). Until recently the intima and endothelium were thought to serve simply as a physical barrier between the blood and the functional, muscular layers of the artery. However, as will be discussed below, during the past thirteen years there have been major discoveries regarding a crucial functional role for the endothelium.

1.1.2 CONTROL OF ARTERIAL TONE.

Systemic blood pressure is predominantly controlled by two main factors :- total peripheral resistance ie. the degree of constriction or relaxation of the arterial tree, and blood volume. Both of these are controlled by multiple mechanism of both neural and humoral origin. Vascular smooth muscle is under tonic control by the sympathetic nervous system ie. there is a constant release of noradrenaline from sympathetic nerves which maintains a relative degree of tone in the arterial wall. This sympathetic tone can be increased or decreased in response to various factors. The renin angiotensin system is another rapidly acting influence on blood vessel tone: if blood pressure falls and blood flow through the kidney is consequently

reduced, the kidney will release an enzyme called renin. This triggers a cascade of events resulting in the formation of angiotensin II which will act to directly constrict arterial smooth muscle. Angiotensin II has another slow, long acting effect to increase water retention and thus increase blood volume.

As mentioned earlier, recent years have seen the emergence of a crucial role for the endothelium in the control of vascular tone. In 1966 a paper was published in the British Medical Journal which described the endothelium as a "cellophane wrapper" of the vascular tree (Florey, 1966). The turning point for the discovery of a role for the endothelium came in 1980 when Furchgott and Zawadski demonstrated that preconstricted rings of rabbit thoracic aorta relaxed in response to acetylcholine and that this relaxation was dependent on the integrity of the vascular endothelium. This lead to the conclusion that a diffusible vasoactive compound produced in the endothelium was responsible for the relaxation of the smooth muscle. This compound became known as endothelium dependent relaxing factor (EDRF). Many subsequent studies have been performed which support the existence of at least one EDRF.

A vasoconstrictor role for the endothelium has also been demonstrated recently. In 1988 Yanigisawa and colleagues characterised an endothelium derived vasoconstrictor peptide named endothelin. Endothelin is able to produce vasoconstriction in isolated arteries and veins and is the most potent vasoconstrictor known (Yanigisawa et al, 1988, Miller et al, 1989).

Thus the endothelium is able to greatly influence the level of arterial tone with the potential to induce either vasoconstriction or relaxation.

1.1.3 CONTRACTION OF VASCULAR SMOOTH MUSCLE.

The contractile response of vascular smooth muscle is regulated by changes in intracellular calcium ion concentration. If intracellular calcium ion levels are elevated, calcium binds to the protein calmodulin. When calmodulin binds calcium this induces a shape change in the protein. The calcium-calmodulin complex will interact with myosin light chain kinase to stimulate the phosphorylation of light chain myosin. Myosin ATP-ase is then able to activate actin. This leads to cross-bridge formation and the development of contraction. If calcium levels fall, the calcium-calmodulin complex will dissociate, myosin light chain kinase will become inactive and consequently the muscle will relax.

Intracellular calcium levels can be increased either by release of intracellular calcium or by influx of calcium from outside the cell. Calcium entry into the cell can be regulated by a number of mechanisms including channels operated by voltage, receptors or second messengers. Voltage operated calcium channels allow calcium to enter the cell during depolarisation. Receptor operated channels allow calcium to enter when specific ligands interact with and activate the receptor. Calcium entry can also be induced by receptors which, when activated, produce second messengers, these second messengers can then act to increase calcium levels.

Noradrenaline and other α_1-adrenoceptor agonists induce contraction in vascular smooth muscle through the production of the second messenger inositol 1,4,5-triphosphate (IP_3). α_1-adrenoceptors are linked through G-proteins to phospholipase C. Agonist binding of the receptor leads to activation of the phosphatidylinositol cycle. Activation of the phosphatidylinositol cycle leads to the enhanced production of IP_3. IP_3 then acts by binding to an IP_3 receptor to mobilise

stored calcium and to promote an influx of external calcium (Berridge, 1993). In fact the phosphatidylinositol cycle is occurring continuously within the cell. In the plasma membrane phosphatidylinositol is phosphorylated to produce phosphatidylinositol 4-monophosphate, and subsequently phosphatidylinositol (4,5) bisphosphate. Phospholipase C acts on phosphatidylinositol (4,5) bisphosphate to produce IP_3, which is released into the cytoplasm and 1,2-diacylglycerol (DAG), which remains in the membrane. IP_3 is sequentially dephosphorylated to inositol, which can then re-enter the cycle by forming phosphatidylinositol. DAG is also recycled to form phosphatidylinositol via a high energy intermediate cytidine diphosphate diacylglycerol. DAG is involved in maintaining the sustained tonic phase of vascular contraction (Danthuluri and Deth, 1986).

1.1.4 RELAXATION OF VASCULAR SMOOTH MUSCLE.

Relaxation of vascular smooth muscle can occur in response to many stimuli. Broadly speaking these stimuli can act directly on the vascular smooth muscle - for example a β_2-adrenoceptor agonist stimulates β_2-adrenoceptors on the vascular smooth muscle cell surface. This activates adenylate cyclase and increases cAMP levels thus inducing relaxation. Other directly acting agents include prostacyclin and atrial natriuretic factor. The second route to induce relaxation, already briefly mentioned earlier, is through the endothelium - a range of agonists including acetylcholine, thrombin, bradykinin and ATP act on the endothelial cell to induce the release of a labile molecule, probably nitric oxide (NO) or a related compound which has been called EDRF.

Whilst the identification of EDRF as either NO (Palmer et al, 1987) or a closely related NO-containing species such as a dinitrosyl-Fe^{2+} complex (Vanin, 1991) or S-nitrosothiol (Myers et al, 1990) is still a matter of debate, it has now been

established that NO is the oxygenated nitrogen species generated from the guanidino nitrogen of L-arginine in the endothelial cell (Moncada et al, 1991; Mülsch et al, 1992). The enzyme responsible for this reaction is termed NO-synthase. NO-synthase is a reduced NADPH-dependent dioxygenase which exists in at least two isoforms. One of the recognised NO-synthase isoenzymes is a calcium independent inducible enzyme which is found in cytokine activated cells - predominantly macrophages and smooth muscle cells but also in the endothelium (Förstermann et al, 1991b). This inducible form seems only to be expressed in the presence of bacterial lipopolysaccharides and / or cytokines (Moncada et al, 1991). The second recognised isoform is constitutive NO-synthase, this isoenzyme is calcium / calmodulin dependent and is found in endothelial and neuronal cells (Lamas et al, 1992; Schini and Vanhoutte, 1992). This constitutive NO-synthase isoenzyme can be activated by a number of different stimuli. Receptor mediated stimuli include acetylcholine, ATP, bradykinin etc. Non-receptor mediated stimuli such as calcium ionophores, polycations and calcium-ATP-ase inhibitors are also potent agents.

EDRF / NO release can also be stimulated by physical factors such as shear stress, pulsatile stretching and a low P_{O_2}. It has been proposed that these physical stimulants may play an important role in mediating the basal release of EDRF / NO and thus regulation of vascular tone (Busse et al, 1993).

Evidence for the basal release of EDRF / NO comes from studies with the compound N^G-monomethyl-L-arginine (L-NMMA). This compound inhibits the production of EDRF / NO from L-arginine. It has been used in systemic, renal, pulmonary and coronary vascular beds to demonstrate the existence of a basal release of EDRF / NO (Hutcheson and Griffith, 1991; Lamontagne et al, 1992).

Constitutive NO-synthase is regulated by a calcium-calmodulin complex and thus agonist stimulated EDRF / NO release is induced by an elevation of intracellular free calcium ions (Busse and Mülsch, 1990; Förstermann et al, 1991a). This agonist induced rise in intracellular free calcium levels involves both a transient IP_3 mediated release of calcium ions from intracellular stores and a more sustained transmembrane flux of calcium from the extracellular space (Busse et al, 1993).

The relaxing actions of EDRF / NO are mediated through guanylate cyclase activation in the vascular smooth muscle cells. The EDRF / NO acts by binding to the ferrohaem moiety of guanylate cyclase, thus stimulating the cyclase and inducing a rise in intracellular cGMP levels. cGMP subsequently activates a cGMP dependent protein kinase, this leads to dephosphorylation of myosin light chains and hence muscle relaxation.

A group of compounds which have been in clinical use as hypotensive agents for some time, act by mimicking NO. The nitrovasodilators such as glyceryl trinitrate and sodium nitroprusside, are chemically degraded in the vasculature to release NO and thus, through a direct action on the smooth muscle cell guanylate cyclase, induce vascular relaxation.

Thus the endothelium is essential for the regulation / maintenance of vascular tone. It plays a crucial role in the supply of blood, and consequently oxygen and nutrients, to various tissues. The endothelium is therefore also involved in the removal of toxic metabolites from tissues, again through the control of vascular tone and blood supply. It has been proposed that dysfunction of the endothelium may contribute to various pathological conditions such as atherosclerosis, ischaemia reperfusion injury and hypertension. The cause of endothelial dysfunction in these instances may well

be multi-factorial. Several factors may make the endothelium more susceptible to attack or injury leading to dysfunction , such as elevated serum cholesterol levels or free radical / reactive oxygen species generation.

1.2 CHOLESTEROL METABOLISM.

1.2.1 CHOLESTEROL SYNTHESIS.

Cholesterol is one of the major component molecules of many biological membranes: it is a key regulator of membrane fluidity, preventing crystallisation of fatty acyl chains by fitting in between them; and conversely, cholesterol can decrease membrane fluidity by acting to sterically block motion of fatty acyl chains. Cholesterol is also the precursor of steroid hormones such as progesterone and cortisol.

Cholesterol is a 27-carbon molecule, all the carbon atoms in cholesterol are derived from the precursor molecule acetate. The first stage in the synthesis of cholesterol is the formation of 3-hydroxy-3-methylglutaryl (HMG) CoA from acetyl CoA (Figure 1.1). The enzyme HMG CoA reductase then acts to reduce HMG CoA to mevalonate - this is the committed, rate-limiting step in cholesterol formation.

Mevalonate is then sequentially phosphorylated and decarboxylated to form isopentenyl pyrophosphate, a five carbon molecule (C_5). A series of condensation reactions occur to form geranyl pyrophosphate (C_{10}), farnesyl pyrophosphate (C_{15}) and squalene (C_{30}). The final stages of cholesterol biosynthesis involve the cyclisation of squalene to lanesterol (C_{30}) and finally the removal of three methyl groups, the reduction of one double bond and the migration of one other double bond to form cholesterol (C_{27}).

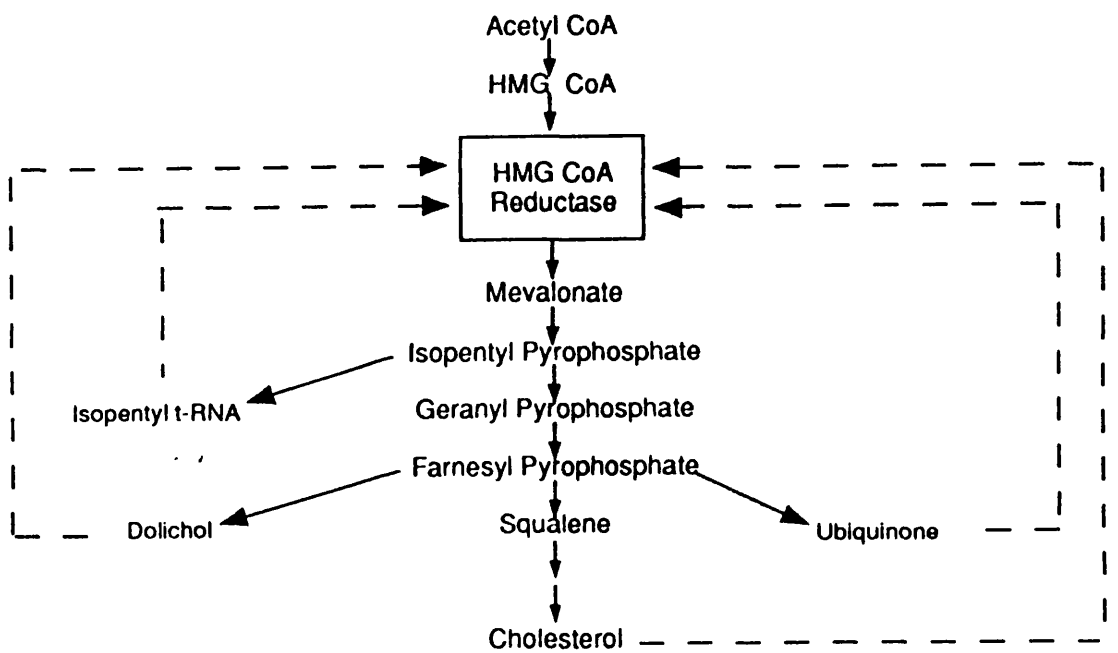


FIGURE 1.1. Schematic representation of the biosynthetic pathway of cholesterol. Dashed lines represent the negative feedback influence on the rate limiting enzyme HMG CoA reductase.

The synthesis of cholesterol requires molecular oxygen. It is interesting to note that cholesterol evolved only after the earths atmosphere became aerobic and thus whilst cholesterol is ubiquitous in eukaryotes it is absent from most prokaryotes.

The control of cholesterol synthesis is multivalent, the intermediates and end products of the pathway : farnesyl pyrophosphate, cholesterol, ubiquinone, dolichol and isopentenyl tRNA act to suppress HMG CoA reductase when present in sufficient concentrations in the cell (Figure 1.1).

1.2.2 CHOLESTEROL METABOLISM AND TRANSPORT.

Cholesterol and other lipids are transported in body fluids by a series of lipoproteins classified according to particle density. Lipoproteins are large globular particles which contain a hydrophobic core of non-polar lipid (cholesteryl esters and triglycerides) surrounded by a polar coat of phospholipids, free cholesterol and apoproteins. Lipoproteins have been classified into six major groups which differ in the relative proportion of cholesteryl esters and triglycerides in the core and the nature of the apoproteins on the surface (Table 1.1). Each of these six classes of lipoproteins has a specific role in the transportation of lipids. Cholesterol can be obtained from external sources, i.e. consumed in the diet, or synthesised *de novo* within the body.

The pathway for lipid transport can be clearly separated into two : dietary lipid absorbed in the intestine is transported in the exogenous pathway, whereas lipid entering the circulation from the liver and other tissues (apart from the intestine) is transported by the endogenous pathway (Figure 1.2).

LIPOPROTEIN CLASS	MAJOR CORE LIPIDS	MAJOR APOPROTEINS	TRANSPORT FUNCTION
Chylomicrons	Dietary triglycerides.	A-1, A-2, A-4, B-48	Dietary cholesterol.
Chylomicron remnants	Dietary cholesterol esters.	B-48, E	Dietary cholesterol.
VLDL	Endogenous triglycerides.	B-100, C, E	Endogenous triglyceride.
IDL	Endogenous cholesteryl esters + triglycerides.	B-100, E	Endogenous cholesterol.
LDL	Endogenous cholesteryl esters.	B-100	Endogenous cholesterol.
HDL	Endogenous cholesteryl esters.	A-1, A-2	Removal of cholesterol from extra-hepatic tissues.

TABLE 1.1. Characteristics of the major classes of lipoproteins in human plasma. VLDL denotes very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL high density lipoprotein. (Modified from Brown and Goldstein, 1992).

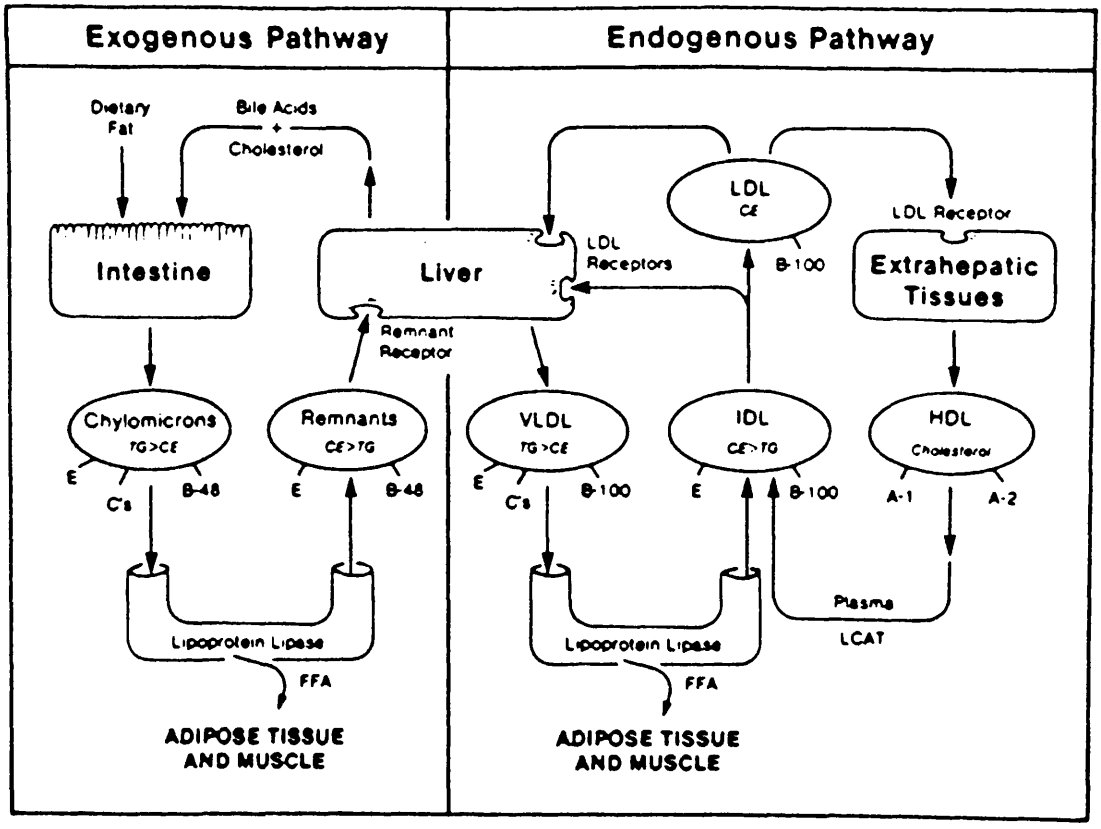


FIGURE 1.2. Model for the metabolism of plasma lipoproteins showing the separate pathways for transport of endogenous and exogenous lipids. VLDL denotes very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL high density lipoprotein; CE , cholesteryl esters; FFA, free fatty acids: TG, triglycerides; LCAT, lecithin:cholesterol acyltransferase. A-1, A-2, B-48, B-100, C's and E represent the apoproteins associated with the indicated lipoprotein particle. (Reproduced from Brown and Goldstein, 1992).

A. Exogenous Cholesterol Transport.

Dietary cholesterol and triglycerides are taken up in the proximal jejunum by pinocytosis of lipid mycelles, esterified in the epithelial cells and subsequently incorporated into the large lipoprotein particles known as chylomicrons. Chylomicrons are then secreted into the lymphatic system and subsequently into the blood stream. The chylomicrons then circulate in the blood until they reach capillaries of muscle or adipose tissue. An enzyme called lipoprotein lipase is bound to the endothelial cells in these capillaries. This enzyme acts on chylomicrons to hydrolyse the triglyceride in the core of the lipoprotein to fatty acids. These fatty acids will then cross the endothelium and enter the muscle or adipose tissue where they may be oxidised for energy requirements or re-esterified for storage. Thus the chylomicron is decreased in size and triglyceride content but the cholesteryl esters remain - this smaller particle is known as a chylomicron remnant.

Chylomicron remnants remain in the circulation until they reach the liver, here they are bound by a remnant receptor, internalised by endocytosis and degraded by lysosomes to liberate free cholesterol. This free cholesterol may be destined for one of several fates :- membrane synthesis, storage, excretion into bile or used to form endogenous lipoproteins which are secreted into the plasma.

B. Endogenous Cholesterol Transport.

Various stimuli, such as a high calorie intake, will stimulate the liver to assemble triglycerides and cholesterol for export and storage in adipose tissue. The lipoproteins formed by this process are called very low density lipoproteins (VLDL). The VLDL are transported in the circulation to the capillaries of adipose tissue where lipoprotein lipase acts to cleave the triglycerides from the VLDL. Note this is the

same enzyme which interacts with chylomicrons. The lipoprotein remnant resulting from this action is intermediate density lipoprotein (IDL). After IDL is released from the endothelium it is subject to one of two possible fates. Approximately 50% of IDL is rapidly cleared by receptor mediated endocytosis into the liver - this is mediated by the low density lipoprotein (LDL) receptor which will bind either apolipoprotein (apo) E or apo B_{100}. The remaining 50% of IDL continues to circulate until it is converted to LDL by removal of the remaining triglyceride. LDL has a relatively long plasma half life of 1.5 days. LDL particles are eventually degraded due to interaction with LDL receptors in the liver or extrahepatic tissues. If a cell requires cholesterol, it will induce LDL receptor synthesis - thus facilitating the uptake of LDL. When requirements are satiated LDL receptor synthesis is down regulated.

The final class of lipoproteins - high density lipoproteins (HDL) are involved in removal of cholesterol from peripheral tissues - as cells die or membranes are replaced free cholesterol is released into the circulation. This cholesterol is adsorbed onto HDL. A plasma enzyme - lecithin:cholesterol acyltransferase (LCAT) acts on this free cholesterol to esterify it with a long chain fatty acid. These newly formed cholesteryl esters are then transferred to VLDL or IDL by a cholesteryl ester transfer protein in plasma. The IDL and VLDL are then processes as above. This sequence of events involving HDL is known as reverse cholesterol transport.

Lipoproteins can also be disposed of by less specific, less regulated pathways in macrophages and other scavenger cells. As plasma levels of lipoproteins increase the relevance of these pathways becomes more apparent. This will be discussed in more detail below.

1.2.3 THE LDL RECEPTOR.

The LDL receptor plays a central role in lipoprotein metabolism and cholesterol homeostasis. At a cellular level the LDL receptor provides an efficient and regulated mechanism for supplying cells with cholesterol as required. At the whole body level the LDL receptor has a major role in controlling total plasma cholesterol concentration. The mature human LDL receptor is a single transmembrane glycoprotein containing 839 amino acids. Five separate domains have been identified:- the ligand binding domain; the EGF precursor homology domain; the O-linked sugar domain; the membrane spanning domain; the cytoplasmic domain. The amino terminal of the receptor has 292 amino acids which form seven copies of a 40 residue repeat - this folds to produce the ligand binding domain, which recognises apo E and apo B_{100} (Innerarity, 1991). The LDL receptors of several mammalian species :- rabbit, rat, hamster exhibit a high degree of homology with the human LDL receptor.

1.3 FREE RADICALS AND REACTIVE OXYGEN SPECIES.

1.3.1 WHAT IS A FREE RADICAL.

Electrons in atoms occupy space known as orbitals. Each orbital can hold a maximum of two electrons, spinning in opposite directions. Free radicals are molecules which contain one or more unpaired electrons, symbolised by a dot ($A^{\cdot}$). They can be positive, negative or neutrally charged. A normal chemical bond consists of a pair of electrons opposite in spin and sharing a single molecular orbit. Hence, due to the presence of a single electron, free radicals are usually highly reactive. If two free radicals meet the two single electrons can pair and form a

covalent bond :-

$$A^{\cdot} + A^{\cdot} \rightarrow A\text{-}A \quad (\text{or } A_2)$$

However if a free radical donates its unpaired electron to another molecule, or abstracts an electron from another molecule in order to stabilise itself, then by definition another free radical is generated.

$$A^{\cdot} + B \rightarrow A + B^{\cdot}$$

This leads to one of the characteristic features of free radicals - the initiation of a chain reaction which may be thousands of events long.

1.3.2. REGULATION OF FREE RADICALS AND REACTIVE OXYGEN SPECIES IN BIOLOGICAL SYSTEMS.

In biological systems the generation of oxygen derived free radicals and reactive oxygen species is of primary interest. Oxygen, although essential to the maintenance of aerobic life, is potentially extremely toxic. Molecular oxygen itself contains two unpaired electrons:-

$$\cdot \underline{\bar{O}} - \underline{\bar{O}} \cdot$$

and can technically be described as a diradical. However, due to quantum-mechanical restrictions, oxygen does not have the extreme reactivity associated with many free radicals. The complete reduction of oxygen to water

requires the addition of four electrons. This process can occur in two ways. Either univalently, producing a series of intermediates known as reactive oxygen species or quadrivalently, through the mitochondrial cytochrome oxidase system and thus avoiding the production of any reactive intermediates. A series of enzymes are present in aerobic cells to scavenge the byproducts of the univalent pathway. Superoxide dismutase catalyses the conversion of the superoxide anion to hydrogen peroxide. The haem containing enzyme catalase is able to transform hydrogen peroxide into water and molecular oxygen. A series of peroxidase enzymes are also able to reduce peroxides.

The ubiquitous presence of these enzymes suggests that the production of reactive oxygen species in aerobic cells is a normal process, but it is one that is under strict control. Another aspect of normal cell regulation which acts to minimise oxygen free radical generation is the control of the intracellular concentration of free transition metal ions. Trace amounts of transition metal ions such as copper or iron can transform hydrogen peroxide into hydroxyl radicals by the Fenton reaction:

$$Fe^{2+} \text{ (or } Cu^{+} \text{)} + H_2O_2 \rightarrow Fe^{3+} \text{ (or } Cu^{2+} \text{)} + OH^{\cdot} + OH^{-}$$

Hence "safe storage" of iron and copper is essential. Iron is stored in a variety of forms in man :- two thirds is stored in haemoglobin, 10% in myoglobin, a small proportion in iron containing enzymes and the transport protein transferrin, the remainder is present in intracellular storage proteins such as ferritin and haemosidderin. Thus the free concentration of iron is virtually zero. Any iron entering the cell, for example, when required for the synthesis of proteins, is immediately stored in ferritin. Ferritin is capable of storing 4,500 molecules of iron per mole of protein. Free copper is bound by albumin. Hydroxyl radical formed by the albumin bound copper immediately reacts with the albumin, thus it has been

suggested that albumin acts as a sacrificial antioxidant. Hence in this controlled environment, biological systems can utilise free radicals and reactive oxygen systems without the risk of initiating uncontrollable chain reactions.

The controlled equilibrium described above maintains a safe environment for the cell such that as and when reactive oxygen species and free radicals are generated automatic defence systems operate preventing any disruption of the cellular environment.

1.4 ATHEROSCLEROSIS.

1.4.1 INTRODUCTION.

The atherosclerotic process represents the normal protective, reparative response of the vasculature following insults to the vascular endothelium and / or the smooth muscle cell of the arterial wall. Under certain circumstances (risk factors) such as hypercholesterolaemia / hyperlipiaemia, cigarette smoking, diabetes and obesity, this process becomes degenerative rather than protective.

Atherosclerosis is not a modern disease - advanced calcific lesions have been found in the arteries of preserved mummies of the ancient Egyptians. However knowledge and understanding of this disease process has been difficult to obtain due to the long time frame of the development of atheroma and the fact that human data usually represents a single time point in any one individual. Recently wide ranging epidemiological studies and the development of appropriate animal models have provided major insights into the pathogenesis of atherosclerosis.

The ubiquity of the atherogenic process is demonstrated by the fact that a recent

study of autopsy specimens revealed that fatty streaks are present in the coronary arteries from half of children aged between 10 and 14 (Starry, 1989) and, that by the third decade of life, advanced plaques are found in the majority of the Western population. Thus atherosclerotic disease is present for a long period before any clear clinical symptoms become evident.

Although atherosclerosis is defined as a systemic disease, the development of plaque occurs in a focal manner. In man, atheroma affects arteries ranging from the aorta down to arteries of 3mm external diameter. The distribution of disease within these arteries is not uniform - some arteries, such as the coronary artery, are at a high risk of developing atheroma, whereas others such as the internal mammary artery are rarely affected. The haemodynamic environment of the area is important in the focal initiation of atheroma - certain sites such as flow dividers, areas of curvature and branching points are more susceptible to the development of atheroma (Schwartz et al, 1993).

Studies in experimental animal models have identified focal 'lesion prone' sites with the protein-binding azo dye Evan's blue. These lesion prone site exhibit increased uptake of Evan's blue in a pattern similar to that of lesion development (McGill et al, 1957; Fry, 1973). Further studies have demonstrated that these sites have increased endothelial permeability to plasma proteins including albumin (Packham et al, 1967; Bell et al, 1974), fibrinogen (Bell et al, 1974) and LDL (Hoff et al, 1983; Feldman et al, 1984); increased endothelial cell turnover (Caplan and Schwartz, 1973); altered endothelial cell morphology - endothelial cells are more polyhedral and the surface glycocalyx is 2-5 fold thinner (Caplan et al, 1974; Gerrity et al, 1977); and under hyperlipidaemic conditions increased monocyte recruitment and accumulation into the subendothelial space is observed in these sites (Gerrity et al, 1979).

1.4.2 THE DEVELOPMENT OF ATHEROMA.

The first recognised lesion of atherosclerosis is known as the "fatty streak". This is characterised by a focal aggregation of lipid rich, monocyte-derived macrophages and T-lymphocytes within the intima of the arterial wall. The lesion causes little if any distortion of the intimal surface. Pathological studies have shown that more advanced lesions tend to develop in sites where fatty streaks are most frequent. However, this does not mean that all fatty streaks will develop into atheromatous plaques. In fact, a post mortem study of infants from a range of geographical locations, demonstrated the presence of fatty streaks in infants from populations where advanced atherosclerosis and coronary artery disease are rarely found (Restrepo et al, 1975).

The appearance of the fatty streak can be considered as a physiological process which has been overcome. Monocytes enter the intima in order to remove excess lipid and maintain a normal environment. However, under conditions such as hyperlipidaemia, the monocyte-derived macrophage becomes overwhelmed by the amount of lipid present in the intima and is consequently unable to re-enter the lumen and dispose of the lipid. Thus foam cell formation occurs and the intended protective system become disruptive (Schwartz et al, 1991).

Progression of the fatty streak depends on the dynamic balance between plasma and intimal lipoproteins. As the fatty streak progresses, monocytes continue to enter the intima and differentiate to tissue macrophages, these macrophages continue to take up intimal lipid and thus form foam cells. Smooth muscle cells migrate into the intima and proliferate within the plaque. Collagen, elastin and proteoglycans are produced by the smooth muscle cells and the plaque increases in size (Davies and

Woolf, 1993).

The microanatomy of the advanced plaque is characteristic :- a core of extracellular lipid forms the centre of the plaque, this extracellular lipid is derived from the death of foam cells. This lipid core is separated from the media by smooth muscle cells and is covered by a thick cap of collagen rich fibrous tissue containing smooth muscle cells. Calcification occurs in the superficial layers of the collagenous cap and also around the extracellular lipid core. Under the area of plaque marked medial thinning occurs possibly due to disuse atrophy (Schwartz et al, 1991). This mature "plaque" derived its name from its pearly white glistening surface which is oil-red-0 resistant (Schwartz et al, 1991). Clinical studies have shown that most cases of sudden death from myocardial infarct are, in fact, due to the rupture of the plaque, resulting in haemorrhage into the plaque, thrombosis and occlusion of the coronary arteries (Davies and Thomas, 1984).

1.4.3 THE RESPONSE TO INJURY HYPOTHESIS.

What initiates the development and progression of the atherosclerotic plaque ? In 1973 Ross and Glomset formally advanced the hypothesis that an injury to the endothelium may precipitate the atherogenic process. This hypothesis has been continually modified to incorporate the new accumulating observations (Ross and Glomset, 1976; Ross and Harker, 1976; Ross, 1981; Ross, 1986; Ross, 1993). This hypothesis proposes that endothelial injury can lead to the dysfunction of the endothelial cell layer. The endothelium has several important roles in the vasculature:- the provision of a non-thrombogenic, non-adherent surface; provision of a permeability barrier; formation and secretion of growth factors and cytokines; maintenance of the basement membrane and the ability to oxidise lipoproteins.

Injury to the endothelium need not involve denudation. Lesions can develop under morphologically intact endothelium. Dysfunction of one or more of the above roles of the endothelium could lead to the development of atheroma. The formation of a fibrous lesion in response to injury is based on the same process as wound healing. However, in atherosclerosis, the source of injury is likely to be prolonged or even chronic - hypercholesterolaemia, hypertension, cigarette smoking, obesity and diabetes do not tend to occur in a transient manner, thus the progression from fatty streak to fibrous plaque is unlikely to be interrupted once initiated.

1.4.4 ROLE OF LDL.

Following the discovery of the LDL receptor, it was assumed that this would be the mechanism which macrophages would use to facilitate the uptake of LDL. It was found, however, that this was not the case.

In 1979, Goldstein and colleagues demonstrated that acetylation of LDL lead to a massive increase in macrophage uptake by a receptor dependent mechanism. This lead to the naming of the macrophage scavenger receptor. Goldstein also proposed that modification of LDL was a prerequisite for macrophage uptake. However it seems that acetylated LDL has little physiological relevance.

A biologically feasible modification was discovered in 1981, when it was shown that oxidised LDL was produced when native LDL was incubated with cultured endothelial cells. This modification led to enhanced uptake of the LDL by cultured macrophages in a saturable and specific manner which could not be inhibited by native LDL (Henriksen et al, 1981). This modification has since been shown with a variety of cell types including cultured vascular smooth muscle cells (Henriksen et

al, 1983, Heinecke et al, 1984) and macrophages (Parthasarathy et al, 1986; Hiramatsu et al, 1987). It has also been observed that this modification can occur *in vivo* (Ylä-Herttuala et al, 1989; Ylä-Herttuala et al, 1990).

The mechanism by which LDL becomes oxidised *in vivo* is still unclear. It has been postulated that intimal cells can produce and secrete free radicals and reactive oxygen species such as the superoxide anion, and that these species are able to initiate the self propagating chain reaction in native LDL which leads to the production of oxidised LDL.

Free radicals have been implicated in this process by several groups. Heinecke et al in 1984, demonstrated that human arterial smooth muscle cells in culture could oxidatively modify LDL and they proposed that free radical peroxidation of lipid was the mechanism of modification. Free radical mediated mechanisms also appear to play an important role in the oxidation of LDL by endothelial cells (Steinbrecher et al, 1984; Steinbrecher, 1988; Morel et al, 1984), and by macrophages (Parthasarathy et al, 1986, Hiramatsu et al, 1987).

Free radicals are certainly involved in the propagation of the chain reaction. Peroxidation is initiated by attack from a chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain of a fatty acid. This, consequently, leaves behind an unpaired electron on the carbon atom. The resulting carbon centred radical can have several fates but in an aerobic environment the most likely one is to undergo molecular rearangement, followed by reaction with molecular oxygen to produce a peroxyradical. This radical can then abstract a hydrogen from an adjacent fatty acid side chain and thus, the reaction is propagated. Hence, a single initiation event can result in the conversion of hundreds of fatty acid side chains into lipid hydroperoxides.

During oxidation there is extensive conversion of lecithin to lysolecithin catalysed by phopshpolipase A_2 activity intrinsic in LDL (Parthasarathy et al, 1985). This will lead to the release of oxidised fatty acids which will further promote the propagation of the chain reaction. Thus, fragmentation of the fatty acids occurs, leading to the formation of highly reactive intermediates such as aldehydes, ketones and oxycholesterol derivatives. These intermediates may complex with apo B. The amino acids of apo B become modified during these processes and thus are no longer recognised by the LDL receptor. However, the newly formed epitopes on apo B are recognised by the scavenger receptor on macrophages (Steinbrecher et al, 1987; Parthasarathy et al,1987). The formation of oxidised LDL does not occur in a coordinated sequential manner. Thus, oxidised LDL is not a single homogeneous entity but a complex, variable particle consisting of oxidised fatty acids and their breakdown products, oxidised sterols, oxidised phospholipids as well as the adducts of these various lipid products with apo B and phospholipids.

It has been proposed that oxidation of LDL is unlikely to occur in the plasma due to the high levels of endogenous antioxidants. Furthermore, any oxidised LDL produced in the plasma would be rapidly removed by scavenger receptors on sinusoidal hepatic cells. Thus, it has been proposed that oxidative modification of LDL occurs primarily in the intima, on the surface of endothelial cells and macrophages - probably in microdomains sequestered from plasma antioxidants (Witztum, 1993). Extensions of macrophage membranes could create suitable loci for this to occur (Heiple et al,1990). Thus, it is primarily the antioxidant content of the actual LDL particle which is able to protect against oxidation.

1.4.5 EVIDENCE FOR THE PRESENCE OF OXIDISED LDL *IN VIVO.*

Epidemiological studies have demonstrated an inverse relationship between plasma concentration of endogenous antioxidants such as vitamin E, vitamin C and β-carotene (Enstrom et al, 1992). It has also been shown that LDL extracted from lesions has the characteristics of oxidised LDL. Immunological studies have shown auto-antibodies for oxidised LDL in both human and animal serum, also that areas of lesion stain intensely with antibodies to oxidised LDL (Palinsky et al, 1989). Experimental studies with antioxidants, such as probucol, have demonstrated inhibition of atherosclerosis in animal models such as the WHHL (Carew et al, 1987). These reports provide support for the hypothesis that oxidative modification of LDL is an important factor in mediating it atherogenicity.

There are several mechanisms by which oxidised LDL may influence the atherogenic process. One of the primary mechanisms is through the enhanced uptake of LDL by the macrophage scavenger receptor, leading to the formation of foam cells in atheroma. Oxidised LDL can induce release of cytotoxic products leading to cellular injury. Also oxidised LDL is chemotactic for monocytes and T-lymphocytes, thus enhancing entry of these cell to the intima. Furthemore oxidised LDL stimulates the synthesis of monocyte chemotactic factor-1 (MCP-1) (Schwartz et al, 1993). Once macrophages are formed the oxidised LDL can act to inhibit their exit from the intima. Oxidised LDL is also able to inhibit EDRF mediated vasodilation and other anti-atherogenic properties of the endothelium.

1.5 DISEASES THAT CAUSE HYPERLIPOPROTEINAEMIA.

Causes of hyperlipoproteinaemia can be broadly separated into two categories :- Primary hyperlipoproteinaemias which have a genetic basis and secondary

hyperlipoproteinaemias where the hyperlipoproteinaemia arises as a result of a more generalised metabolic disorder.

1.5.1 PRIMARY HYPERLIPOPROTEINAEMIA.

There are several genetic disorders known to cause an elevation of the level of one or more of the plasma lipoproteins. There are five monogenic disorders - caused by a single inherited gene defect, which follows standard Mendelian inheritance patterns. These disorders are as follows :-

Familial lipoprotein lipase deficiency - this disorder occurs in around one in a million of the population. Affected homozygotes fail to produce lipoprotein lipase and are thus unable to catabolise dietary triglyceride contained in chylomicrons This leads to an elevation in plasma chylomicrons and clinically leads to formation of xanthoma and pancreatitis.

Familial type-III hyperlipoproteinaemia. This disorder affects approximately one in one hundred of the population. Patients have inherited two mutant genes at the locus for apo E. This apoprotein is necessary for the binding of chylomicron remnants and IDL to hepatic receptors. In this disorder, homozygosity itself does not lead to hyperlipidaemia, another aggravating factor is required such as hypothyroidism. Thus, only one in one hundred homozygotes develop hyperlipidaemia.

Familial Hypercholesterolaemia. People with familial hypercholesterolaemia have an elevation of lipoproteins that carry endogenous cholesterol. One in 500 of the population is heterozygous for this condition. Heterozygote subjects have approximately half the normal number of LDL receptors. In consequence cholesterol

is cleared from the plasma at two thirds of the normal rate - largely through receptor independent pathways. An overproduction of LDL has been observed in these patients. Lipoproteins which are responsible for the transport of dietary cholesterol - chylomicrons and chylomicron remnants are found to be normal in familial hypercholesterolaemia. People with heterozygote familial hypercholesterolaemia have a 2-3 fold increase in plasma LDL concentrations from birth. The elevation of LDL leads to symptomatic atherosclerosis in these patients, usually between the ages of 40 and 50 (Goldstein et al, 1983). People who are homozygote for familial hypercholesterolaemia have two defective LDL receptor genes and are found to produce few, if any, functional LDL receptors. Thus, LDL is removed from the plasma at one third of the normal rate, almost exclusively through receptor independent pathways. This combined with an overproduction of LDL results in a 6-8 fold increase in plasma concentrations of LDL from as early as the twentieth week of gestation and will subsequently lead to symptomatic coronary atherosclerosis in these subjects before 20 years of age (Goldstein et al, 1983). It has been shown that in people with familial hypercholesterolaemia, the LDL receptor is produced, however it is non-functional due to mutations in the structural gene encoding for the receptor (Telleshaug et al, 1983).

Two other monogenic disorders are classified in which the actual genetic defect has not been identified :-

Familial hypertriglyceridaemia - in which elevated plasma VLDL levels are found.

Multiple lipoprotein-type hyperlipidaemia, also known as familial combined hyperlipidaemia - in this disorder both VLDL and LDL are elevated.

Other genetic disorders, which are multifactorial have been identified. The actual causes of these disorders have not been established however it is postulated that subtle genetic abnormalities predispose subjects to these forms of disease. Additional factors such as obesity and / or a diet high in saturated fat and cholesterol are thought to play a significant role in these disease states. Two multi-factorial disorders have been identified :-

Polygenic hypercholesterolaemia - leading to an elevation of LDL.

Hypertriglyceridaemia - where an elevation in VLDL is observed.

1.5.2 SECONDARY HYPERLIPIDAEMIA.

Secondary hyperlipidaemia can result from several relatively common clinical disorders. These include :-

Diabetes mellitus - due to increased secretion and delayed catabolism of VLDL the plasma concentration of this lipoprotein is elevated.

Hypothyroidism - leads to suppression of LDL receptors and thus, an elevation of plasma LDL.

Nephrotic syndrome - leads to an increase in secretion and decrease in catabolism of VLDL and LDL, elevating the plasma concentrations of both of these lipoproteins.

Uremia - decreases catabolism of VLDL, increasing the plasma concentration of VLDL.

Alcoholic hyperlipidaemia results from increased concentrations of VLDL in individuals genetically predisposed to hypertriglyceridaemia.

The oral contraceptive pill can similarly increase VLDL secretion in subjects genetically predisposed to hypertriglyceridaemia.

Thus, varying forms of hyperlipoproteinaemias can occur as a result of a wide range of disorders. Hyperlipiproteinaemia or hypercholesterolaemia are recognised as major risk factors for the development of atherosclerosis. The atherogenic process described earlier is obviously more likeky to occur in a hyperlipidaemic environment and once initiated the potential for progression of the plaque is much greater if plasma lipoproteins are elevated. Additionally, the lipoprotein itself can be atherogenic and thus, increase the likelyhood of initiation.

1.6 EFFECTS OF ATHEROSCLEROSIS ON THE CARDIOVASCULAR SYSTEM.

Clinical atherosclerotic disease can be manifested in several ways. In the coronary circulation, stable or unstable angina, acute myocardial infarction or sudden cardiac death can occur. In the cerebral circulation transient ischaemic attack and cerebral ischaemia may result. Atherosclerosis of the abdominal or leg arteries can result in acute or chronic ischaemia of the extremities.

Rupture of advance plaque is often the cause of the acute syndromes. However, the chronic effects may be due to alterations in the vasoreactivity of the surrounding vessel. A range of studies in both humans and in animal models of atherosclerosis have examined vascular reactivity under atherosclerotic conditions. These are discussed in detail in later chapters. Briefly it has been proposed that atherosclerotic

vessels exhibit decreased endothelium dependent responsiveness. The effects of atherosclerosis on contractile function seems to depend on the stage of atherosclerosis, the agonists studied and the particular model / tissue examined - both increased and decreased vasoconstriction have been reported.

1.7 FREE RADICALS AND ISCHAEMIA REPERFUSION INJURY.

With regard to the cardiovascular system and free radicals / reactive oxygen species, one of the most interesting areas of research in recent years has been that of ischaemia reperfusion injury. As early as 1960, Danforth et al detailed studies of glycolytic changes during ischaemia and reperfusion, whilst Jennings et al (1960) reported electrophysiological and structural changes during reperfusion following ischaemia. If the supply of oxygen to a tissue is lost the tissues sustain injury. After a period of time this injury becomes irreversible and the cells will die. The period of time a tissue can survive without oxygen varies depending on the tissue type. Skeletal muscle can be ischaemic for several hours without sustaining any serious injury, whereas brain tissue will survive only a matter of minutes. Cardiac tissue is able to withstand approximately one hour in an ischaemic environment.

When tissues become ischaemic it is therefore essential to restore oxygenated blood flow as soon as possible, hence the use of thrombolytic agents in the treatment of myocardial infarction.

However, over the past decade many studies have shown that it is possible to decrease myocardial damage if antioxidants are administered during or shortly following reperfusion. On first consideration, this observation is surprising - one would not expect elevated production of oxygen derived free radicals during a period of ischaemia. Yet a series of events occur during hypoxia which, when oxygen is

reintroduced, can lead to the generation of a range of free radicals and reactive oxygen species. Thus initiating self propagating tissue injury.

Normal healthy tissue contains an enzyme known as xanthine dehydrogenase (XD). This enzyme can reduce nicotinamide-adenine dinucleotide (NAD^+) as follows :-

$$\text{Xanthine} + H_2O + NAD^+ \xrightarrow{XD} \text{uric acid} + NADH + H^+$$

It has been known for some time that xanthine dehydrogenase could, under certain conditions, be converted to the enzyme xanthine oxidase (XO) as a result of sulphydryl oxidation or limited proteolysis (Battelli et al, 1972; Della Corte & Stirpe, 1972). The enzyme xanthine oxidase can use molecular oxygen instead of NAD^+ to oxidise xanthine, resulting in the production of the superoxide anion ($O_2^{\cdot-}$).

$$\text{Xanthine} + H_2O + 2O_2 \xrightarrow{XO} \text{uric acid} + 2O_2^{\cdot-} + 2H^+$$

Xanthine oxidase was the first documented biological source of the superoxide anion (McCord & Fridovich, 1968). Roy and McCord demonstrated in 1983 that this conversion of xanthine dehydrogenase to xanthine oxidase does in fact occur in ischaemic tissues. They hypothesised that the process begins when blood flow decreases sufficiently to limit the oxygen supply for ATP production. As the energy charge of the cell decreases, ionic gradients across the membrane are disrupted leading to a redistribution of calcium ions. Roy and McCord proposed that this elevation of intracellular calcium activates a protease capable of converting the dehydrogenase to the oxidase.

Depletion of cellular ATP stores during the period of ischaemia will lead to an

elevation of the intracellular concentration of AMP. The AMP is subsequently catabolised to adenosine, inosine and then hypoxanthine. The build up of hypoxanthine in ischaemic tissue has been demonstrated (DeWall et al, 1971) and also during haemorrhagic shock (Jones et al, 1968). Hypoxanthine is an alternative oxidizable purine substrate for either xanthine dehydrogenase or xanthine oxidase.

Hence, during a period of ischaemia, two crucial events occur - the appearance of a new enzyme and concomitantly the generation of one of its' substrates (Figure 1.3). Then, when reperfusion occurs, the other substrate for xanthine oxidase is supplied - molecular oxygen. These factors when combined lead to a burst of superoxide radical and hydrogen peroxide production (Figure 1.3). These two species can then take part in the Haber-Weiss reaction to produce hydroxyl radicals:

$$H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + OH^{\cdot} + OH^-$$

In biological systems this reaction is normally catalysed by iron:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

with the latter reaction being known as the Fenton Reaction (see above).

As discussed earlier, intracellular free iron concentrations are virtually zero. However, at pH6 iron will dissociate from the storage protein ferritin. In hypoxic tissues, this level of acidosis can occur. Iron may also be released from ferritin in a reductive environment, for example in the presence of the superoxide anion.

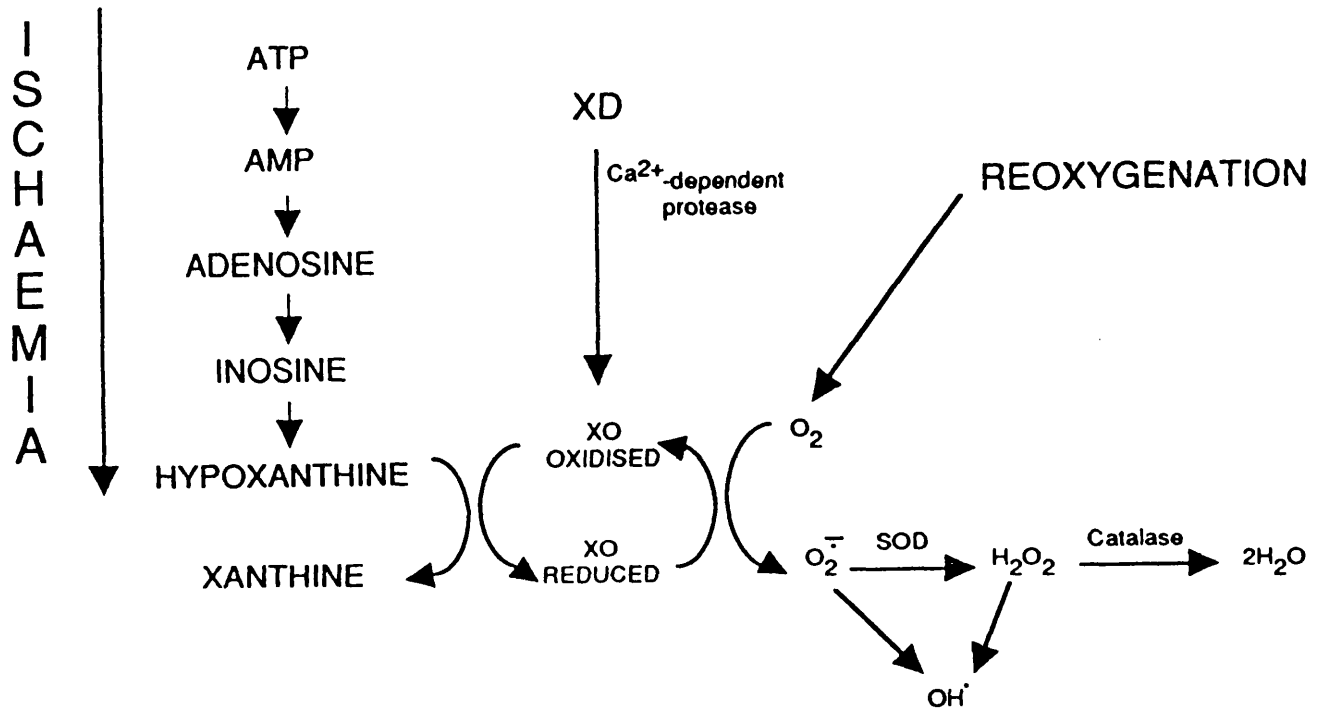


FIGURE 1.3. Schematic representation of the events occuring during a period of ischaemia followed by reperfusion. XD denotes xanthine dehydrogenase; XO, xanthine oxidase; SOD, superoxide dismutase.

Hence, when a tissue becomes ischaemic and is subsequently reperfused, there is the potential for all these factors to interact and produce a burst of free radicals and reactive oxygen species. Direct evidence for the presence of free radical species during reperfusion is hard to obtain due to the highly reactive and transient nature of these species. Arryo and colleagues (1987) demonstrated the presence of oxygen centred radicals in ischaemic canine myocardium using electron spin trapping techniques. Another study in dogs used electron paramagnetic resonance and a spin trapping agent to detect a burst of free radical formation, peaking at 2 minutes, in venous effluent from dogs subjected to 15 minutes coronary occlusion and reperfusion (Bolli et al, 1988).

Thus, following a period of ischaemia, the tissue is then vunerable to attack from this burst of reactive oxygen species and free radicals when oxygen is reintroduced. Due to the highly reactive nature of these species, all cellular components are susceptible to attack. Oxidant injury can affect structural contractile and transport proteins, enzymes, receptors, membrane lipids, glycosaminoglycans and nucleic acids. This can lead to major ultrastructural damage, cell swelling, massive cytosolic enzyme release and prolonged depression of contractile function. In cardiac tissue, reperfusion can also induce a number of electro-physiological abnormalities that may produce potentially lethal arrythmias (Hearse, 1977).

The clinical manifestations of ischaemia reperfusion injury largely depend upon the duration of ischaemia. Short periods of myocardial ischaemia are accompanied by a reduction in contractile function termed myocardial 'stunning'. The myocardium is able to recover from this stunning - the time course for recovery being dependent on the duration of ischaemia. After one minute of coronary occlusion, active shortening in the affected area returns within twenty seconds and is complete by thirty minutes (Braunwald & Kloner, 1985). Regional loss of myocardial function following fifteen

minutes of ischaemia may take sixty minutes to recover. Even after prolonged ischaemia some return of myocardial function may be seen as late as one or two weeks after the event.

Recent work has suggested that important changes in endothelial function occur during ischaemia reperfusion. It has been demonstrated that brief periods of ischaemia followed by reperfusion in canine coronary artery causes the loss of endothelium dependent vasodilation and enhanced vasoconstrictor response together with increased permeability of the coronary endothelium (Dauber et al, 1990; Kim et al, 1992). These functional changes occur without any evidence of structural injury.

1.8 AIMS.

Hence it seems that free radicals / reactive oxygen species may be involved in two aspects of cardiovascular disease. Firstly, in atherosclerotic disease :- oxidation of LDL, which increases its atherogenicity by several mechanisms, is likely to involve free radicals / reactive oxygen species; free radicals / reactive oxygen species may also be involved in mediating the initial injury to the endothelium which subsequently induces the development of an atherosclerotic plaque (Henning and Chow, 1988; Ross, 1986). Secondly, free radicals / reactive oxygen species are likely to be involved in mediating ischaemia reperfusion injury.

There is evidence that vascular reactivity is altered during hyperlipidaemia / atherosclerosis and also following ischaemia and subsequent reperfusion. The aim of this work was, therefore, to examine the vasoactive properties of the vasculature in response to elevations in serum cholesterol and exposure to free radicals / reactive oxygen species using appropriate animal models.

Firstly, a classic model of atherosclerosis was examined - the cholesterol fed New Zealand White rabbit. It has been shown that cholesterol feeding in rabbits can induce a form of atherosclerotic disease and lead to alterations in vascular function. Thus, the aim was to assess the changes in vasorelaxation following a period of increased dietary cholesterol and to subsequently assess the possible effects of normalisation of the dietary cholesterol intake. The responses of the thoracic aorta were examined. The thoracic aorta was used in this and other parts of the study for practical reasons such as its size, its ease of preparation and its reproducability of responses.

The second part of this work involved a relatively new animal model of atherosclerosis - the Watanabe Heritable Hyperlipidaemic (WHHL) rabbit. This model was first developed in the 1970's and it has since been shown that the WHHL rabbit is genetically deficient in LDL receptors and as such is a appropriate model for human familial hypercholesterolaemia. These animals have been studied by several groups since their discovery, however as detailed in later chapters, many of these studies are limited due to study design and group size. Thus, the aim of this section of my work was to investigate the contraction and relaxation of the thoracic aorta, aortic arch and the carotid artery of these animals, at periodic intervals up to one year of age, thus producing a detailed study of the sequential changes occuring during the development and progression of atherosclerosis. One further aspect of this investigation was to broaden the study to examine not only the homozygous WHHL rabbits, as has been done previously, but also to incorporate the heterozygous animals as well. From a clinical point of view these animals would be especially interesting as the heterozygous form of familial hypercholesterolaemia is obviously much more common than the homozygous form.

Having established the pattern of changes in vasoreactivity during the development of atherosclerosis in these rabbits, it was then possible to study a potentially beneficial, cholesterol lowering therapy. The effects of the HMG CoA reductase inhibitor Simvastatin were examined in both young and old WHHL rabbits of homozygote and heterozygote characteristics.

The third set of studies presented in this thesis are designed to investigate an *in vitro* model of free radical / reactive oxygen exposure. Here, using thoracic aorta from New Zealand White rabbits, vessels were exposed to a xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system. The effects of this system were examined on adrenergic contraction; muscarinic, endothelium dependent relaxation; and SNP, non-endothelium dependent relaxation of the thoracic aorta. Subsequent studies were carried out in order to elucidate which of the potentially detrimental species produced by the xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system were responsible for the observed alterations in vascular response.

CHAPTER TW0.

GENERAL METHODS.

2.1 STUDIES IN CHOLESTEROL FED NEW ZEALAND WHITE AND WHHL RABBITS.

2.1.1 DETERMINATION OF SERUM CHOLESTEROL LEVELS.

A. Preparation of Samples.

Blood samples (2-5mls) were taken from the marginal ear vein or, if at the time of study, from the left ventricle of the heart. The blood was immediately transfered into Sterilin lithium heparin tubes. The plasma was separated by centrifugation (2000 rpm, 5 minutes, 4°C, and stored at 4°C until assay.

B. Spectrophotometric Determination of Serum Total Cholesterol.

Cholesterol was measured by the Boehringer Mannheim Cholesterol C-system which involves the CHOD-PAP method. This is a standard enzymatic colourimetric assay, involving the cleavage of the cholesterol esters by cholesterol esterase and the oxidation of the free cholesterol by cholesterol oxidase.

$$\text{cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \Delta^4\text{-cholestenone} + H_2O_2$$

The reaction is quantified photometrically by the use of a H_2O_2 - dependent colour forming reaction :-

$$2\,H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{4-(}p\text{-benzoquinone-monoimino)-phenazone} + 4\,H_2O$$

20µl of the sample were incubated for 10 minutes at 20 - 25°C with 2mls of the reagent solution of the following composition :- Tris buffer: 100 mmol/l, pH 7.7;

magnesium aspartate: 50 mmol/l; 4-aminophenazone: 1 mmol/l; sodium cholate: 10 mmol/l; phenol: 6 mmol/l; 3,4-dichlorophenol: 4 mmol/l; hydroxypolyethoxy-*n*-alkanes: 0.3%; cholesterol esterase 0.4 U/ml; cholesterol oxidase 0.25 U/ml; peroxidase 0.2 U/ml. Absorbance of the sample (A_{sample}) was read at Hg 546 nm against a reagent blank within one hour. Concentration of cholesterol (C) in the sample is calculated using the following equation :-

$$C \text{ (mmol/l)} = 22.1 \times A_{sample}$$

The assays were carried out by the Biochemistry Department at Stobhill Hospital.

2.1.2 DETERMINATION OF FUNCTIONAL PROPERTIES OF RABBIT BLOOD VESSELS.

Rabbits were killed with an overdose of sodium pentobarbitone (50 mg/Kg), administered intravenously in the marginal ear vein. The thoracic aorta, aortic arch and carotid arteries were immediately dissected out and immersed in ice-cold Krebs bicarbonate buffer of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, CaEDTA 0.05, glucose 11.1, pH 7.4. 17ß oestradiol (10^{-5}M), cocaine hydrochloride (10^{-5}M) and indomethacin (10^{-5}M) were added to the Krebs buffer to block extraneuronal and neuronal adrenergic uptake, and prostaglandin synthesis, respectively. The tissues were trimmed free of fat and adhering connective tissue. 2mm wide transverse rings were cut and suspended between two stainless steel hooks in a 10ml organ bath, filled with Krebs buffer at 37°C, continuously gassed with 95% O_2, 5% CO_2. Isometric tension was recorded using a force transducer (Grass model FT03), connected to a chart recorder (Grass Polygraph model 7B). Each ring was set individually at the optimal point of

its length tension relationship as determined by repeated exposure to phenylephrine (10^{-7}M). Tissues were then allowed to equilibrate for 1 hour. Cumulative concentration curves to phenylephrine (10^{-8}M - 10^{-5}M) were obtained. The baths were washed out and the tissues were allowed to re-equilibrate for 45 minutes, they were then recontracted to between 50 and 70% of the maximum contraction to phenylephrine as determined from the full concentration response curve. Once a plateau contraction response was established, cumulative concentration response curves to carbachol (10^{-8} - 10^{-5}M) were obtained. Before the baths were washed, each ring was exposed to 10^{-4}M sodium nitroprusside (SNP). At the end of the assay each ring was blotted dry and weighed.

2.1.3 DETERMINATION OF STRUCTURAL PROPERTIES OF RABBIT AORTIC TISSUE.

Changes in the structural properties of the tissue were assessed in four different ways.

A. Sudan Red Staining.

Gross staining with Sudan red was used to allow accurate detection and estimation of fatty deposits in the arterial intima. A section of aorta adjacent to that used in the functional study was opened longitudinally and pinned out flat, with the intimal side uppermost, in a petri dish containing silicon gel. The tissues were washed with 70% alcohol and then immersed in Herxheimer's Solution (5g Sudan IV, 500ml 70% ethanol, 500ml acetone) for 15 minutes at room temperature, the petri dish was periodically agitated to avoid irregular staining. The tissues were differentiated in 80% alcohol for 20 minutes and then washed in running tap water for 1 hour. Accurate drawings of each sample were made using a microscope with a drawing arm attachment. The percentage of the intimal surface stained with sudan red dye can

then be measured using image analysis.

The other methods of assessing structural changes in the aorta used histological sections. These were prepared in collaboration with Dr George Lindop in the Department of Pathology at the Western Infirmary.

Preparation of Histological Sections.

Segments of thoracic aorta were fixed in formalin and embedded in parafin wax. They were subsequently sectioned and stained with four standard histological stains :- haematoxylin and eosin (H&E); alcian blue / periodic acid-Schiff (AB / PAS); Van Gesin (VG); elastic / matius scarlet blue (EMSB) . The sections were examined and photographed under a light microscope (Leitz Orthoplan).

Electron micrographs were prepared from tissue fixed in gluteraldehyde and embedded in araldite. Both scanning and electron micrographs were prepared using standard techniques.

B. % Intimal Involvement.

Using image analysis the internal circumference of the aortic section was measured using the internal elastic lamina as a guide line. The proportion of the intimal circumference involved in atheroma (X + Y) was also measured (Figure 2.1). The percentage of the intima involved in atheroma was then calculated :-

$$\frac{X+Y}{\text{Internal Circumference}} \times 100 = \% \text{ Intimal Involvement.}$$

C. Ratio of Intima to Media.

Again using image analysis the area of the media and the area of the intima were

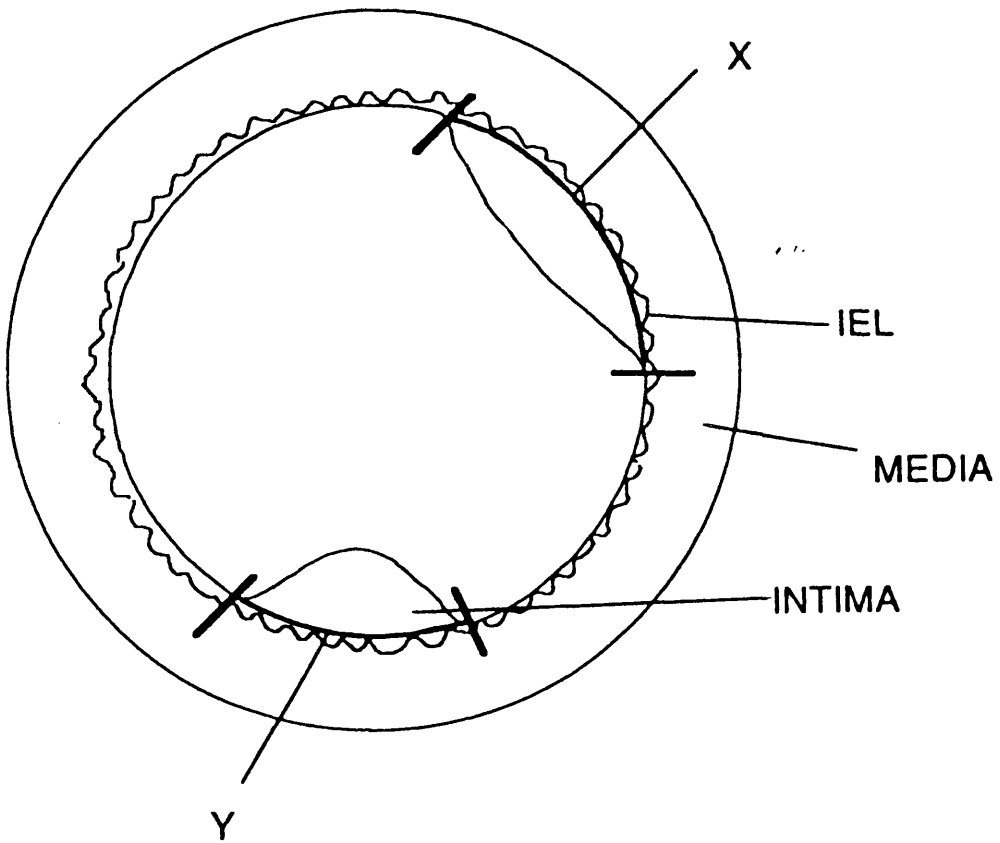


FIGURE 2.1. Diagramatic representation of measurement of percentage intimal involvement. Distance under area of plaque (X + Y) measured by image analysis and expressed as a percentage of the internal circumference of the artery using the internal elastic lamina as a guide line. IEL represents internal elastic lamina.

measured using the internal elastic lamina as the separation line (Figure 2.2). The ratio of the two was then calculated:-

$$\frac{\text{INTIMA}}{\text{MEDIA}} \times 100 = \% \text{ Ratio of Intima to Media.}$$

In a section from a control animal or an animal with no detectable signs of atherosclerosis, using our techniques it is not possible to quantify the intima. Thus under these circumstances this parameter would apparently be zero.

D. Assessment of Histological Changes.

The histological sections were examined and assessed in a randomised, blind manner. Each section was scored for a range of histological / pathological changes:-

Intimal changes : Presence of plaque, foam cells, smooth muscle cells, extracellular lipid, proteoglycans, collagen and activation of the endothelium.

Medial changes : Presence of extracellular lipid, collagen, proteoglycans both under areas of plaque and distant to plaque, separation of elastic laminae and alteration in the orientation of smooth muscle cells.

2.1.4 IMAGE ANALYSIS.

Image analysis was performed using VIDS - V. VIDS - V is an advanced semi-automatic image analysis system designed specifically for the IBM PS/2 and compatible computers. The system uses a high performance television camera to provide direct imaging of samples viewed with a microscope or macro lens. VIDS - V displays an image of the sample on a high resolution monitor and allows the user to dot or outline features of interest using the digitising tablet and cursor.

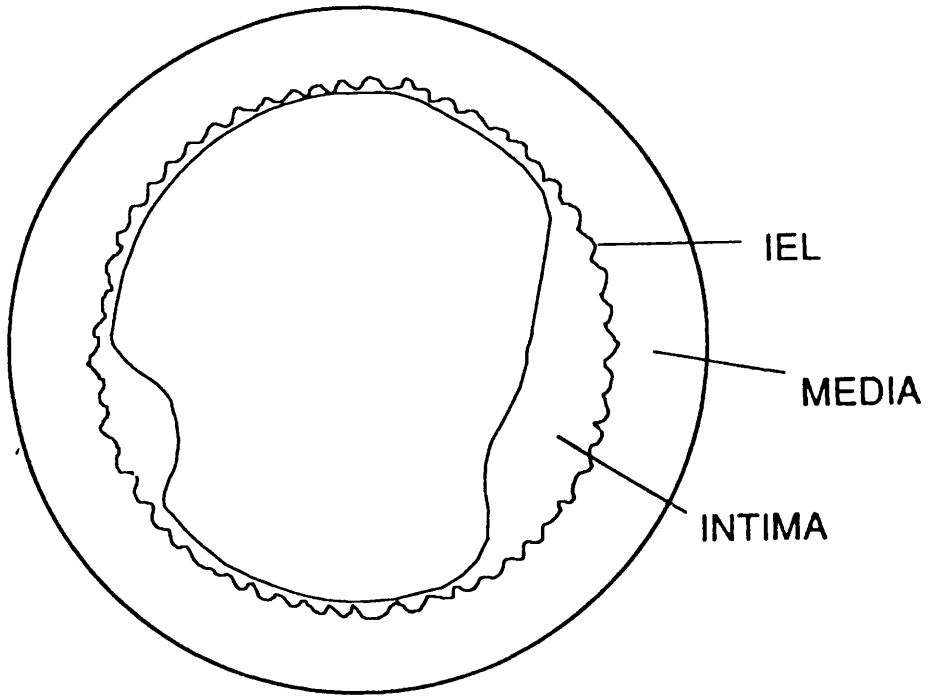


FIGURE 2.2. Diagramatic representation of measurement of percentage ratio of area of intima to area of media. Area of intima is measure by image analysis and expressed as a percentage of the are of media. IEL represents internal elastic lamina.

The soft ware provides several drawing modes allowing distance and area calculations. Measurements are displayed on the computer monitor together with updated statistics.

2.2 STUDIES OF FREE RADICALS / REACTIVE OXYGEN SPECIES IN NEW ZEALAND WHITE RABBITS.

2.2.1 PROTOCOL FOR THE STUDY OF THE EFFECTS OF FREE RADICALS / REACTIVE OXYGEN SPECIES.

Male New Zealand White rabbits weighing 2-2.5Kg were used in this study. Rings of thoracic aorta were obtained and prepared as described above. Up to six rings were obtained from each animal. Control concentration response curves to either:- (A) phenylephrine (10^{-8}M - 10^{-5}M), (B) phenylephrine (10^{-8}M - 10^{-5}M) and carbachol (10^{-8}M - 10^{-5}M) or (C) phenylephrine (10^{-8}M - 10^{-5}M) and SNP (10^{-8}M - 10^{-5}M) were obtained. For the carbachol and SNP concentration response curves, the tissues were precontracted to between 50 and 70% of the maximum response to phenylephrine as determined from the full concentration response curve. The baths were then washed and allowed to re-equilibrate for 15 - 30 minutes. Rings were then exposed to either vehicle, the xanthine oxidase / hypoxanthine (XO/HX) system or hydrogen peroxide for 30 minutes. The tissues were then washed and allowed to equilibrate for 10- 15 minutes. Concentration response curves to (A) phenylephrine, (B) carbachol or (C) SNP were then repeated. In experiments studying the responses to carbachol, where there was evidence of loss of EDRF mediated relaxation then SNP (10^{-4}M) was added to the bath before it was washed.

In order to investigate which of the products of the XO/HX system were primarily

responsible for the observed alterations in tissue function a range of "protective" agents were used: Two SH-containing compounds, captopril and N-(2-mercapto-propionyl)-glycine (MPG), were examined. Both these compounds have been shown to be effective free radical scavengers in vitro and have also been shown to be protective against postischaemic myocardial dysfunction (Mak et al. 1990, Koerner, Anderson & Dage. 1991). Mannitol was used as a specific hydroxyl radical scavenger. Superoxide dismutase (SOD) (EC 1.15.1.1), an endogenous enzyme which catalyses the dismutation of superoxide to hydrogen peroxide in most eukaryotic cells, was used to scavenge $O_2^{\cdot-}$ radicals. Catalase (EC 1.11.1.6), which is also an endogenous enzyme found in most aerobic cells, catalysing the degradation of hydrogen peroxide to water and oxygen, was used to investigate the involvement of H_2O_2. Each "scavenging" agent was added to the bath for 5 minutes before the addition of either the xanthine oxidase/hypoxanthine (XO/HX) system, H_2O_2 or vehicle for 30 minutes. In addition to this, in one set of experiments MPG was added to the bath for 60 minutes prior to the XO/HX system to allow more effective distribution of the MPG into the cytoplasm of the cells.

2.2.2 THE XO/HX FREE RADICAL / REACTIVE OXYGEN SPECIES GENERATING SYSTEM.

Hypoxanthine (250μM), EDTA (37.5μM) and $FeCl_3$ (25μM) were added to the bath, followed by xanthine oxidase (20mU/ml) (EC 1.1.3.22). The possible products of this reaction are shown in Figure 1.3.

2.3 DATA ANALYSIS.

Data are expressed as means ± standard deviation (s.d.) unless otherwise stated in the text. Statistical analysis of cumulative concentration response curve data was carried

out using repeated measures ANOVA, differences were considered to be significant when $p < 0.05$, and Bonferroni multiple comparisons were used where appropriate. Statistical analysis of cholesterol levels, structural parameters and changes in baseline tension were analysed using t-tests, allowing for multiple comparisons where necessary, differences were considered to be significant when $p < 0.05$.

2.4 MATERIALS.

The materials used in this thesis were obtained from the following suppliers :-

New Zealand White rabbits - Interfauna, Wyton, Huntingdon, U.K.
WHHL rabbits - bred and maintained in house at Stobhill Hospital, Glasgow.
Sodium nitroprusside and cocaine hydrochloride - Pharmacy, Western Infirmary, Glasgow.

All other chemicals and reagents were obtained from Sigma Chemical Company, Dorset, U.K.

CHAPTER 3.

EFFECTS OF PERIODS OF ELEVATED DIETARY CHOLESTEROL ON THE FUNCTION OF THE THORACIC AORTA FROM NEW ZEALAND WHITE RABBITS.

3.1 INTRODUCTION.

Cholesterol feeding in rabbits was first shown to induce hypercholesterolaemia and subsequently atherosclerosis in 1913 (Goldstein et al, 1983). As a model for human hyperlipidaemia, this model is imperfect as the human conditions are usually the result of genetic or acquired abnormalities in cholesterol / lipid metabolism, with dietary cholesterol only an aggravating influence.

However, there is substantial evidence for an association between diet and morbidity / mortality from coronary heart disease. A positive correlation between coronary heart disease and dietary fat (cholesterol, saturated and polyunsaturated fatty acids) has been observed in several epidemiological studies (Keys, 1970; Keys et al, 1986; Shekelle et al, 1981; Kushi et al,1985). The unfortunate circumstances in both World War I and World War II demonstrated that severe restriction of calorific intake from all sources, including fat, can reduce the incidence of coronary atherosclerosis. Diet deprivation during World War I resulted in a fall in athersclerosis found at autopsy in Germany (Aschoff, 1924) and World War II lead to similar findings in Scandinavia (Malmros, 1950; Strom and Jensen, 1951; Schettler, 1979).

More recently, studies have shown that controlling fat intake can prevent progression of atherosclerotic disease (Arntzenius et al, 1985; Blankenhorn et al, 1990). Thus, in the clinical situation, where atherosclerosis has become symptomatic, dietary modification is often the first line of therapy. Dietary intervention is recommended for all patients with an LDL level more than 160mg/dl, regardless of additional risk factors. For subjects in a high risk category intervention is recommended when plasma LDL concentration exceeds 130mg/dl. The logic for dietary intervention is two fold :-

First, and most obviously, a proportion of the cholesterol in the body is obtained from the diet, thus reducing intake will consequently reduce availability.

Secondly, it is known that lipoprotein production in the liver is related to calorie intake (see chapter 1), thus a reduction in fat intake will reduce calorie intake (unless substituted by other low fat food) and thus, the liver will consequently decrease lipoprotein production.

However, studies in man are limited. It is hard, practically and ethically, to assess functional changes of the vasculature *in situ*, some studies have been carried out using forearm blood flow measurements. However, in order to obtain an overall assessment, many studies are carried out in animal models of atherosclerotic disease. In the cholesterol fed rabbit model, hypercholesterolaemia develops as a result of the normal mechanisms of lipoprotein clearance being overloaded with dietary cholesterol. This leads to an elevation of serum cholesterol levels and subsequently a form of atherosclerosis. In hypercholesterolaemic rabbits, morphological or functional changes in vascular tissues have been noted (Imai et al, 1966; Goode et al, 1977; Ingerman-Wojenski et al, 1983). Various studies have examined the effect of hypercholesterolaemia and atherosclerosis in rabbits on endothelium dependent relaxation. These studies have generally shown the endothelium dependent relaxation is impaired following a period of elevated dietary cholesterol (Hof and Hof, 1988; Senaratne et al, 1989; Simonsen et al, 1991).

Having shown that diet induced hyperlipidaemia is able to impair endothelium dependent relaxation, it is obviously of interest to examine whether subsequent dietary restriction of cholesterol is able to reverse this phenomenon. The information in the literature regarding this possibility is contradictory. Jayakody et al (1987)

demonstrated that the effects of a 2% diet cholesterol feeding for 6 weeks in New Zealand White rabbits could not be reversed even after 32 weeks of return to normal rabbit chow. However, other studies in rabbits (Kappagoda et al, 1990) and monkeys (Harrison et al, 1987) have demonstrated recovery of endothelium dependent relaxation following return to standard diet.

3.2 AIMS.

The aims of this study were to examine the effects of dietary induced hyperlipidaemia (0.3% cholesterol diet for 20 weeks) on the functional responses of the thoracic aorta. Both phenylephrine induced contraction and carbachol induced relaxation. Secondly, where alterations in vascular function are observed - to examine the effect of subsequent removal of excess cholesterol from the diet.

3.3 PROTOCOL FOR DIETARY TREATMENT.

The protocol for diet induced hypercholesterolaemia is shown in Figure 3.1. Seven month old New Zealand White rabbits were randomly allocated to 3 groups. Group 1 were maintained on standard rabbit chow for 20 weeks. Group 2 were fed a 0.3% cholesterol diet for 20 weeks. Group 3 were fed a 0.3% cholesterol diet for 20 weeks at which point they were returned to standard rabbit chow for a further 20 week period.

Blood samples were taken for cholesterol quantification in Group 1 after 20 weeks standard diet, in Group 2 after 20 weeks of cholesterol diet and in Group 3 at 16, 18,

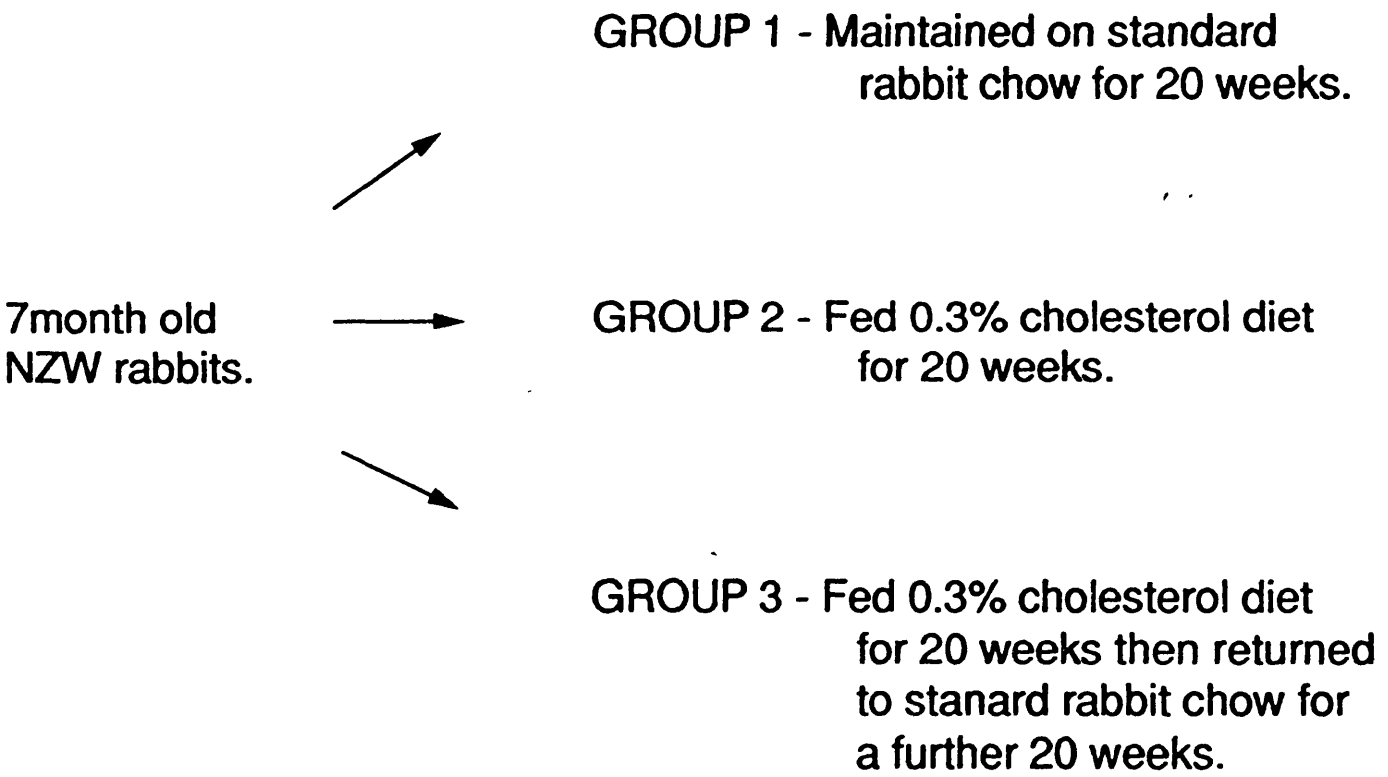


FIGURE 3.1. Protocol for dietary manipulation in New Zealand White rabbits.

and 20 weeks of cholesterol diet and every 4 weeks during the maintenance of standard diet.

Following the allocated dietary treatment, the animals were studied as detailed in chapter 2.

3.4 RESULTS.

3.4.1 CHOLESTEROL LEVELS.

Cholesterol levels in Group 1, which received no additional dietary cholesterol were normal, mean = 0.53 (sd =0.20, n=7). In the two groups receiving dietary cholesterol (Groups 2 & 3), cholesterol levels did not rise in the uniform manner expected (Table 3.1). Initially, it was suspected that an error in the allocation of the diet had occurred, however on investigation and expansion of the study it was found that the cholesterol levels were correct. At this time, no obvious cause for this wide variability has been found.

Following return to standard chow the cholesterol levels of the rabbits in Group 3 all returned to normal within the 20 week period (Figure 3.2).

Animal No.	G1	G2	G3
1	0.4	18.2	33.1
2	0.4	18.5	33.0
3	0.9	18.9	3.5
4	0.6	22.2	3.6
5	0.3	5.8	0.7
6	0.5	2.2	
7	0.6	3.4	

TABLE 3.1. Serum cholesterol levels following the initial 20 week dietary modification period. G1 - Group 1 were maintained on standard rabbit chow. G2 & G3 - Groups 2 and 3 were fed a 0.3% cholesterol diet for the 20 week period.

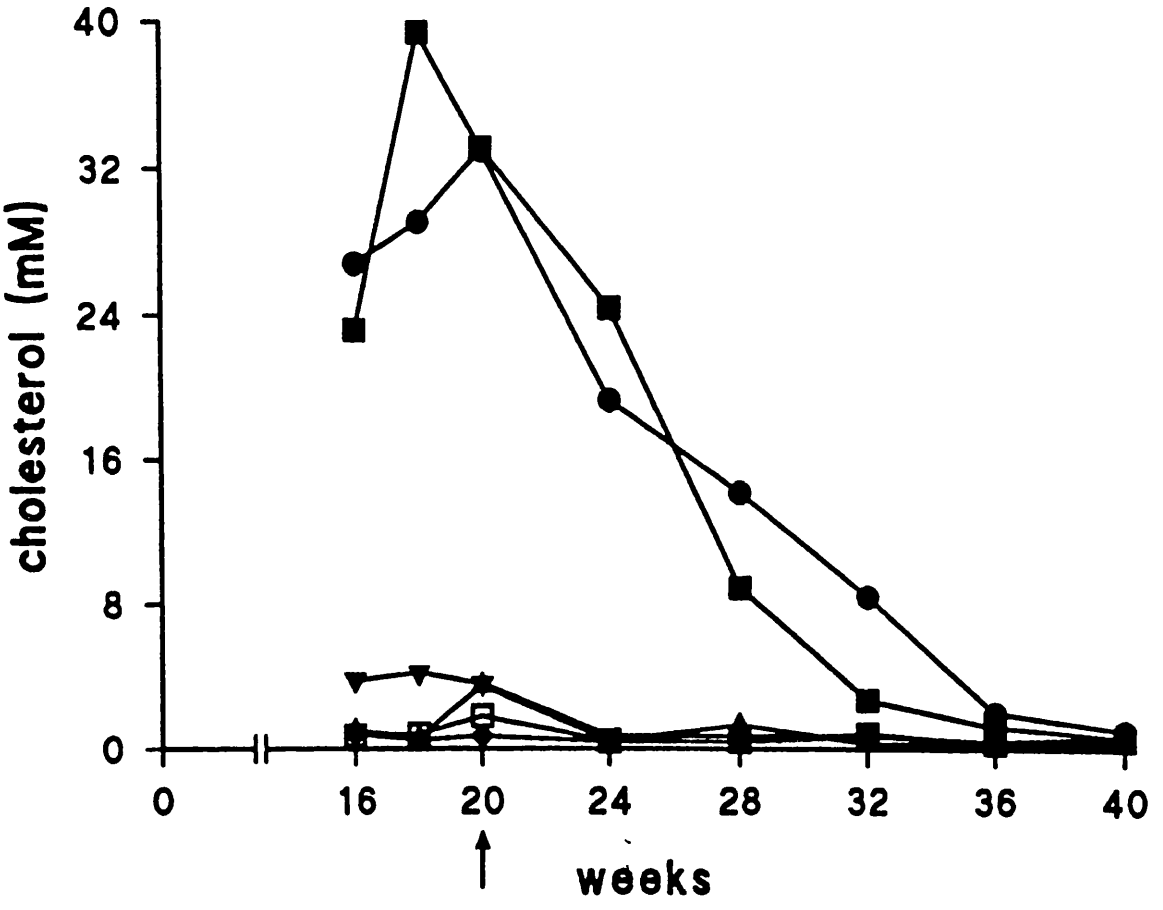


FIGURE 3.2. Serum cholesterol levels in Group 3 New Zealand White rabbits during the 20 week period of return to standard diet, subsequent to 20 weeks on a 0.3% cholesterol diet. Data points represent individual animals at the time points indicated. Arrow denotes the point at which the rabbits were returned to the standard diet.

3.4.2 FUNCTIONAL STUDIES IN THE THORACIC AORTA OF CHOLESTEROL FED NEW ZEALAND WHITE RABBITS.

A. Phenylephrine induced contraction.

The response of the thoracic aorta to the α_1-adrenoceptor agonist phenylephrine was examined in rings from control New Zealand White rabbits (Group1) and rabbits fed cholesterol diet (Group 2). No difference was found in the concentration response curve to phenylephrine in thoracic aorta from Group 1 and Group 2 (Figure 3.3).

B. Carbachol induced endothelium dependent relaxation.

The response of the thoracic aorta to the muscarinic agonist carbachol was examined in rings from control New Zealand White rabbits (Group 1) and rabbits fed cholesterol diet (Group 2). The cholesterol fed rabbits (Group 2) clearly showed an impairment of endothelium dependent relaxation when compared to the untreated control (Group 1) (Figure 3.4). It can also be seen that in the group returned to standard diet (Group 3), there is a partial restoration of endothelium dependent relaxation towards normal levels.

At all stages of the study, 10^{-4}M SNP was able to induce 100% relaxation when administered following the carbachol concentration response curve.

Normal statistical analysis of this data would be subject to many problems due to the wide variation in the serum cholesterol levels achieved. In order to obtain a satisfactory analysis of the data an alternative approach was taken:-

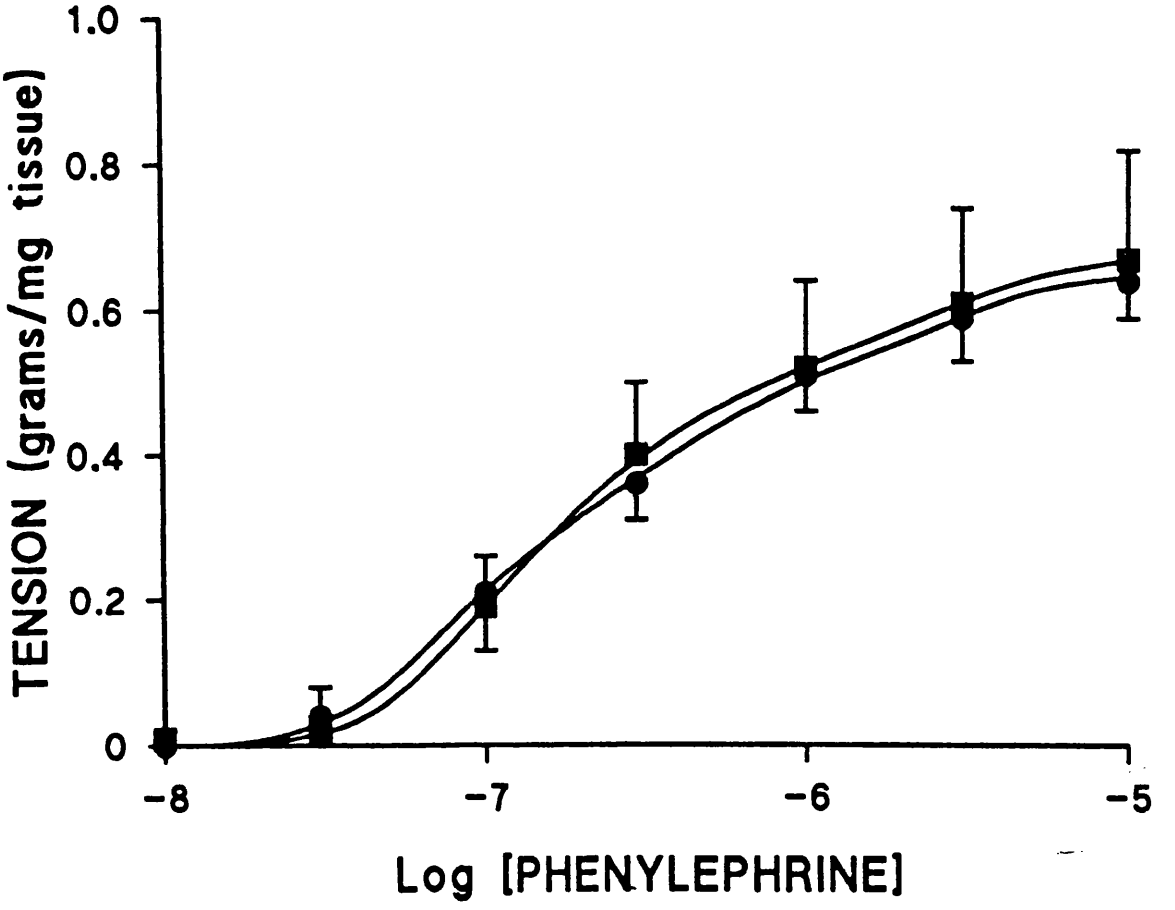


FIGURE 3.3. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses are expressed in grams tension / mg tissue. Data is mean ± s.d.

■ - Group 1 - control. n=7.
● - Group 2 - 20 week 0.3% cholesterol diet. n=7.

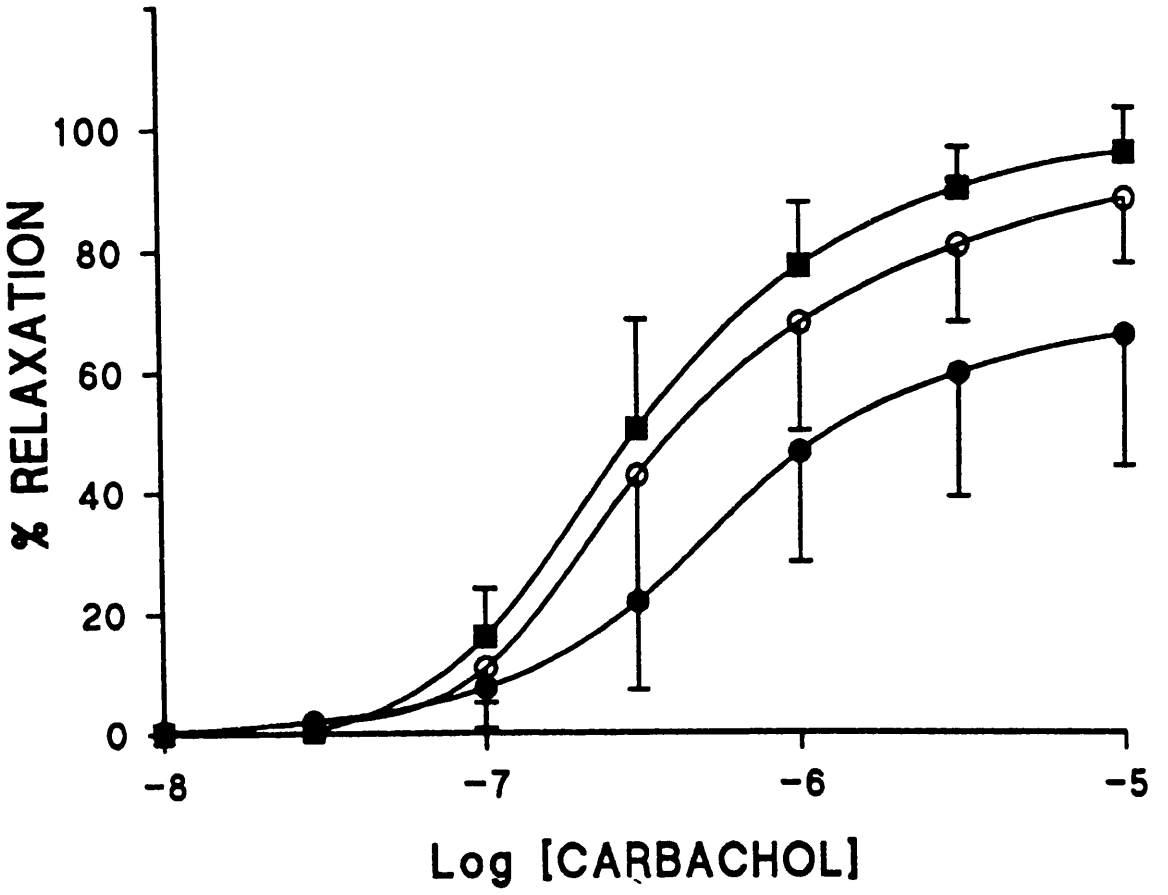


FIGURE 3.4. Cumulative concentration-response curves to carbachol (10^{-8}M - 10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses are expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

■ - Group 1 - control. n=7.
● - Group 2 - 20 week 0.3% cholesterol diet. n=7.
○ - Group 3 - 20 week 0.3% cholesterol diet followed by 20 week standard diet. n=5.

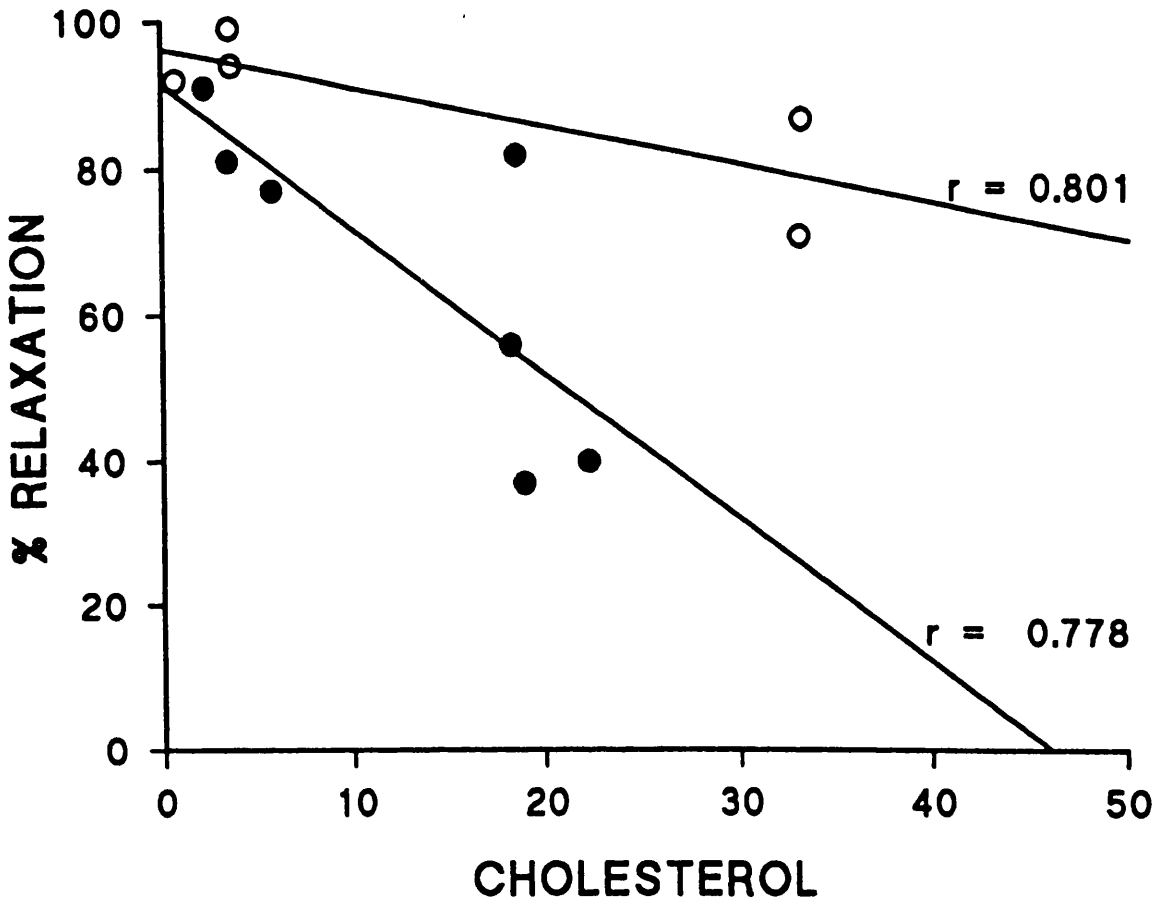


FIGURE 3.5. Bivariate plots of the maximum carbachol (10^{-5}M) induced relaxation of phenylephrine induced tone at study versus serum cholesterol levels following 20 weeks 0.3% cholesterol diet.

● - Group 2 - 20 week 0.3% cholesterol diet. n=7.
○ - Group 3 - 20 week 0.3% cholesterol diet followed by 20 week standard diet. n=5.

Where appropriate the correlation and fitted line are shown.

First, examining the data from Group 2 (cholesterol fed). Maximum carbachol induced relaxation (10^{-5}M) was plotted versus cholesterol level obtained following the 20 week period. On analysis it was found that a linear relationship existed between these two variables, r=-0.778. This relationship was negative, thus as serum cholesterol levels increased relaxation decreased. The gradient of the slope was 3.28 (Figure 3.5).

The same principle can be applied to the data from Group 3. Maximum carbachol induced relaxation was plotted versus the cholesterol level which was obtained following the 20 week cholesterol feeding period (NB. Not the level following return to normal diet). If the endothelium dependent relaxation had continued to decline or stayed the same after withdrawal of cholesterol, then the line of regression would be steeper or the same as for Group 2. If, however, return to the standard diet had induced recovery of endothelium dependent relaxation, the line of regression will become less steep i.e. the gradient will decrease.

On analysis of the Group 3 data, this latter situation was found to be the case. A linear relationship was found to exist between the two variable, r=-0.801. The gradient of the line was 0.800 (Figure 3.5).

Statistical analysis of this data using the F-test for model comparison (General linear test) ($p < 0.05$) showed that the two lines of regression were significantly different, and that two individual plots produced a better fit the one overall plot.

Thus within the limitations of the data from this study, these results show that a period of dietary cholesterol elevation produces an impairment of endothelium dependent relaxation in the thoracic aorta of New Zealand White rabbits. Furthermore, it can be seen that return to a normal diet for an equivalent period can

initiate recovery of the endothelium dependent response towards normal.

It had been proposed that pathological assessment of sections of thoracic aorta from these animals would be carried out. However due to the failure of the dietary treatment to produce consistent, sustained elevations in serum cholesterol levels valid interpretation of pathological studies would be difficult.

3.5 DISCUSSION.

The experiments described in this chapter were designed to examine the effects of hypercholesterolaemia in New Zealand White rabbits induced by dietary manipulation, on the vasoactive properties of the thoracic aorta. Also, to subsequently examine the effects of returning the rabbits to a normal diet for an equivalent period of time.

Surprisingly, when the rabbits were fed the 0.3% cholesterol diet, the serum levels did not rise in the expected manner. Unfortunately, the serum cholesterol levels were not measured until the end of the dietary supplementation period, by this stage it was to late to increase the number of animals included in the study, which would, presumably, have increased the number of rabbits with significantly elevated serum cholesterol levels.

There have been other reports in the literature regarding "hypercholesterolaemia resistant" rabbits (Roberts et al, 1974; Van Zutphen et al, 1981). In fact, Overturf and colleagues (1989) actually describe the development of a strain of New Zealand White rabbit which have an inheritable hypercholesterolaemia resistance. Similarly

in humans, significant differences in response to cholesterol challenge have been noted between individuals (Katan and Beyen, 1987).

Obviously, due to the varied serum cholesterol levels obtained, interpretation of this study is limited. In the rabbits fed cholesterol diet for 20 weeks no change in phenylephrine induced contraction was found in the thoracic aorta. Reports in the literature are contradictory - increased (Heric and Tackett, 1985) and decreased (Ibengwe and Suzki, 1986) responses to adrenergic agonists have been reported.

Carbachol induced endothelium dependent relaxation was found to decrease in a linear manner as cholesterol increased thus supporting previous studies documenting this fact. Return to normal diet for a 20 week period was found to cause a significant regression in the loss of endothelium dependent relaxation. As stated in the introduction reports are available to both support and contradict this finding.

The nature of the loss of endothelium dependent relaxation is still unclear - the loss may be due to a modification of the EDRF / NO pathway. This theory is supported by the fact that all rings retained the ability to relax 100% when exposed to the directly acting nitrovasodilator SNP, even when carbachol induced responses are severely impaired. This modification could affect either the synthesis or release of EDRF or may be due to the physical barrier of atheroma. More detailed studies could be conducted to investigate the modification of the response.

Removing the elevation of serum cholesterol by returning the rabbits to a standard diet enabled the vasculature to initiate repair mechanisms. It has been shown that intimal thickening remains following the regression of atherosclerosis (Harrison et al, 1987) indicating that the initial loss of endothelium dependent relaxation is not due to a physical barrier to the EDRF, preventing access to the underlying smooth

muscle.

So in summary, this study has shown that cholesterol feeding in rabbits does not alter phenylephrine induced contraction of the thoracic aorta, but carbachol induced endothelium dependent relaxation is impaired. Following return to a normal dietary regime endothelium dependent relaxation is, at least partially, restored towards normal.

CHAPTER FOUR.

DEVELOPMENT AND PROGRESSION OF ATHEROSCLEROSIS IN HOMOZYGOUS AND HETEROZYGOUS WHHL RABBITS IN THE THORACIC AORTA, AORTIC ARCH AND CAROTID ARTERY.

4.1 INTRODUCTION.

4.1.1 Watanabe Heritable Hyperlipidaemic (WHHL) Rabbits.

In 1973, Yoshio Watanabe, a vet at Kobe University in Japan, noted that a rabbit in his colony had an abnormally elevated serum cholesterol level. The cholesterol level of this male rabbit was 11.6mM - more than ten times the normal level of the colony. Watanabe bred this animal with a normal female to produce a first filial generation, which had slightly elevated serum cholesterol levels. This litter were then bred with each other and also back crossed with the original male. The resulting offspring had a mixture of mild and severe hypercholesterolaemia. The segregation pattern of the cholesterol levels identified the disease as a simple mendelian trait with partial expression in the heterozygous state and complete expression in the homozygous state - a pattern similar to that of familial hypercholesterolaemia (Kondo and Watanabe, 1973).

The parallel with familial hypercholesterolaemia was not immediately recognised due to a disparity in the lipid profiles of these rabbits and humans with familial hypercholesterolaemia. Watanabe's rabbits were found not only to have elevated cholesterol levels but also elevated triglyceride levels (Watanabe, 1980). In the human condition of familial hypercholesterolaemia, triglyceride levels are found to be normal. This disparity is due to a difference between normal human and rabbit LDL. Normal human LDL has low triglyceride levels whereas normal rabbit LDL has high triglyceride levels. Thus, in rabbits an elevation in circulating LDL leads to an increase in the triglyceride level.

Early pathological studies in these homozygous WHHL rabbits revealed that atherosclerotic lesions developed in the aorta as early as three months of age,

progressing to diffuse aortic atherosclerosis by one year of age. After one year, tendon xanthomas were found to develop on the planar aspects of both the fore and hind limbs.

The parallel between familial hypercholesterolaemia and Watanabe's rabbits was recognised in 1980 following studies in cultured skin fibroblasts from the homozygous rabbits. These fibroblasts were shown to have less than 5% of the expected number of high affinity binding sites for LDL (ie. LDL receptors). The fibroblasts were also unable to degrade LDL or stimulate cholesterol esterification. Similarly, when ^{125}I-labelled LDL was injected into the homozygous rabbits, the clearance of cholesterol was very low (Tanzawa et al, 1980). These finding were confirmed by studies in membranes prepared from liver and adrenal glands from homozygous WHHL rabbits, which were shown to lack the characteristic high affinity receptors for LDL normally found in preparations from normal rabbits (Kita et al, 1981).

Further studies using cultured hepatocytes from homozygous WHHL rabbits demonstrated that these cells lacked the normal ability to degrade LDL at low concentrations in the culture medium (Attie et al, 1981).

These initial studies could not exclude the possibility that the observed lack of LDL receptor activity reflected a metabolic suppression of the receptor due to the elevated serum LDL levels. However, a genetic deficiency of the receptor was a more likely explanation for at least two reasons:- firstly, these animals did not have abnormal tissue cholesterol in organs such as the liver and secondly, because when hepatocytes from homozygote WHHL were cultured in lipoprotein deficient serum for 24 hours receptor activity remained undetectable.

In 1983, work was published reporting that the LDL receptor is synthesised in WHHL rabbits but that it is glycosylated at a markedly reduced rate (Schneider et al, 1983). It was shown that up to 25% of the receptor could be converted to the glycosylated form, yet binding of LDL to the receptor in cells or membranes remained undetectable. This finding led to the proposal that this abnormal receptor must bind LDL poorly - this finding is consistent with the demonstration that a human LDL receptor which has been similarly poorly glycosylated, is able to reach the cell surface. Here, it will react with a monoclonal antibody to the human LDL receptor, but it is unable to bind LDL efficiently.

Genetic studies were carried out in WHHL rabbits in order to identify the mutation responsible for the malfunction of the LDL receptor. In 1986, Yamamoto and colleagues showed an in-frame deletion of 12 nucleotides eliminated 4 amino acids at residues 115-118 of the mature rabbit protein. The normal rabbit LDL receptor protein contains 829 amino acids as deduced from its cDNA sequence. The four deleted amino acids (asp-gly-ser-asp) are part of a highly conserved sequence in the third of seven cysteine rich repeat units of the ligand binding domain of the receptor. These conserved sequences in the repeat units are negatively charged amino acids and are thought to be directly involved in the binding of apo B_{100} and apo E - the ligands for the LDL receptor. Western blot analysis showed that the receptor was present entirely in its poorly glycosylated form of M_r~100,000 compared with that of the normal mature rabbit LDL receptor protein of M_r~130,000. Northern blot analysis demonstrated that mRNA was present in amounts and distribution similar to normal rabbits in the adrenal gland, liver, lower intestine, testis, lung and kidney. These studies led to the conclusion that, although the receptor is translated in a normal manner, the deletion leads to abnormal folding. This could impair the transport and ligand binding properties of the receptor protein (Havel et al, 1989).

WHHL rabbits have a selective elevation of all lipoproteins containing apo B_{100} - one of the particles which is recognised and bound by the LDL receptor. Thus elevated levels of LDL, IDL and VLDL are found. The level of HDL is found to be decreased. The levels of chylomicrons and chylomicron remnants, the lipoproteins responsible for the transport of exogenous cholesterol, are found to be normal (Havel et al, 1982). Due to the lack of functional LDL receptors in WHHL rabbits, the clearance of IDL, LDL and VLDL is abnormally low - approximately one third of the normal value. However this is insufficient to account for the twenty fold increase in plasma LDL levels. There must also be a six fold over production of LDL. One possible explanation emerged from studies of the precursor-product relationship between VLDL and LDL (Kita et al, 1982). In normal rabbits VLDL is converted to IDL, more than 90% of this IDL is then cleared to the liver - a through receptor mediated endocytosis modulated by the LDL receptor. Hence, less than 10% of circulating IDL is actually converted to LDL (Figure 4.1). In WHHL rabbits, VLDL is converted to IDL normally. However, due to the functional deficit of LDL receptors, this IDL is not cleared from the plasma into the liver but remains in the circulation. Thus, there is an abnormally high level of IDL available for conversion to LDL by what is termed the "Shunt Pathway" (Figure 4.1). Thus the decrease in hepatic LDL receptor number leads to impaired catabolism and an increase in production of LDL.

However, turnover studies in WHHL rabbits or human with familial hypercholesterolaemia cannot reveal whether all the observed increase in LDL production is due to the shunt pathway or if there is some direct secretion of LDL into the circulation from another source.

Thus the WHHL rabbit is an appropriate animal model for studying the human

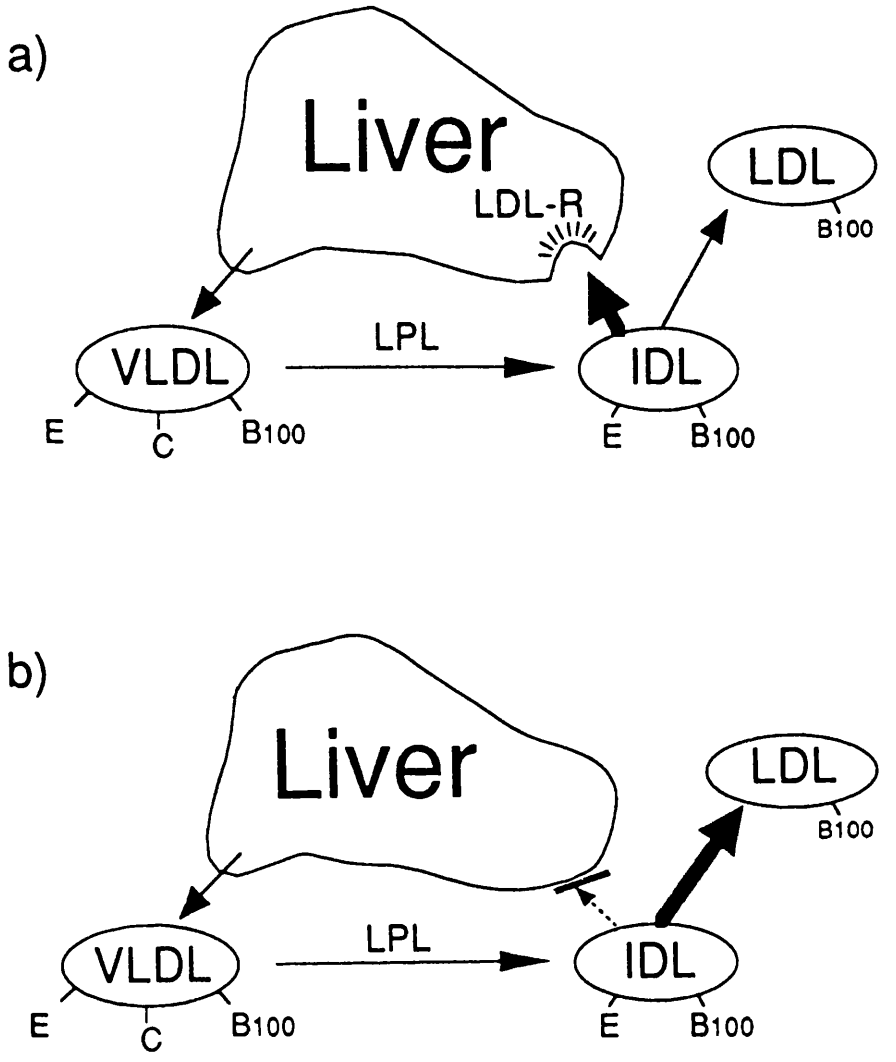


FIGURE 4.1. The Shunt Pathway. a) represents normal lipoprotein metabolism by the liver - a high proportion of IDL is cleared from the plasma by receptor mediated endocytosis modulated by the LDL receptor (LDL-R) in the liver. b) represents the LDL-R deficient state found in WHHL rabbits and human familial hypercholesterolaemia. In the absence of LDL-R IDL is converted to LDL in the plasma thus leading to an elevation in serum LDL levels. VLDL represents very low density lipoprotein; IDL - intermediate density lipoprotein; LDL - low density lipoprotein; LPL - lipoprotein lipase; E, C, B_{100} - are the apoproteins associated with the indicated lipoprotein particle.

condition of familial hypercholesterolaemia. The two disease states are qualitatively similar with regard to deficiency of functional LDL receptors and the resultant altered lipid profiles.

The differences between the two models reflect species differences between humans and rabbits. As discussed above, rabbits have a much higher proportion of triglyceride in LDL than humans, resulting in a hypertriglyceridaemia in WHHL rabbits but not in human familial hypercholesterolaemia. Other differences include the fact that, in normal humans, little IDL enters the liver when compared to rabbits - a large proportion escapes uptake and is converted to LDL, in familial hypercholesterolaemia even less IDL is taken up by the liver thus leading to an elevation in LDL production by the shunt pathway as discussed above; a decrease in hepatic lipase activity; a lower conversion of VLDL to LDL and an increase in activity of cholesterol ester transfer protein in rabbits (Ha and Barter, 1982). However, there are some important species similarities which are not found with some other mammalian species:- apo B_{100} is the major form of apo B secreted by the liver (Hornick et al, 1983), an active mechanism is present for the transfer of cholesterol esters between lipoprotein particles and the LDL receptor in the rabbit can be readily regulated by diet and drugs as in humans (Chao et al, 1982).

Since their discovery and development nearly twenty years ago, WHHL rabbits have been widely studied. Many different aspects that may be associated with the development of atherosclerosis have been investigated. A wide range of blood vessels have been used to assess changes in vasoactive function :- the aorta (Tagawa et al, 1991; Chinellato et al, 1991; Chinellato et al, 1992; Hirata et al, 1992; Kolodgie et al, 1990; Wines et al, 1989; Ragazzi et al, 1989), the basilar artery (Stewart-Lee et al, 1991a), the hepatic artery (Brizzolara et al, 1992), the superior mesenteric artery (Dalessandri et al, 1989, Stewart-Lee et al, 1991b) and the coronary, femoral and

carotid arteries (Yokoyama et al, 1983; Corr et al, 1993). Studies have also assessed changes in the structure of the vasculature - these studies have predominantly examined changes in the structure of the aorta (Fukuo et al, 1991; Daugherty et al, 1991; Wu et al, 1988; Nagano et al, 1992; Gallagher et al, 1988). The structure of the carotid, coronary and femoral arteries has also been examined (Giurato et al, 1993; Yokoyama et al, 1983).

Other studies, beyond the scope of this thesis, include work on such diverse topics as coagulation of platelets (Mori et al, 1989), effects of atherosclerosis on blood pressure (Katsuda et al, 1992) and energy metabolism and purine turnover (Pandolfo et al, 1989).

Previous studies have examined many aspects in WHHL rabbits, however, due to poor breeding and maintenance characteristics, WHHL rabbits are commonly studied in small groups. Alternatively, wide age bands are used to produce larger groups. In our study we have achieved reasonable n-numbers in most of the groups studied and used specific age points rather than broader age bands.

Most studies in WHHL rabbits have focused on the homozygote animals. In these animals cholesterol levels are dramatically elevated and thus any changes associated with the development of atherosclerosis would be clearly demonstrated. However these WHHL rabbits are studied as an animal model of the human condition of familial hypercholesterolaemia. Subjects with homozygote familial hypercholesterolaemia do occur in the population, but in these cases the hypercholesterolaemia is so overwhelming that coronary atherosclerosis develops before 20 years of age and transplant surgery is often the resulting treatment.

Heterozygote familial hypercholesterolaemia subjects are much more common - as

stated earlier occurrence is one in 500 of the population, with atherosclerosis developing around the age of 40-50. Hence, the study of heterozygous WHHL rabbits has a much greater clinical significance. If these animals are studied - the functional and pathological changes occurring can be documented. Therapeutic strategies can then be investigated and developed towards controlling the hypercholesterolaemia and minimising the development of disease.

4.2 AIMS.

The aims of this study were to provide an overview of the changes associated with the development and progression of atherosclerotic disease in WHHL rabbits. Three broad aspects of the disease were examined in this study:- Cholesterol levels, vascular function and structural changes, in both homozygous and heterozygous WHHL rabbits, between the ages of 3 and 12 months.

4.3 METHODS.

1. Cholesterol levels :- Cholesterol levels were measured at weaning (8-10 weeks), 3, 6, 9 and 12 months of age to provide a cholesterol profile for the duration of the study. On the basis of the results, the animals were allocated to one of two groups - if the serum cholesterol level was between 2 and 5mM the animals were designated as heterozygote, if the serum cholesterol was elevated above 10mM the animals were designated as homozygote (Figure 4.2).

2. Vascular function :- three arterial tissues were studied the thoracic aorta, the aortic arch and the carotid artery. These vessels were studied at four age points of 3, 6, 9 and 12 months. Isolated arterial rings were studied in organ bath experiments. The response to the α_1-adrenoceptor agonist phenylephrine was studied to assess

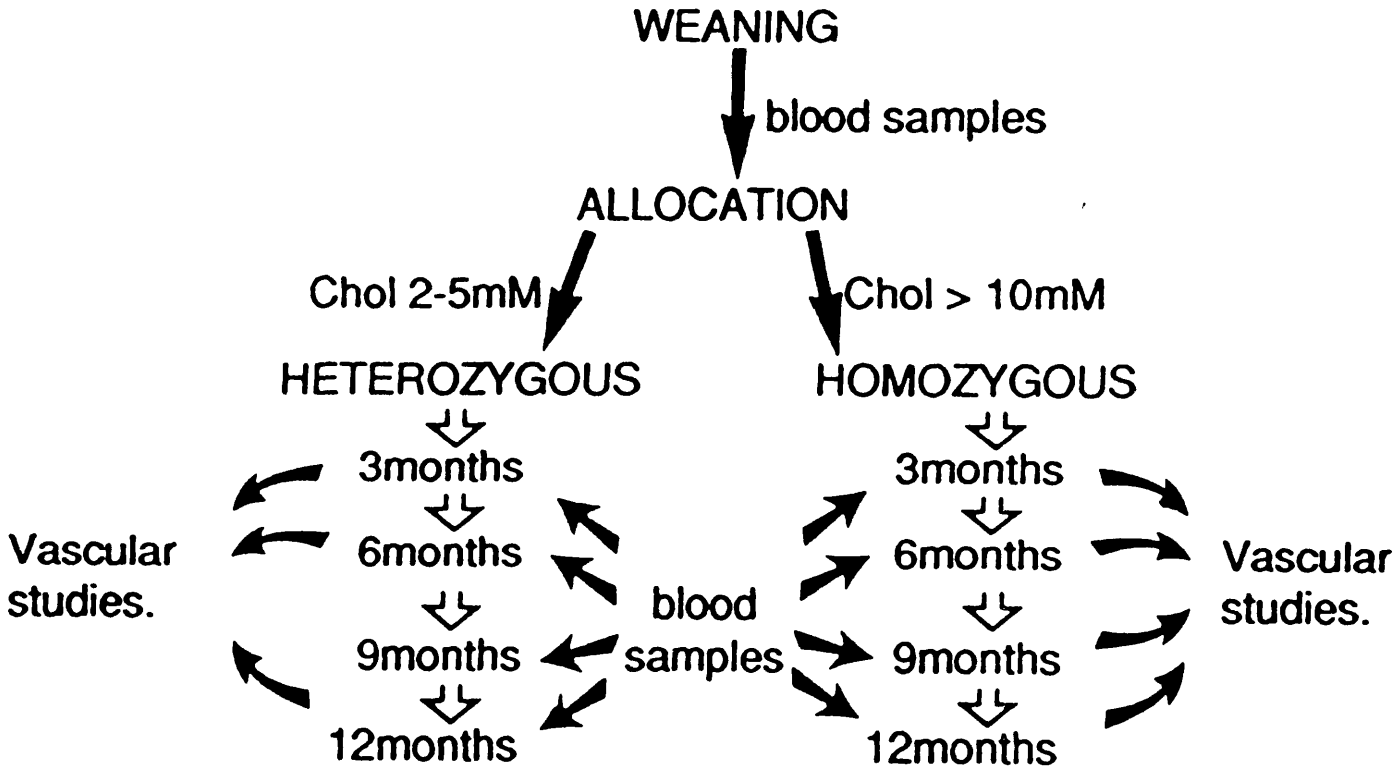


FIGURE 4.2 Flow chart depicting studies in WHHL rabbits. Chol represent Cholesterol.

contractile function of the arterial smooth muscle. The response to the muscarinic agonist carbachol was also examined in order to assess endothelium dependent relaxation of the arterial rings.

3. Structural development:- a range of histological techniques, both macro- and microscopic, were used to follow the development and progression of atherosclerosis in the thoracic aorta at the four age points of 3, 6, 9 and 12 months. Pathological changes in vascular structure were quantified using percentage sudan staining, percentage of the internal circumference involved in atheroma and the percentage ratio of the area of intima to the area of media, and a visual assessment of the development of atheroma was also conducted.

The above studies were carried out in both homozygous and heterozygous WHHL rabbits. New Zealand White rabbits were also studied to allow comparison with a normocholesterolaemic control rabbit strain.

Hence, this study has been conducted to provide a better understanding of the development and progression of atherosclerotic disease in heterozygote as well as homozygote WHHL rabbits.

4.4 RESULTS.

4.4.1 CHOLESTEROL LEVELS IN WHHL RABBITS.

The mean serum cholesterol levels at the age of 8-10 weeks of all the rabbit groups included in the study are shown in Figure 4.3. It can be seen that the homozygote WHHL rabbit cholesterol level is massively elevated when compared with the heterozygote WHHL rabbit cholesterol. When compared to the serum cholesterol

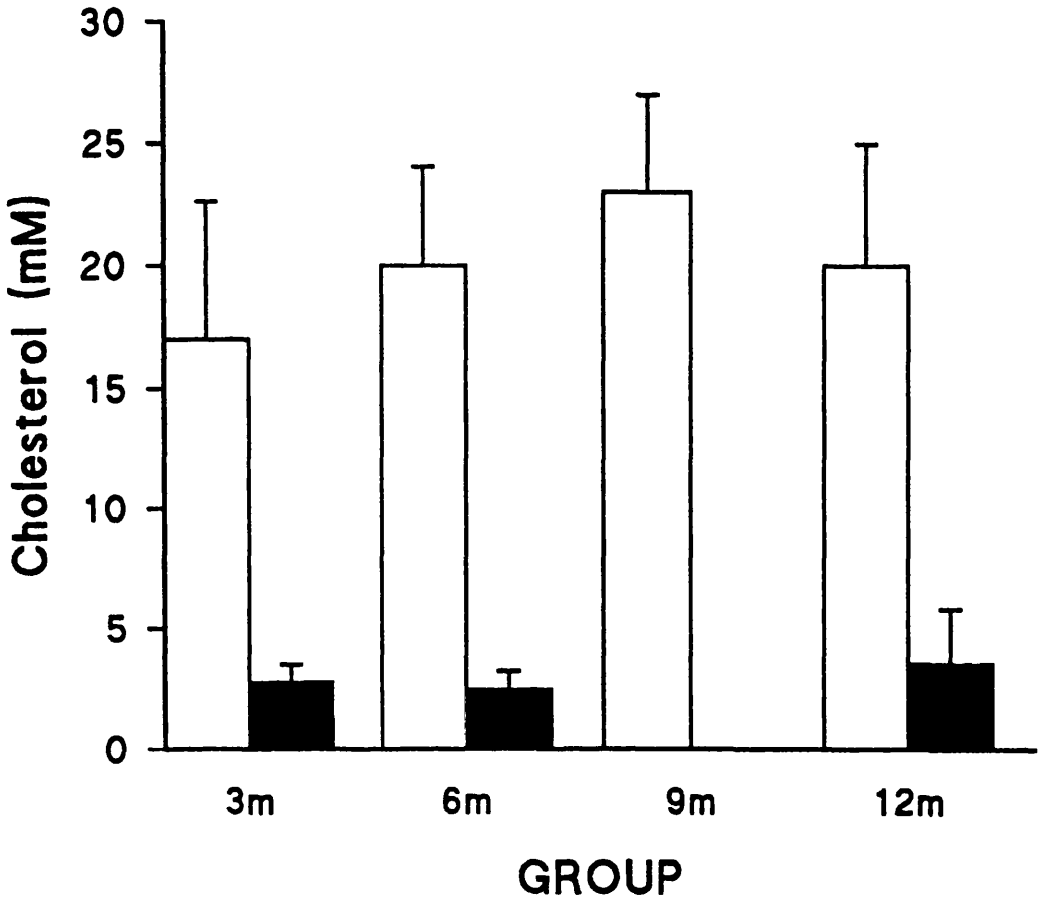


FIGURE 4.3. Serum cholesterol levels at the age of 8-10 weeks. Open bars are homozygous (hh) WHHL rabbits, solid bars are heterozygous (hH) WHHL rabbits. Data is mean ± s.d. n=4-7.

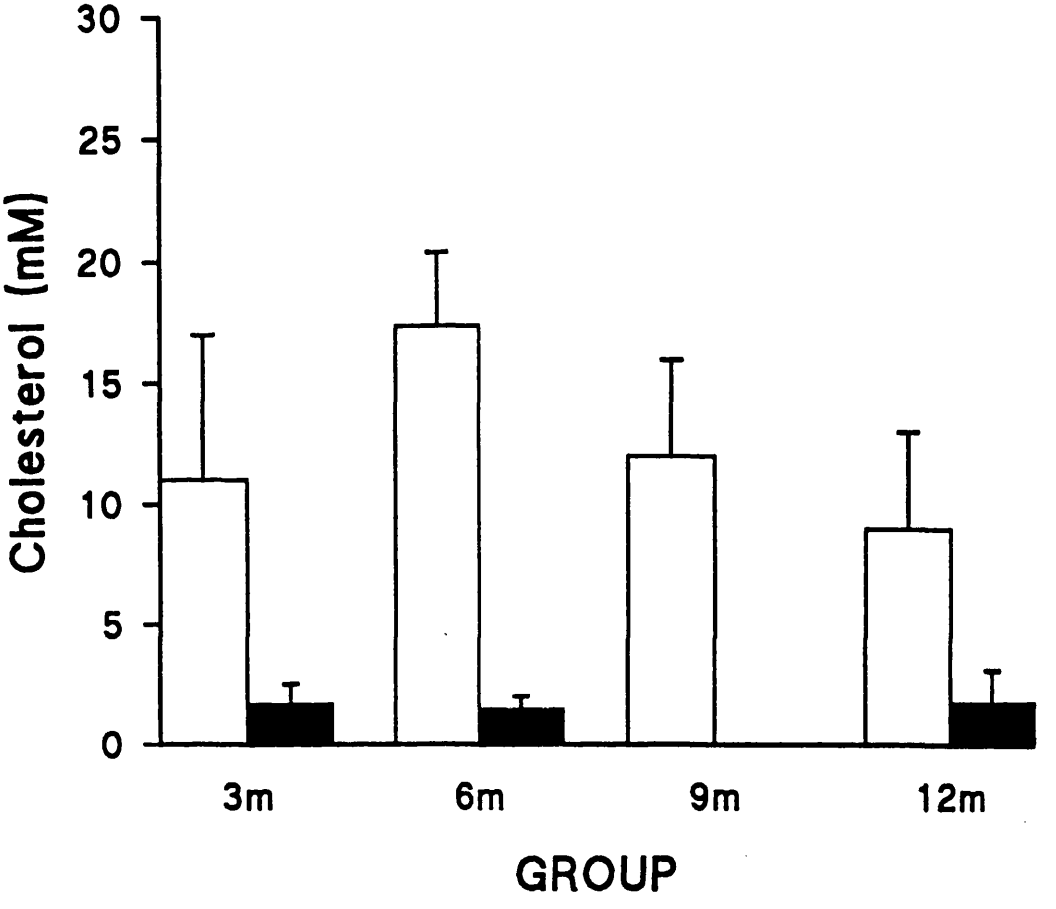


FIGURE 4.4. Serum cholesterol levels at the age of study. Open bars are homozygous (hh) WHHL rabbits, solid bars are heterozygous (hH) WHHL rabbits. Data is mean ± s.d. n=4-7.

levels found in New Zealand White rabbits, both the heterozygous and homozygous groups are significantly elevated. The cholesterol level at the age of 8-10 weeks did not statistically differ between the four homozygous WHHL groups studied. Nor was there any statistically significant difference in initial serum cholesterol levels between the three groups of heterozygous WHHL rabbits studied.

Cholesterol levels were also measured at the time that each rabbit was included in the study (Figure 4.4). When compared with Figure 4.3 it can be seen that serum cholesterol levels fall with age in both the homozygous and heterozygous WHHL rabbits. There is an apparently large fall in serum cholesterol levels, between weaning and study, in the 3 month old homozygote rabbit group. This reflects the fact that the three month old homozygous group had a slightly lower initial serum cholesterol level, and unfortunately is relatively small. This is due to the fact that, for practical reasons, the 3 month group were not started until towards the end of the study and due to problems with breeding and rearing of the rabbits we had a limited number of rabbits from which to select those for inclusion in the study.

4.4.2 FUNCTIONAL STUDIES IN THE THORACIC AORTA OF WHHL RABBITS.

A. Changes in Phenylephrine Induced Contraction with Increasing Age.

The response of the thoracic aorta to the α_1 adrenoceptor agonist phenylephrine was examined in rings from both homozygous and heterozygous WHHL rabbits.

In rings of thoracic aorta from homozygous WHHL, there was a progressive decrease in the level of tension obtained in response to phenylephrine as the age of the animals increased. Looking first at absolute levels of contraction observed

(Figure 4.5). It can be seen that there is a small decrease in response to phenylephrine associated with increasing age. There was no statistically significant decrease in response from 3 to 6 months of age, however by the age of 9 months there was a significant decrease in the level of contraction at 10^{-7}M and 3 x 10^{-7}M phenylephrine. At the age of 12 months, there was a significant difference at 10^{-7}M phenylephrine when compared to the 3 month group.

When studying this type of response, it is more usual to express the results in grams tension / mg tissue, to take into account the relative amount of muscle contributing to the contraction. If the results are expressed this way, there is a more dramatic change in response associated with age (Figure 4.6). There was a statistically significant decrease in the levels of contraction in response to phenylephrine from the age of 3 months to the age of 6 months. The difference was statistically significant from 10^{-7}M to 10^{-5}M. There was a further decrease in the level of contraction at both 9 and 12 months of age.

In rings of thoracic aorta from heterozygous WHHL, there was also a decrease in contractility associated with increasing age. As with the responses of the homozygous rabbits, the contraction can be expressed in two ways. Again, looking initially at the absolute level of contraction measured, there was a decrease in response associated with increasing age (Figure 4.7), this difference was found to be statistically significant by the age of 6 months. Significant differences were found at 10^{-7}M and 3 x 10^{-7}M when the 6 month group were compared to the 3 month group. There was no further change by the age of 12 months.

A similar pattern is seen when the results are expressed in grams tension / mg tissue (Figure 4.8). There was a statistically significant decrease in response to phenylephrine from the age of 3 to 6 months. This was found to be marginally

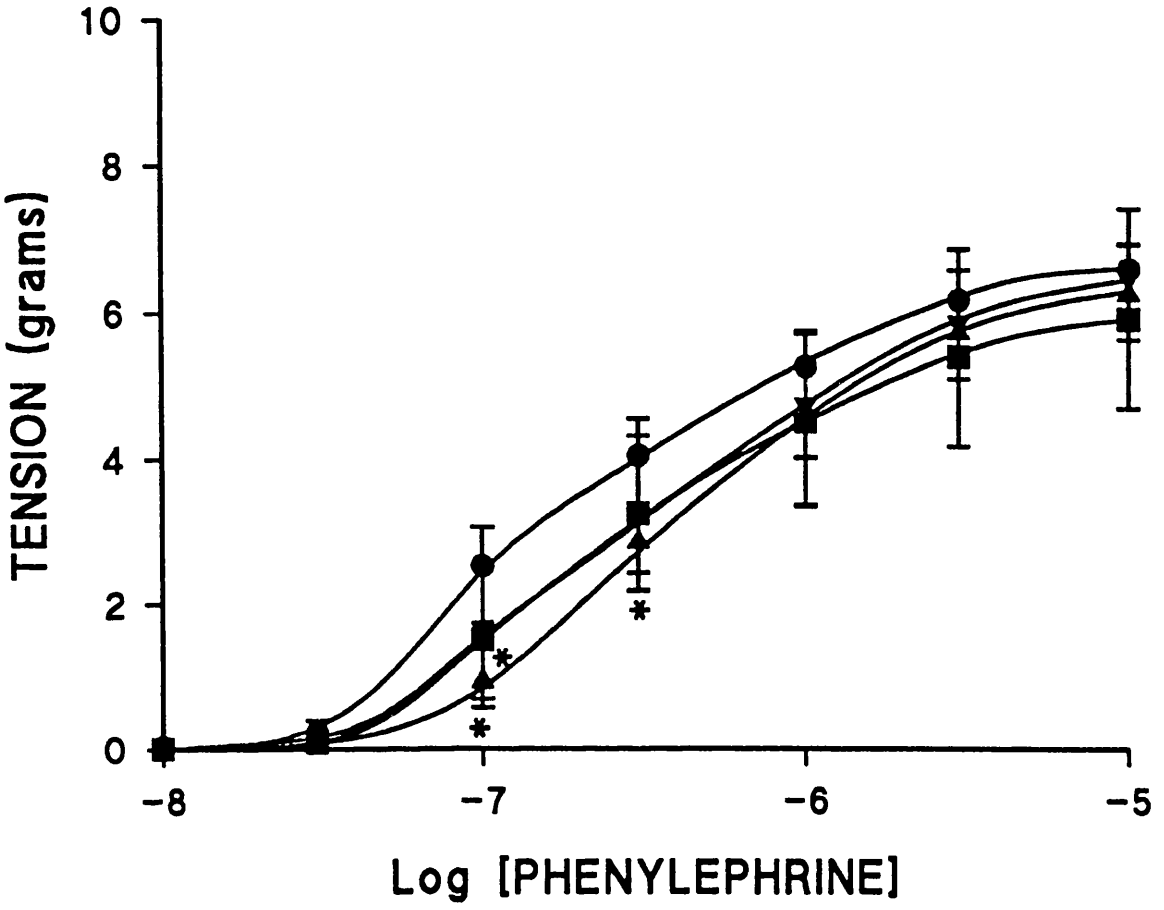


FIGURE 4.5. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed in grams tension. Data is mean ± s.d.

● - 3 months. n=4
▼ - 6 months. n=5
▲ - 9 months. n=6
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

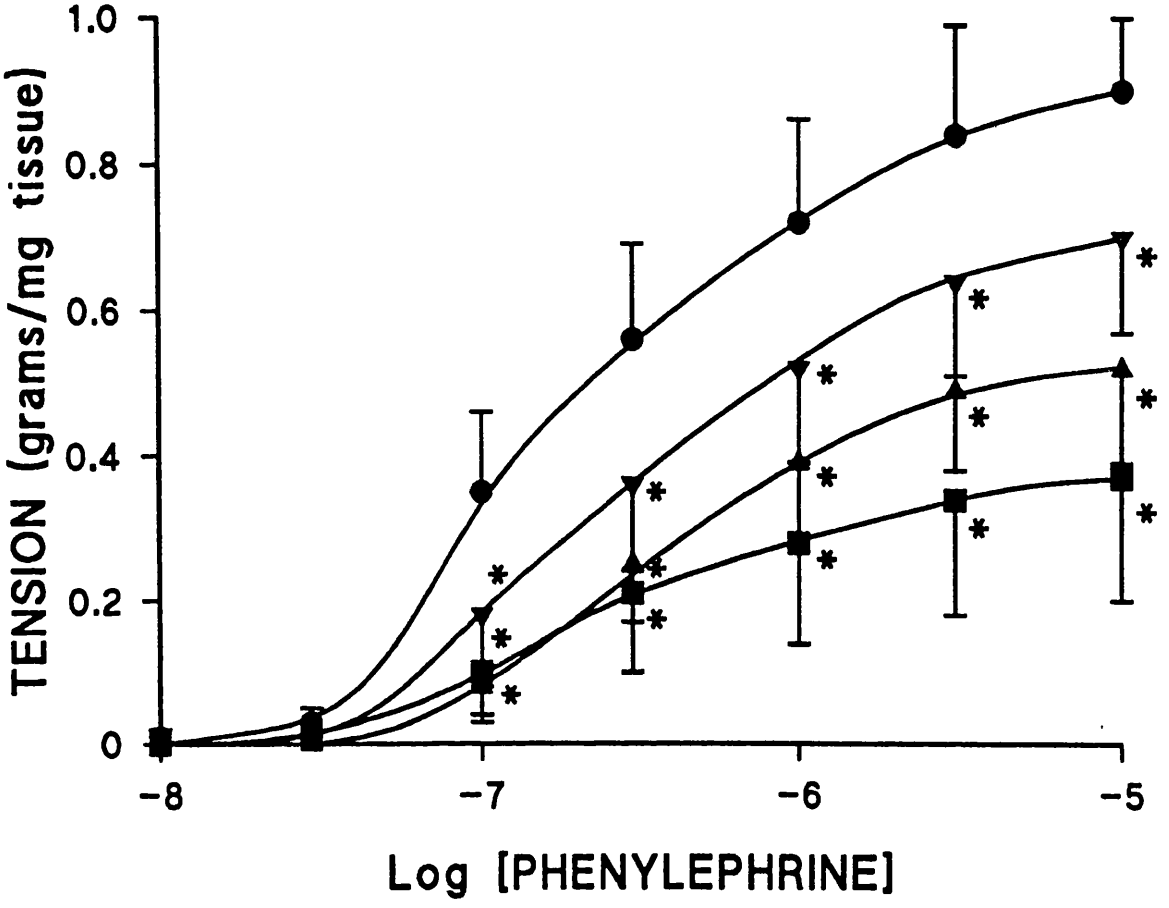


FIGURE 4.6. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months.	n=4
▼ - 6 months.	n=5
▲ - 9 months.	n=6
■ - 12 months.	n=7

* - $p < 0.05$ compared to 3 month group.

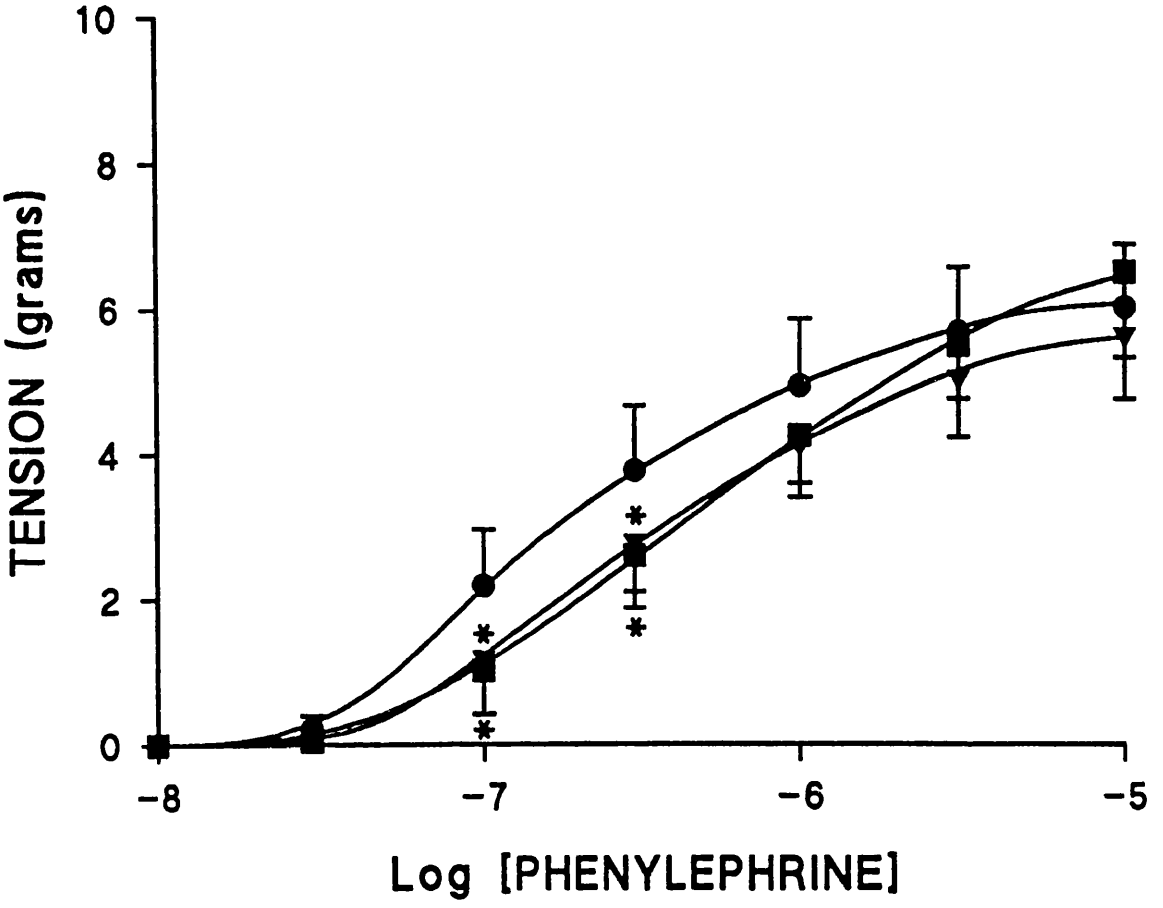


FIGURE 4.7. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed in grams tension. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

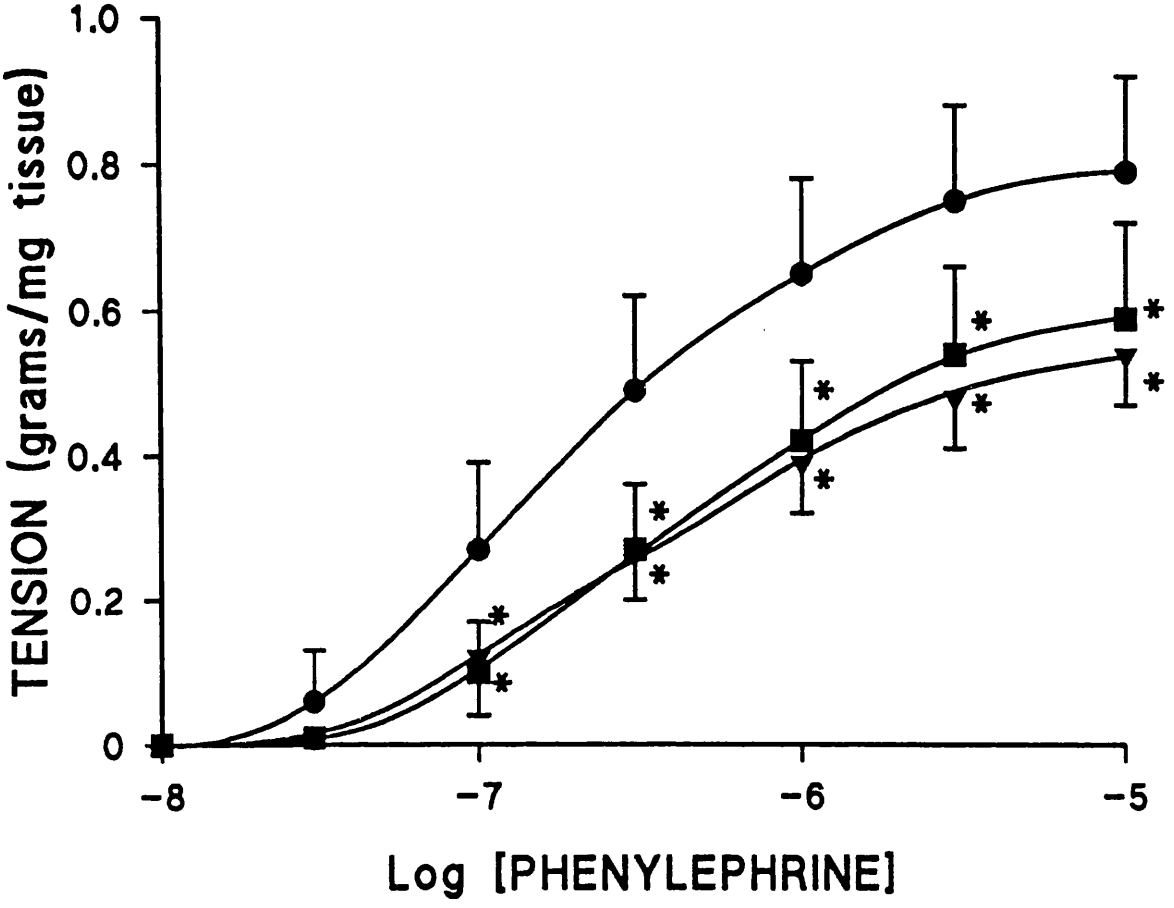


FIGURE 4.8. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

significant at 10^{-7}M phenylephrine 95%, C.I. (-0.249,-0.059) and more clearly significant from 3 x 10^{-7}M to 10^{-5}M. There was no further change in the concentration response curve to phenylephrine from the age of 6 to 12 months.

In order to allow comparison with a "normal control", rings of thoracic aorta from New Zealand White rabbits were also studied. When the absolute levels of contraction are analysed, it can be seen that there is a significant decrease in phenylephrine induced contraction from 3 to 6 months of age (Figure 4.9). Significant differences were found from 10^{-7}M to 10^{-5}M. There was little further change by the age of 12 months, where analysis showed significant differences, from 10^{-7}M to 10^{-5}M phenylephrine, when compared with the 3 month group.

When the responses were expressed in grams tension / mg tissue these differences were found to be similar. There was a small decrease in the levels of contraction in the thoracic aorta in response to phenylephrine through out the concentration response curve as the age of the New Zealand White rabbits increased (Figure 4.10). By the age of 6 months, this difference was found to be statistically significant at 10^{-7}M, 3 x 10^{-7}M, 3 x 10^{-6}M and 10^{-5}M when compared to the response of the 3 month group. The difference was marginally non-significant at 10^{-6}M, 95% C.I. (-0.154,0.008). By the age of 12 months significant differences were observed from 10^{-7}M to 10^{-5}M phenylephrine when compared with the response at 3 months. This change in phenylephrine induced contraction is taken to be representative of normal maturation.

B. Changes in Endothelium Dependent Relaxation with Increasing Age.

Carbachol induced endothelium dependent relaxation was examined in rings from homozygous WHHL rabbit thoracic aorta. There was a progressive loss of the ability

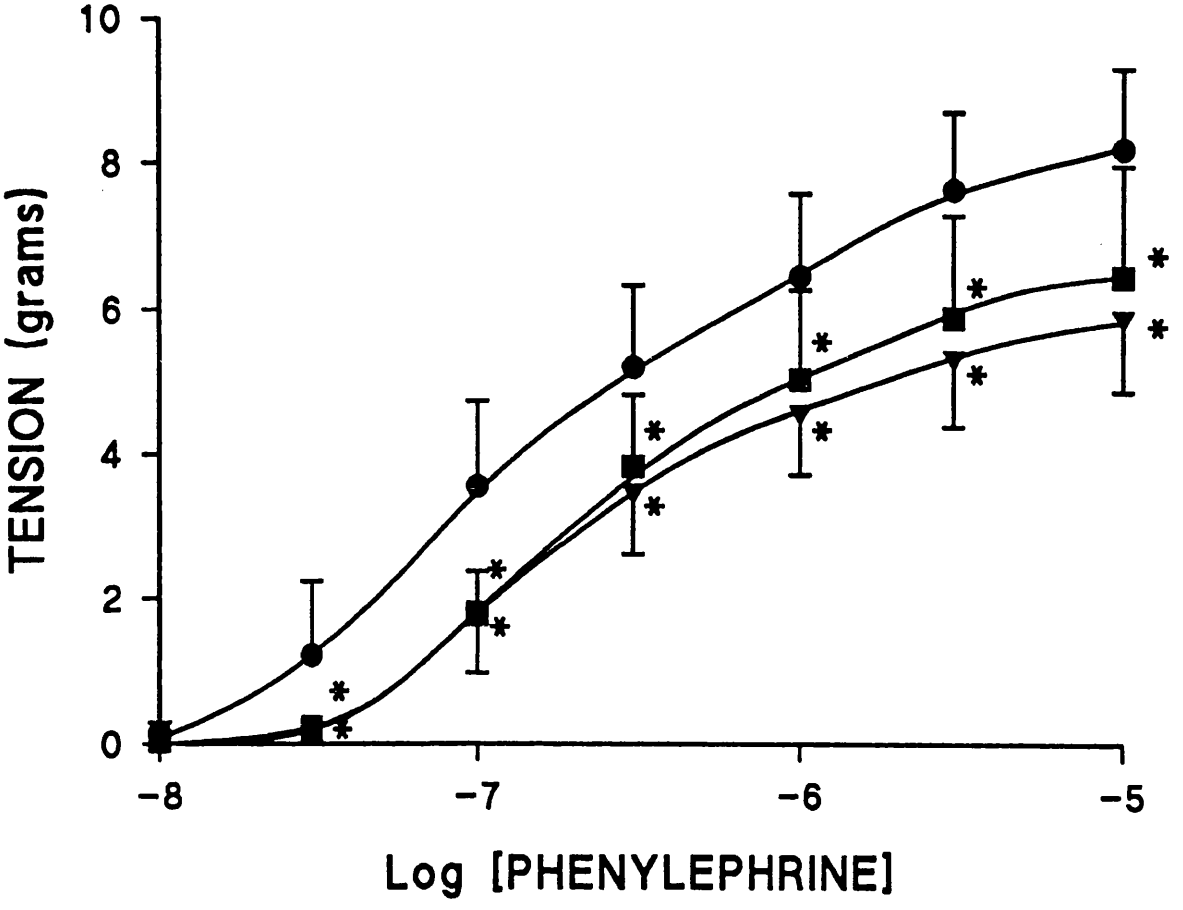


FIGURE 4.9. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

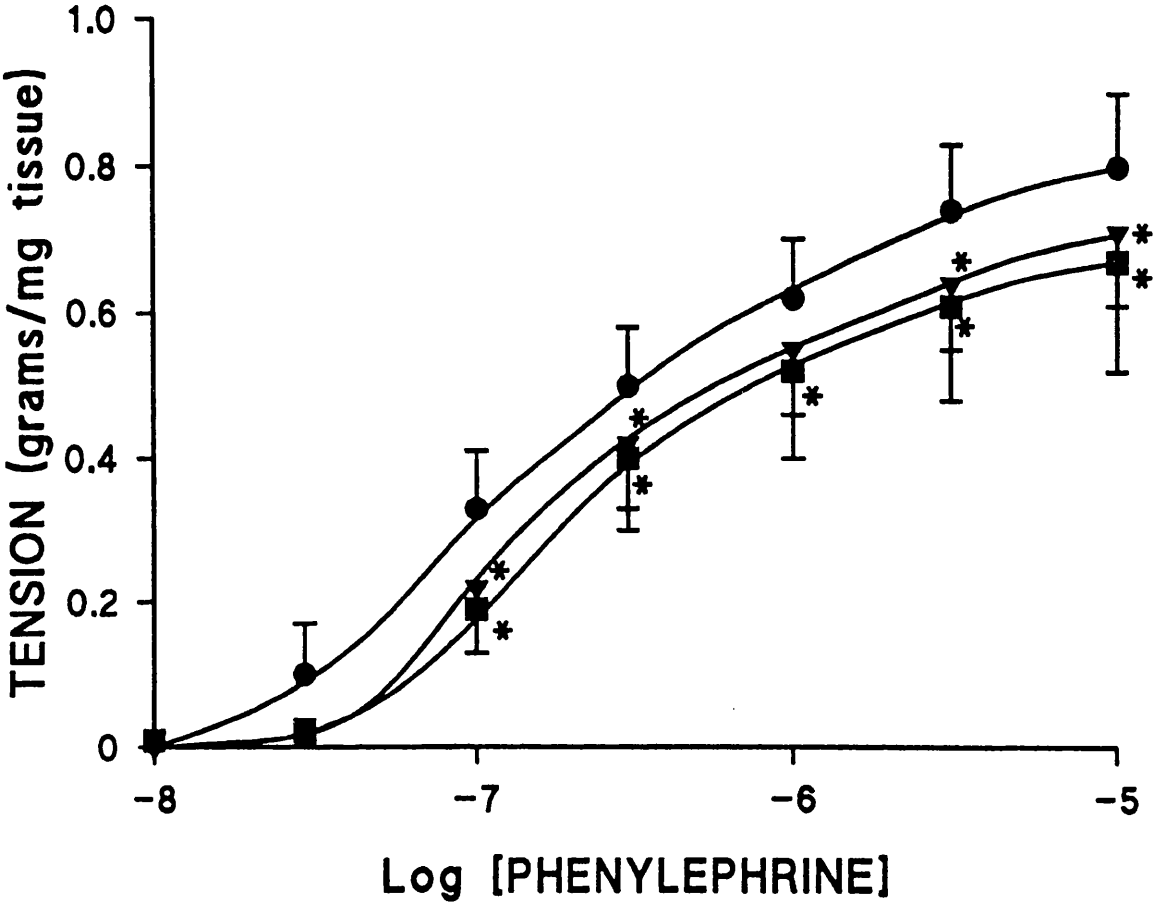


FIGURE 4.10. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

of this tissue to respond to carbachol as the age of the animal increased (Figure 4.11). The decrease in relaxation observed from 3 to 6 months of age was marginally significant at 10^{-6}M and 3 x 10^{-6}M carbachol, 95% C.I. (-40.701,-0.098 and -41.544,-0.941 respectively), and marginally non-significant at 10^{-5}M carbachol, 95% C.I. (-37.721,2.882). By 9 months of age, the response was statistically significantly decreased from 10^{-6} - 10^{-5}M when compared to the response of the 3 month homozygous WHHL. There was a slight progressive decrease to 12 months of age, at which point the responses were also significantly different from those of the 3 month old WHHL rabbits from 10^{-6}M - 10^{-5}M carbachol.

At all ages, rings of thoracic aorta from homozygous WHHL rabbits were able to relax fully to 10^{-4}M sodium nitroprusside.

Carbachol induced endothelium dependent relaxation was also examined in heterozygous WHHL rabbit thoracic aorta. As with the homozygous animals there was a decrease in the ability of the rings to relax in response to carbachol with increasing age (Figure 4.12). The decrease in vasorelaxation in response to carbachol at 6 months of age was found to be statistically significant at 3 x 10^{-7}M and 10^{-6}M and marginally non-significant at 3 x 10^{-6}M and 10^{-5}M, 95% C.I. (-25.864,0.549 and -22.944,3.469 respectively), when compared to the 3 month group. There was little further change in the response of the 12 month old group which was found to be significantly different to the 3 month old group at 10^{-6}M carbachol.

As with the homozygous animals all rings retained the ability to relax 100% when stimulated by 10^{-4}M sodium nitroprusside.

Due to the absence of any difference between the 6 month and 12 month

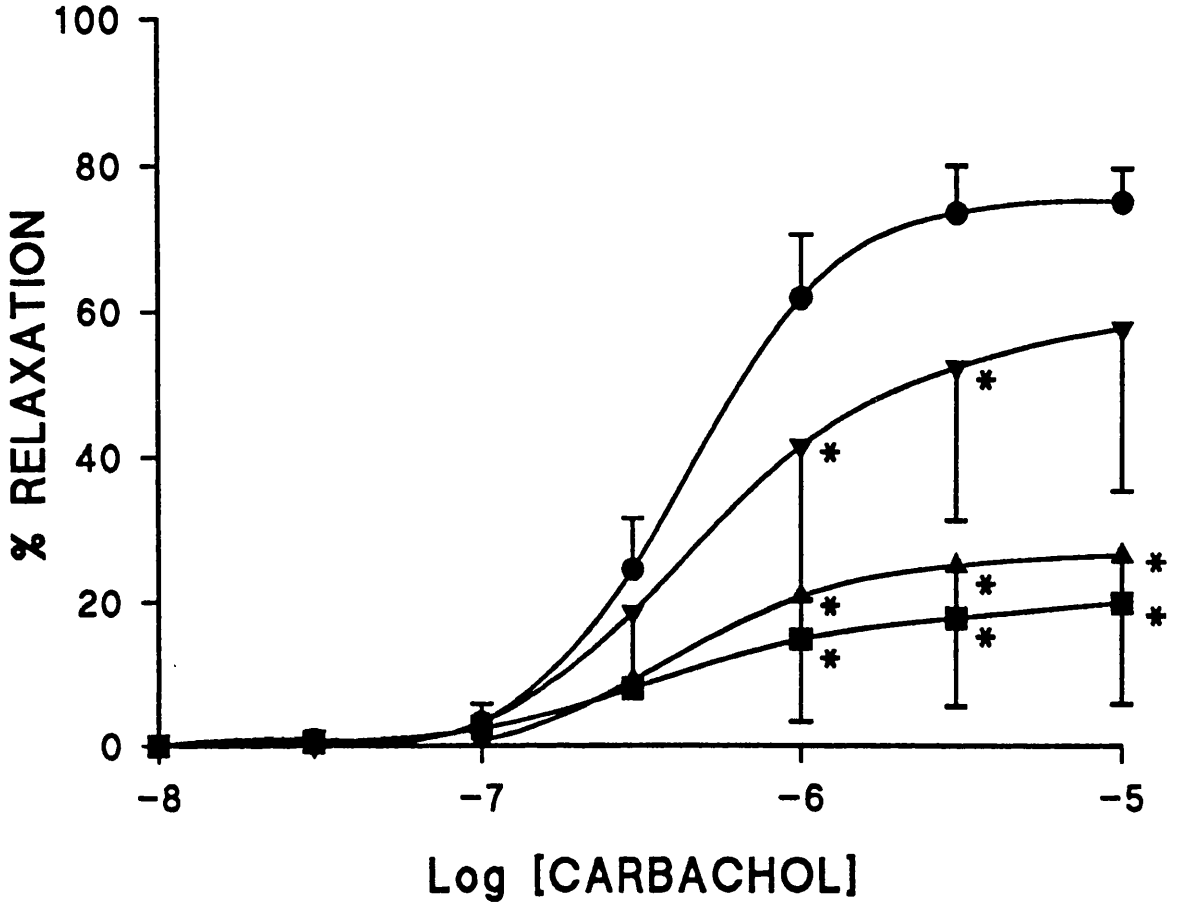


FIGURE 4.11. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=4
▼ - 6 months. n=5
▲ - 9 months. n=6
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

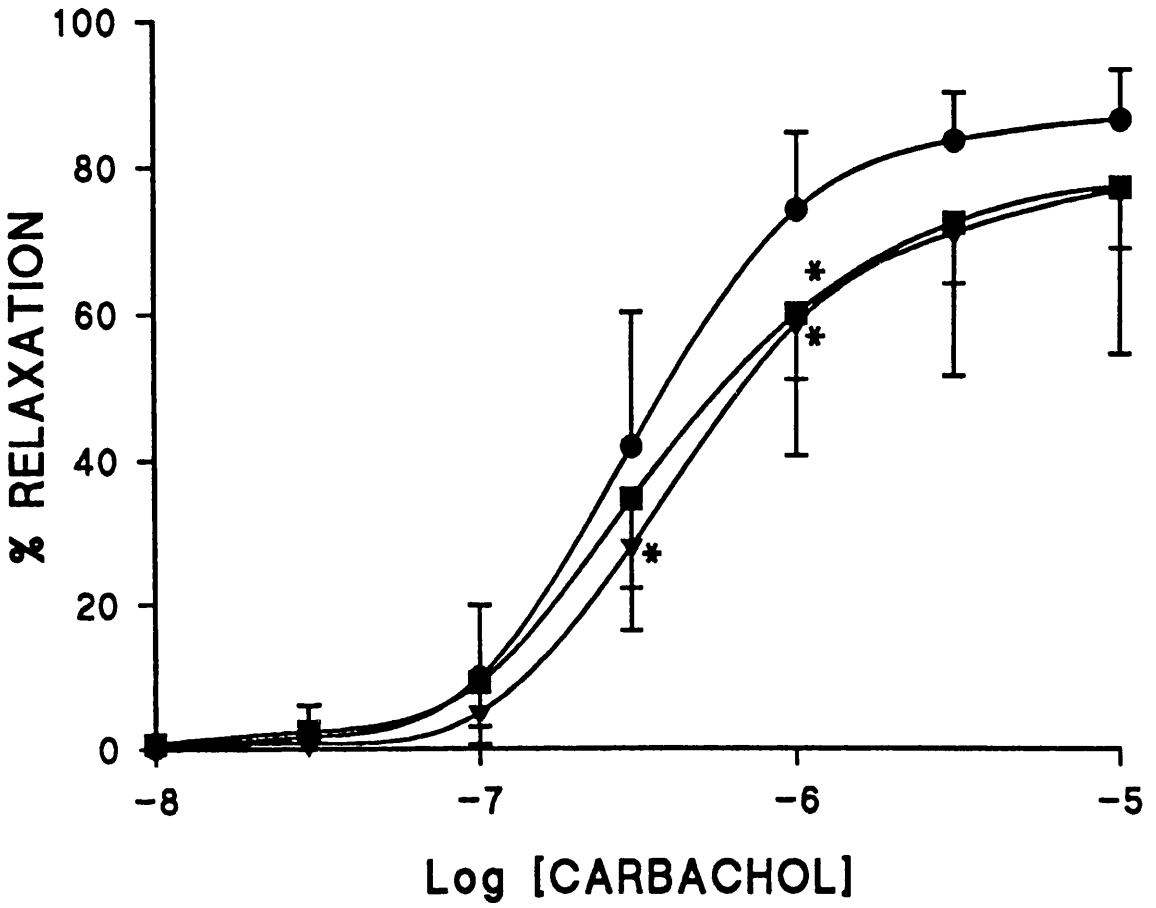


FIGURE 4.12. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

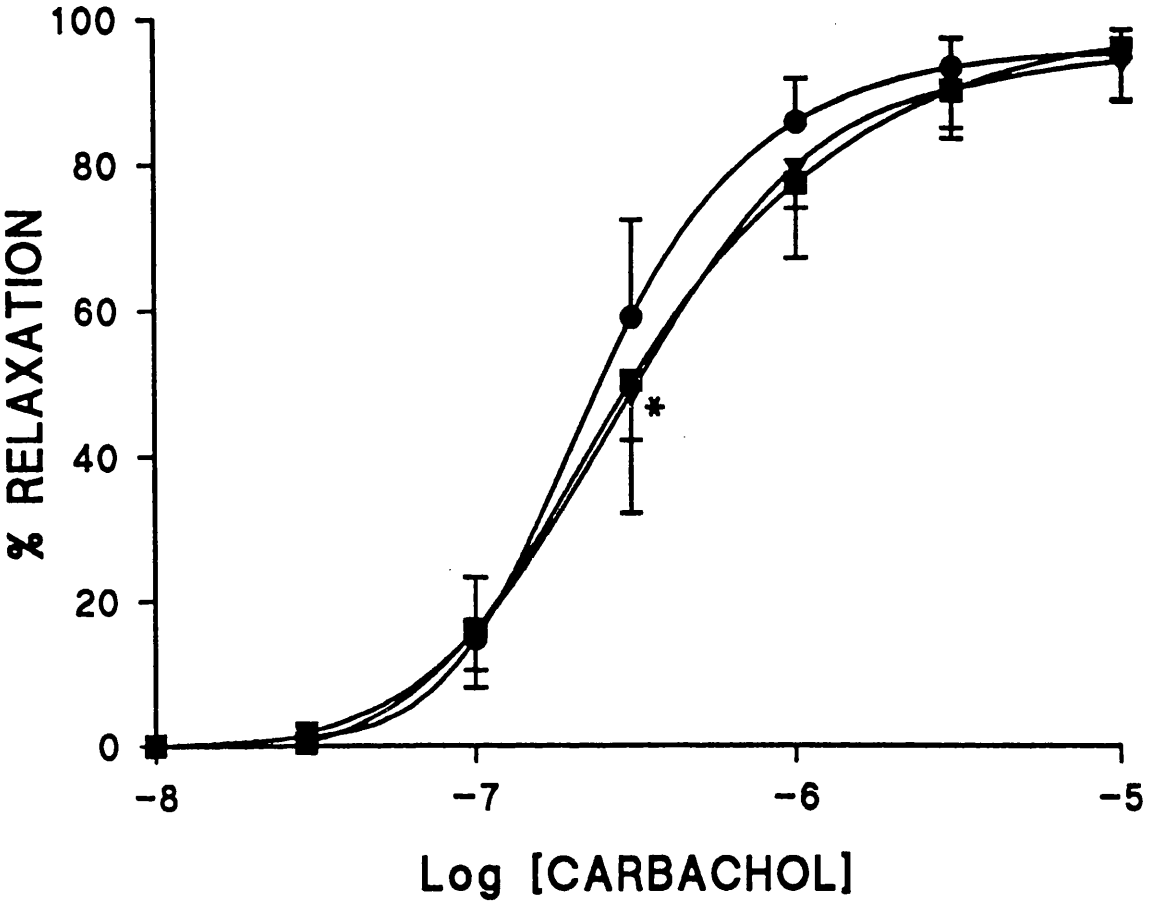


FIGURE 4.13. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

heterozygous groups a 9 month old heterozygous group was not studied.

There was little or no change in carbachol induced endothelium dependent relaxation in New Zealand White rabbits of 3, 6 and 12 months of age (Figure 4.13). There was only one statistically significant difference found between the responses from 3 and 6 month old New Zealand White rabbits at 3 x 10^{-7}M carbachol. No statistically significant difference were found between responses from the 3 and 12 month old groups.

C. Effects of Disease Status on Phenylephrine Induced Contraction.

In order to study the relative effects of the genetic LDL-receptor deficiency in WHHL rabbits the response to phenylephrine was compared in control New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at three age points:- 3, 6 and 12 months.

i. Comparison between phenylephrine induced contraction in New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at 3 months of age.

There were no statistically significant differences between the phenylephrine concentration response curves for the rings of thoracic aorta from the control new Zealand White rabbits, the heterozygous WHHL rabbits and the homozygous WHHL rabbits at the age of three months (Figure 4.14). However, from 10^{-7}M - 10^{-5}M phenylephrine, the responses from homozygous WHHL thoracic aorta were consistently higher than those from either the control New Zealand White rabbits or the heterozygous WHHL rabbits.

ii. Comparison between phenylephrine induced contraction in New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at 6 months of age.

At the age of 6 months there was no statistically significant difference in the responses of the thoracic aorta rings to phenylephrine between the control New Zealand White rabbits and the homozygous WHHL rabbits. The responses of the rings of thoracic aorta from heterozygous WHHL rabbits were consistently lower than those from both the control New Zealand White rabbits and the homozygous WHHL rabbits (Figure 4.15). The difference between the New Zealand White rabbit and the heterozygous WHHL rabbit responses were statistically significant from 10^{-7}M to 10^{-5}M phenylephrine. The difference between the homozygous WHHL rabbit and heterozygous WHHL rabbit responses were statistically significant from 3 x 10^{-7}M to 10^{-5}M phenylephrine.

iii. Comparison between phenylephrine induced contraction in New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at 12 months of age.

At the age of 12 months, the response of rings of thoracic aorta from the heterozygous WHHL rabbits to phenylephrine were consistently less than those from New Zealand White rabbits throughout the concentration response curve, however, this difference was only statistically significant at one concentration (3 x 10^{-7}M) (Figure 4.16). The responses of rings of thoracic aorta from homozygous WHHL to phenylephrine were consistently less than the responses from both the heterozygous WHHL rabbits and the New Zealand White rabbits. The differences between the phenylephrine induced contraction in thoracic aorta rings from New Zealand White rabbits and homozygous WHHL rabbits was statistically significant from 10^{-7}M to

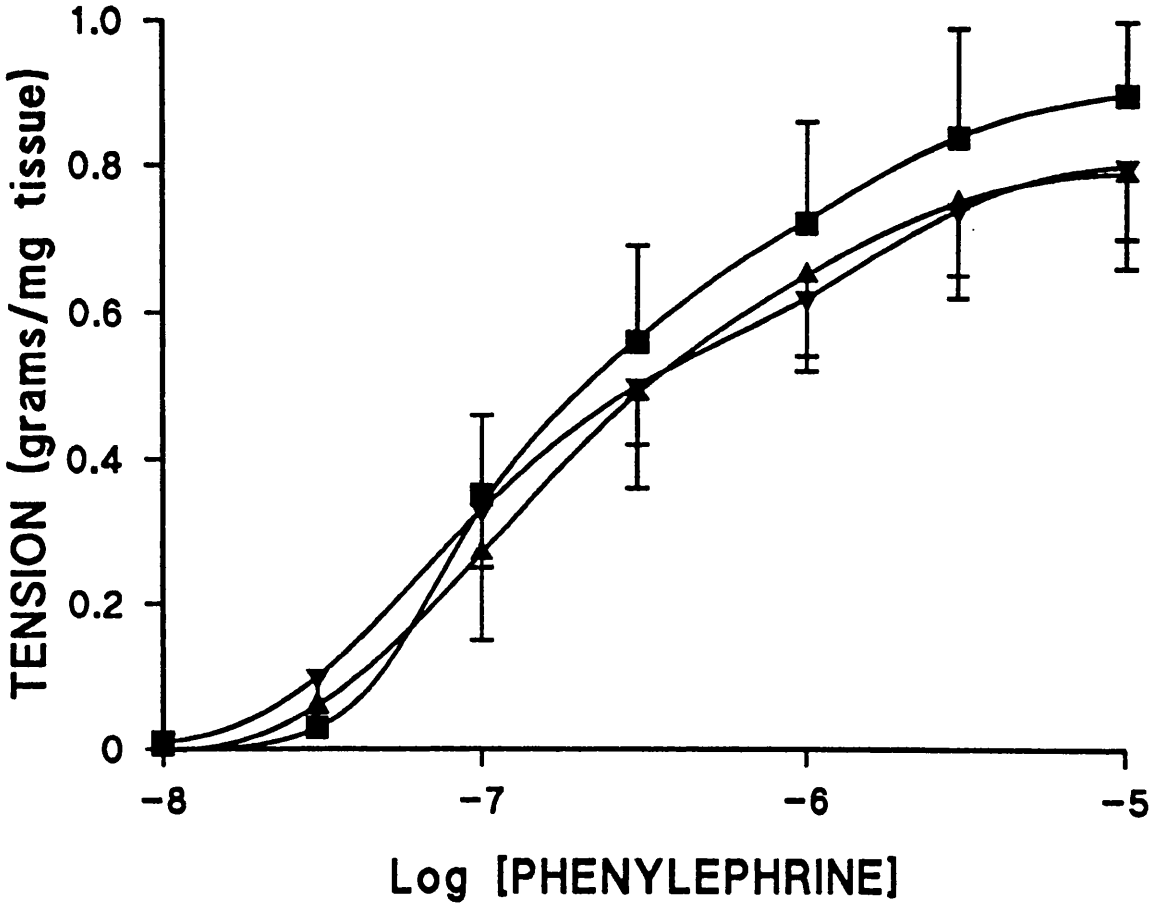


FIGURE 4.14. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from 3 month old rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

■ - homozygote WHHL. n=4
▲ - heterozygote WHHL. n=7
▼ - New Zealand White. n=7

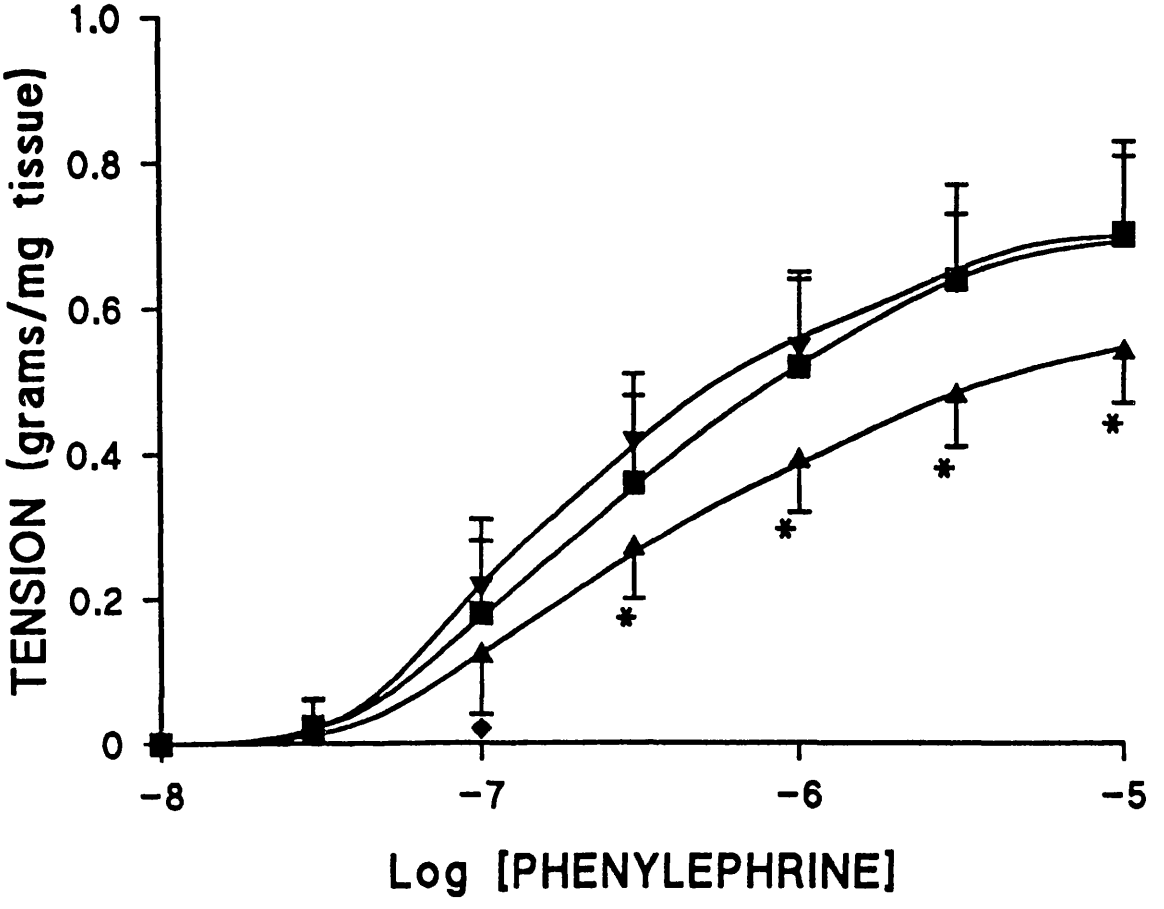


FIGURE 4.15. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from 6 month old rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

■ - homozygote WHHL. n=5
▲ - heterozygote WHHL. n=7
▼ - New Zealand White. n=7

* - $p < 0.05$ compared to New Zealand White.
◆ - $p < 0.05$ compared to homozygote WHHL.

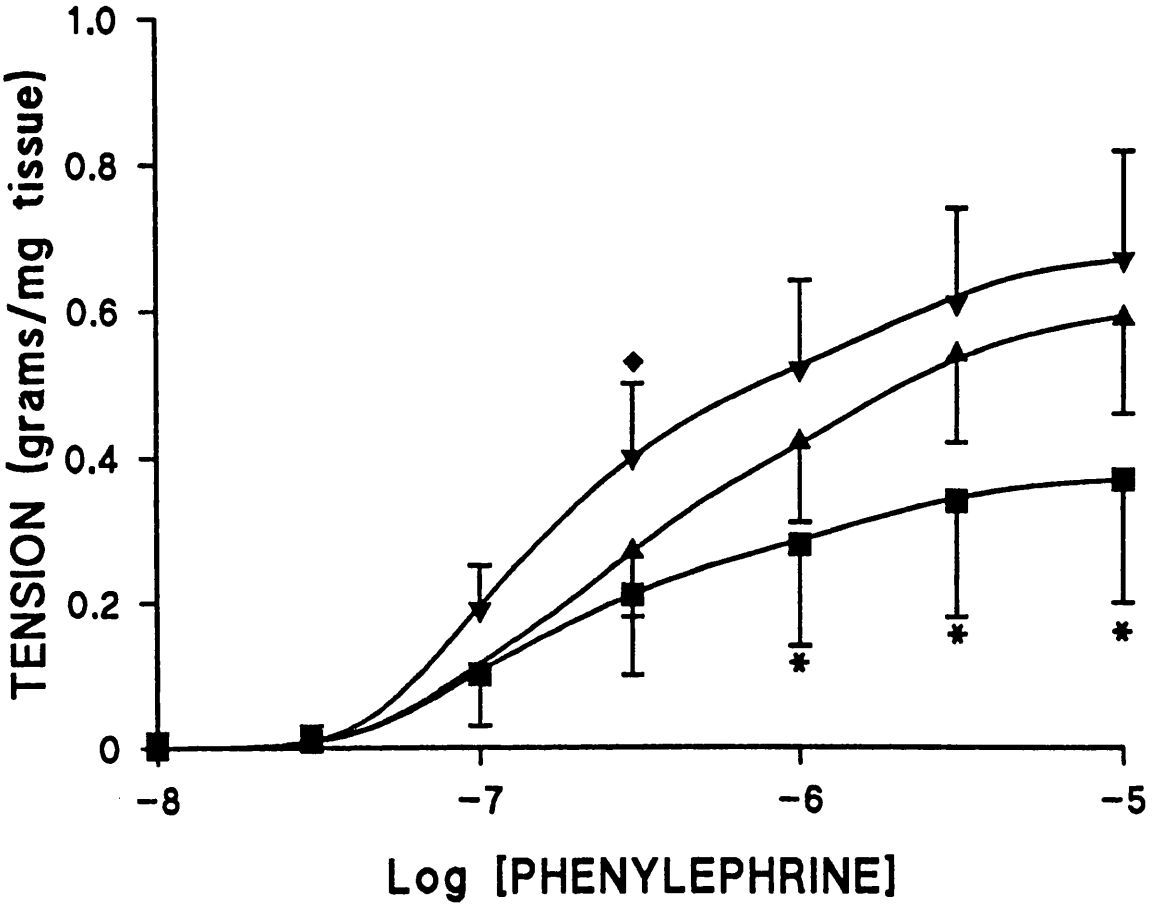


FIGURE 4.16. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from 12 month old rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

■ - homozygote WHHL. n=7
▲ - heterozygote WHHL. n=7
▼ - New Zealand White. n=7

* - $p < 0.05$ compared to New Zealand White.
◆ - $p < 0.05$ compared to heterozygote WHHL.

10^{-5}M phenylephrine. In rings of thoracic aorta from heterozygous WHHL rabbits and homozygous WHHL rabbits the difference was statistically significant from 10^{-6}M to 10^{-5}M phenylephrine.

D. Effects of Disease Status on Endothelium Dependent Relaxation.

In order to examine the relative effects of the genetic defects in WHHL rabbits carbachol induced endothelium dependent relaxation in control New Zealand White rabbits, the heterozygous WHHL rabbits and homozygous WHHL rabbits was compared at three age points:- 3 months, 6 months and 12 months.

i. Comparison between New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at 3 months of age.

The responses of the 3 months old heterozygous WHHL rabbits were consistently less than the 3 month New Zealand White rabbits throughout the concentration response curve. Relaxation in the 3 month old heterozygote rabbit aorta was significantly less than that in the 3 month old New Zealand White rabbit aorta at 3 x 10^{-7}M, 10^{-6}M and 3 x 10^{-6}M carbachol. The responses from the 3 month old homozygous WHHL rabbits were consistently less than both the 3 month New Zealand White control rabbits and the 3 month heterozygous WHHL rabbits throughout the concentration response curve. Relaxation in the 3 month old homozygote WHHL rabbit aorta was significantly less than that in aorta from 3 month New Zealand White rabbits from 10^{-7}M through to 10^{-5}M carbachol, and significantly less than relaxation of rings of thoracic aorta from 3 month old heterozygote WHHL rabbits at 3 x 10^{-7}M, 10^{-6}M and 10^{-5}M carbachol (Figure 4.17).

ii. Comparison between New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at 6 months of age.

The responses of the 6 month old heterozygous WHHL rabbits were consistently less than the 6 month New Zealand White rabbits throughout the concentration response curve. Relaxation in the 6 month old heterozygote rabbit aorta was significantly less than that in the 6 month old New Zealand White rabbit aorta from 10^{-7}M through to 10^{-5}M carbachol. The responses from the 6 month old homozygous WHHL rabbits were consistently less than both the 6 month New Zealand White control rabbits and the 6 month heterozygous WHHL rabbits throughout the concentration response curve. Relaxation in the 6 month old homozygote WHHL rabbit aorta was significantly less than that in aorta from 6 month New Zealand White rabbits from 10^{-7}M through to 10^{-5}M carbachol, and significantly less than relaxation of rings of thoracic aorta from 6 month old heterozygote WHHL rabbits from 10^{-6}M through to 10^{-5}M carbachol (Figure 4.18).

iii. Comparison between New Zealand White rabbits, heterozygous WHHL rabbits and homozygous WHHL rabbits at 12 months of age.

The responses of the 12 months old heterozygous WHHL rabbits were consistently less than the 12 month New Zealand White rabbits throughout the concentration response curve. Relaxation in the 12 month old heterozygote rabbit aorta was significantly less than that in the 12 month old New Zealand White rabbit aorta from 10^{-6}M through to 10^{-5}M carbachol. The responses from the 12 month old homozygous WHHL rabbits were consistently less than both the 12 month New Zealand White control rabbits and the 12 month heterozygous WHHL rabbits throughout the concentration response curve. Relaxation in the 12 month old homozygote WHHL rabbit aorta was significantly less than that in aorta from 12

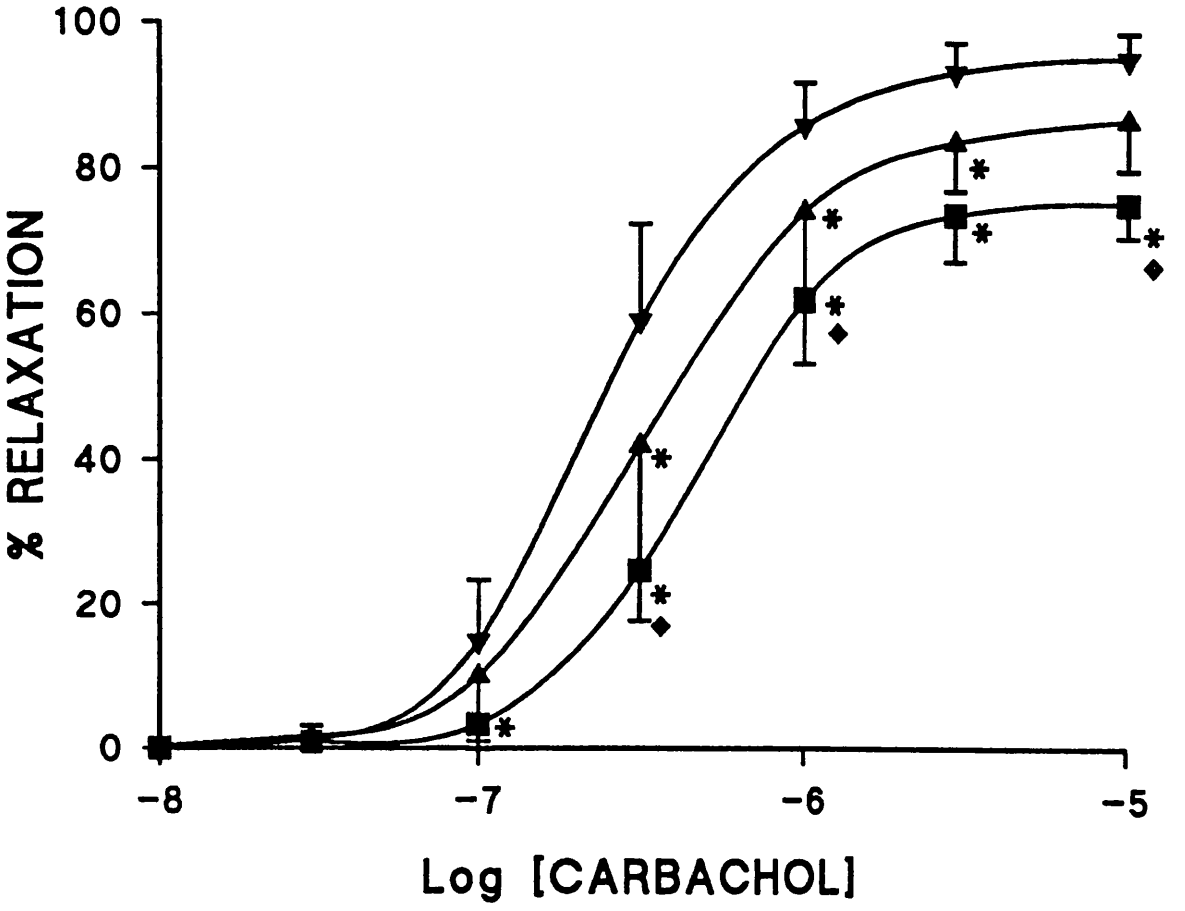


FIGURE 4.17. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from 3 month old rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

■ - homozygote WHHL. n=4
▲ - heterozygote WHHL. n=7
▼ - New Zealand White. n=7

* - $p < 0.05$ compared to New Zealand White.
◆ - $p < 0.05$ compared to heterozygote WHHL.

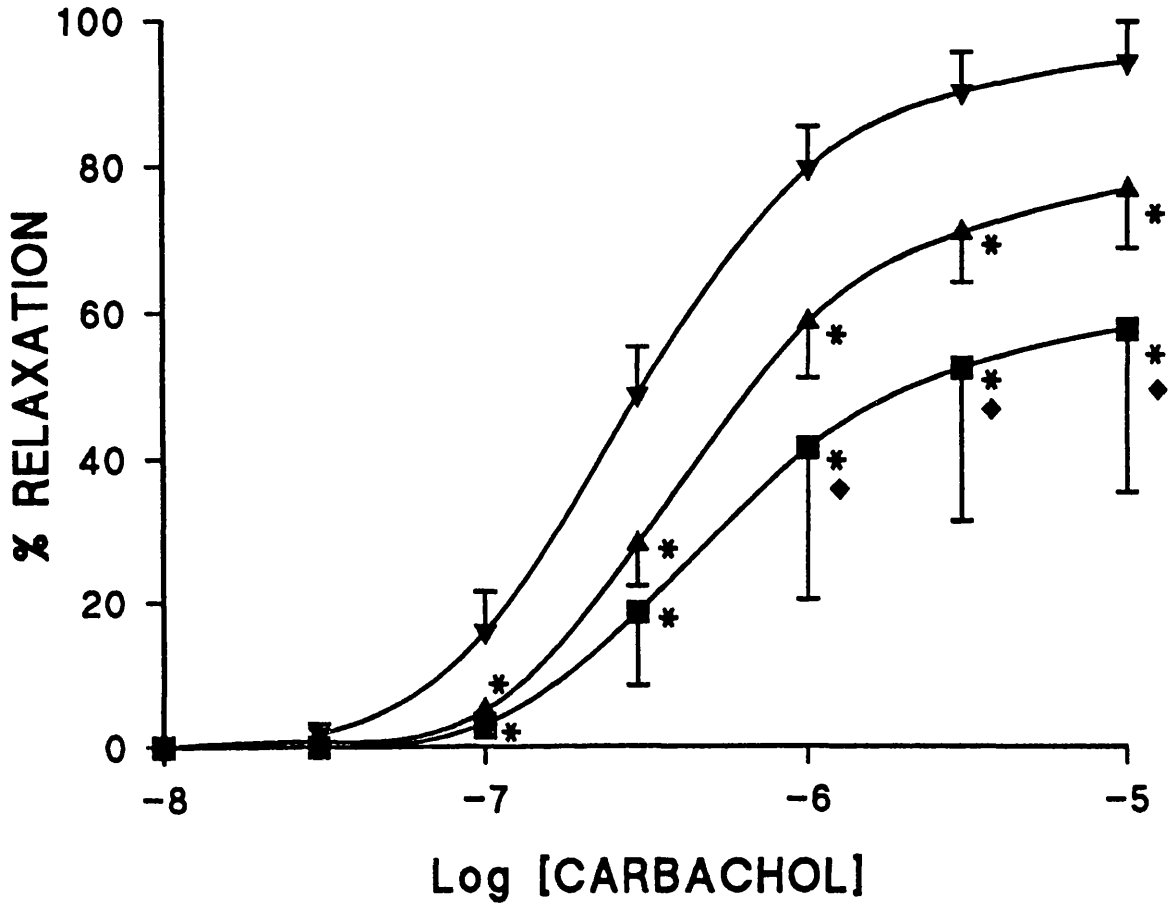


FIGURE 4.18. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from 6 month old rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

■ - homozygote WHHL. n=5
▲ - heterozygote WHHL. n=7
▼ - New Zealand White. n=7

* - $p < 0.05$ compared to New Zealand White.
◆ - $p < 0.05$ compared to heterozygote WHHL.

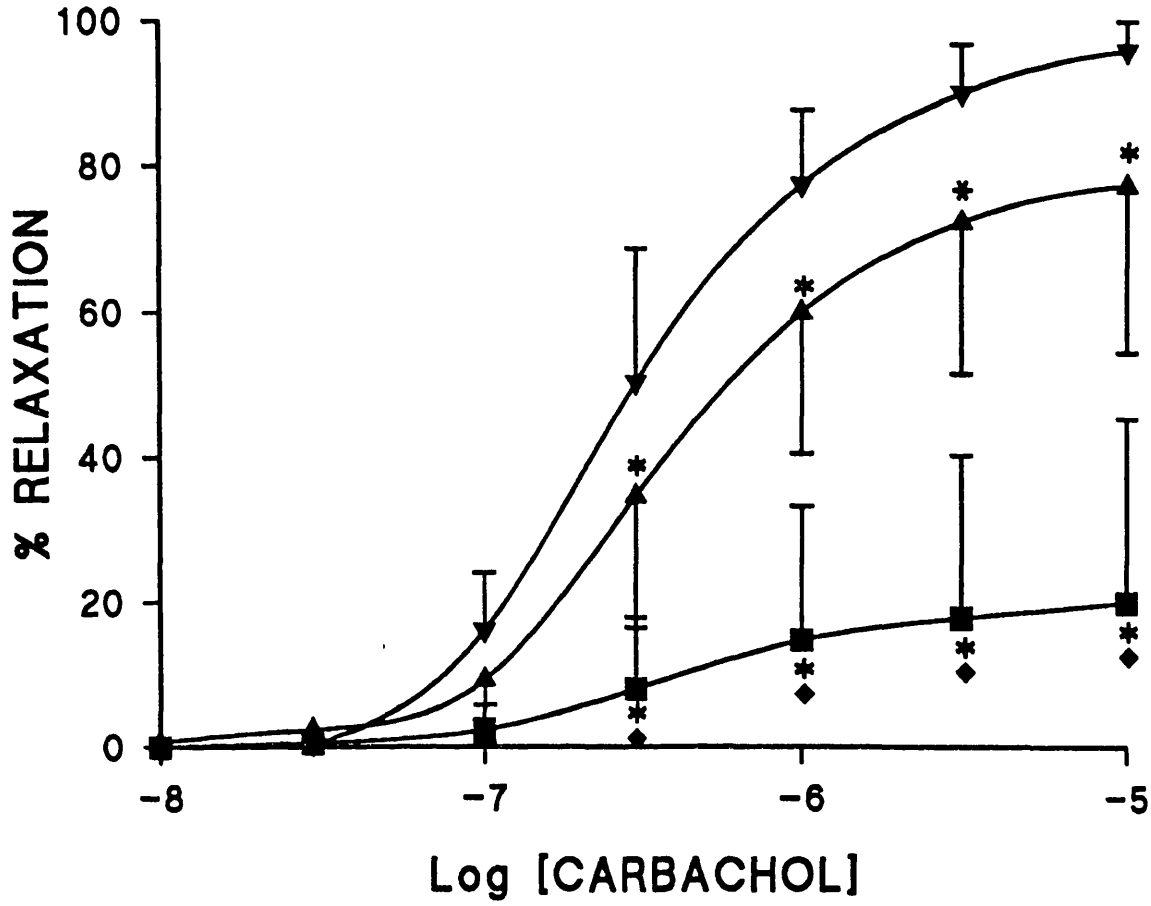


FIGURE 4.19. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from 12 month old rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

■ - homozygote WHHL. n=7
▲ - heterozygote WHHL. n=7
▼ - New Zealand White. n=7

* - $p < 0.05$ compared to New Zealand White.
◆ - $p < 0.05$ compared to heterozygote WHHL.

month New Zealand White rabbits from 3 x 10^{-7}M through to 10^{-5}M carbachol, and significantly less than relaxation of rings of thoracic aorta from 12 month old heterozygote WHHL rabbits from 3 x 10^{-7}M through to 10^{-5}M carbachol (Figure 4.19).

4.4.3 FUNCTIONAL STUDIES IN THE AORTIC ARCH OF WHHL RABBITS.

A. Phenylephrine Induced Contraction in the Aortic Arch of WHHL rabbits.

The contractile function of rings of aortic arch in response to phenylephrine was examined in both homozygous and heterozygous WHHL rabbits.

In rings of aortic arch from homozygous WHHL there was a decrease in the levels of contraction throughout the phenylephrine concentration response curve associated with increasing age. The decrease in contractile function was most prominent between the ages of 6 and 9 months (Figure 4.20). No statistically significant difference was found between the responses from the 3 and 6 month old rabbits. By the age of 9 months, there was a statistically significant decrease in phenylephrine induced contraction from 10^{-7}M through to 10^{-5}M phenylephrine when compared to the responses from the 3 month old rabbits. There was a slight further, non-significant, decrease in contractile function by the age of 12 months of age.

In the heterozygous WHHL rabbits, there was also a decrease in the level of contraction to phenylephrine throughout the concentration response curve in rings of aortic arch (Figure 4.21). There was a statistically significant decrease in response to phenylephrine at 6 months of age from 10^{-7}M to 10^{-5}M phenylephrine when compared to the response of the 3 month old group. There was no further change by 12 months of age.

B. Endothelium Dependent Relaxation in the Aortic Arch of WHHL rabbits.

The ability of rings of aortic arch to respond to carbachol was examined in both homozygous and heterozygous WHHL.

In homozygous WHHL rabbits there was a progressive decrease in the amount of relaxation of phenylephrine induced tone in response to carbachol (Figure 4.22). A small decrease in relaxation was observed from 3 to 6 months, however this was not found to be statistically significant. By the age of 9 months, the decrease in relaxation was found to be statistically significant from 3 x 10^{-7}M to 10^{-5}M carbachol, when compared to the responses obtained at 3 months of age. No further changes were observed by 12 months of age.

As with the rings of thoracic aorta, all these tissues were able to relax fully when exposed to 10^{-4}M SNP.

In the heterozygous WHHL, the response of the aortic arch did not seem to be affected by increasing age (Figure 4.23).

As with the rings from homozygous WHHL rabbits, these rings of aortic arch were fully relaxed following exposure to SNP (10^{-4}M).

4.4.4 FUNCTIONAL STUDIES IN THE CAROTID ARTERY OF WHHL RABBITS.

A. Phenylephrine Induced Contraction in Carotid Artery from WHHL rabbits.

Contractile function in response to phenylephrine was examined in rings of carotid

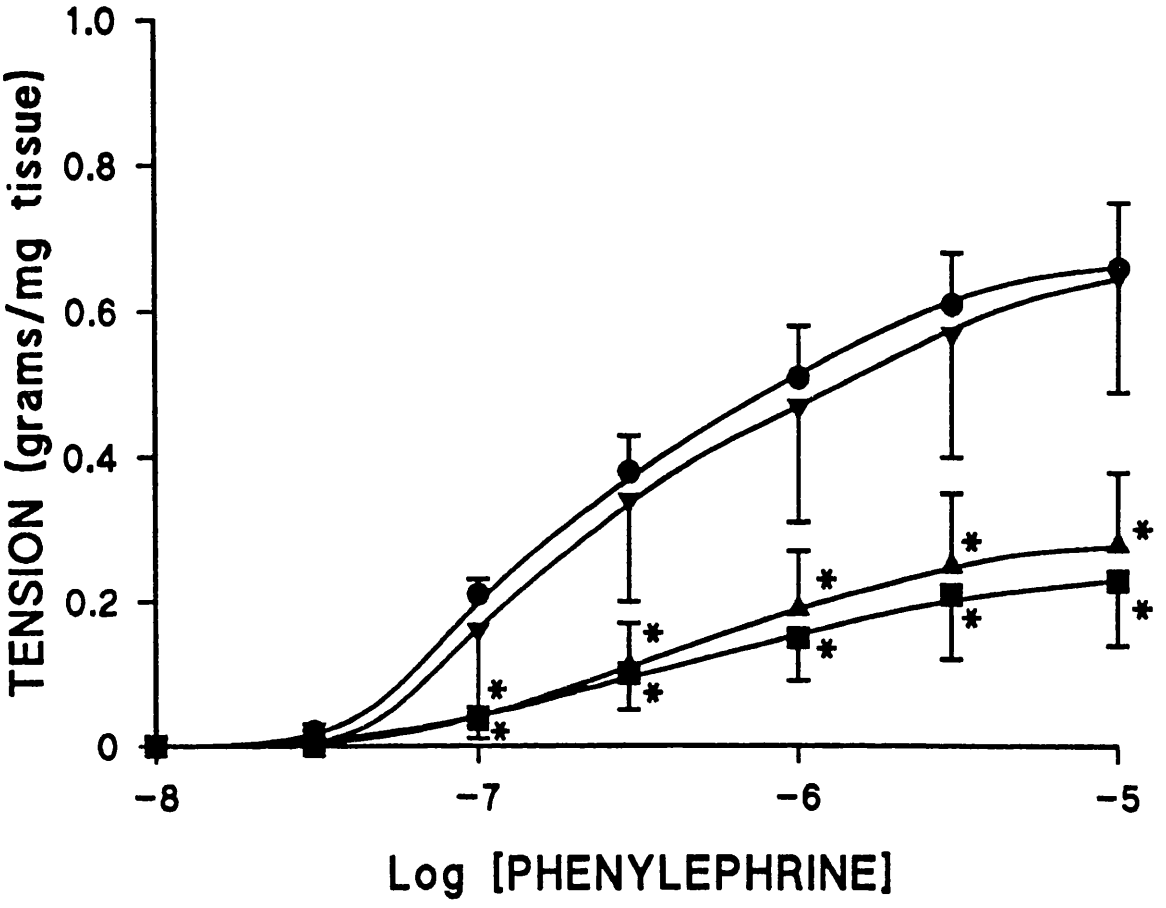


FIGURE 4.20. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of aortic arch from homozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months. n=4
▼ - 6 months. n=5
▲ - 9 months. n=6
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

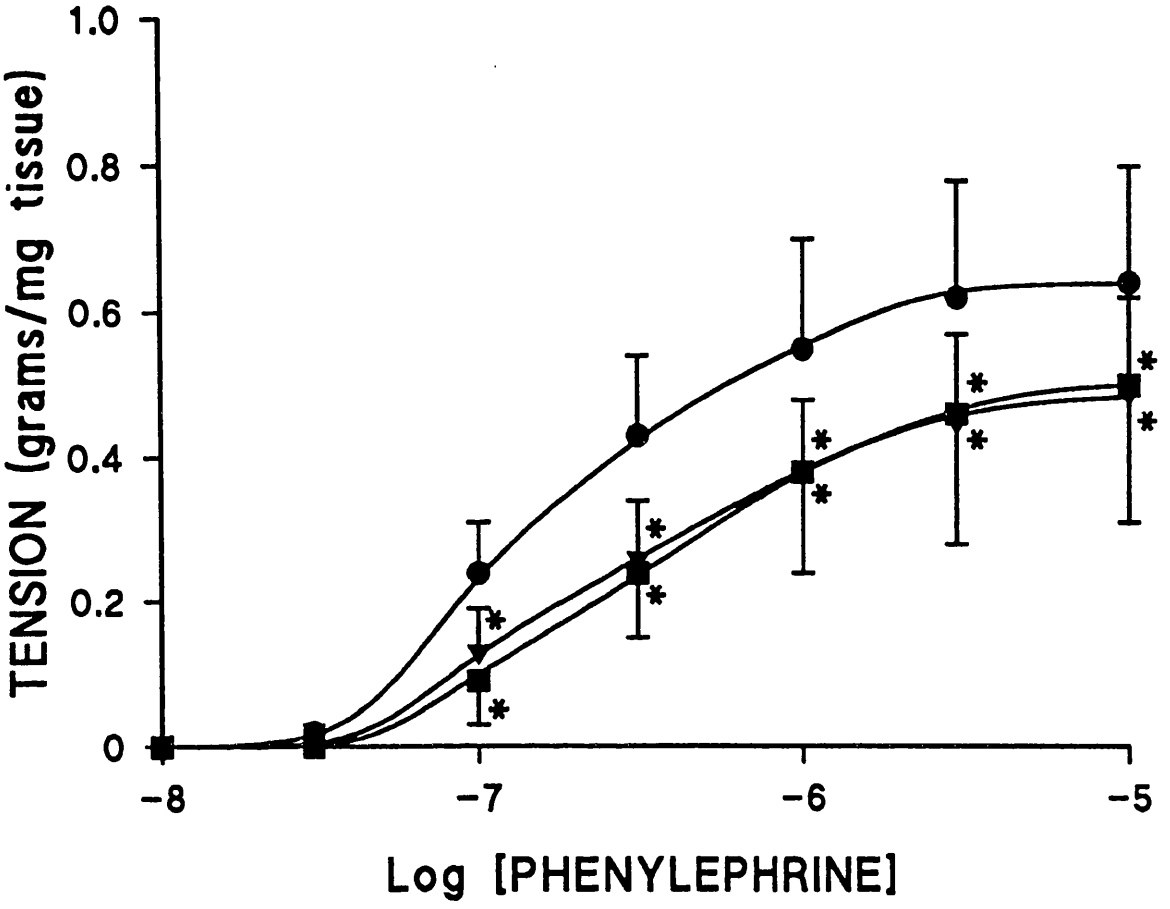


FIGURE 4.21. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of aortic arch from heterozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

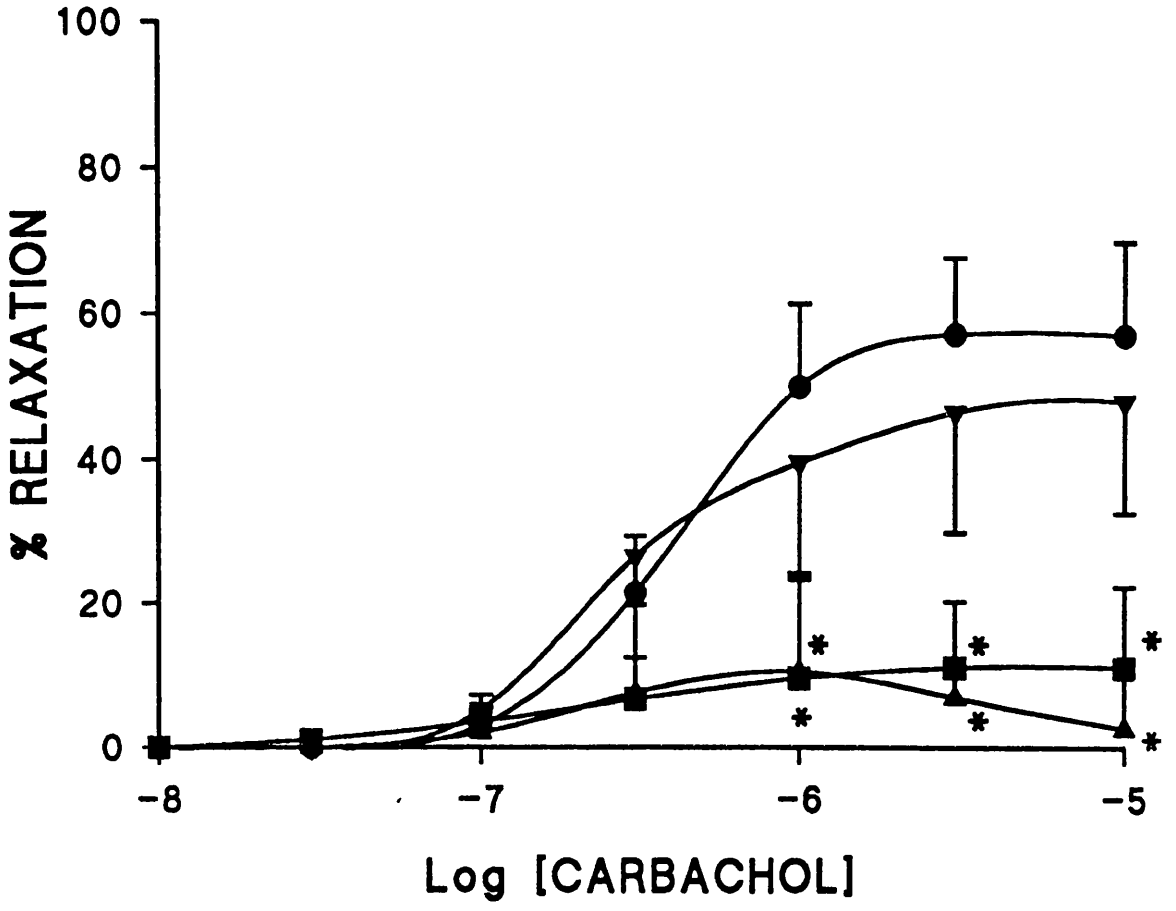


FIGURE 4.22. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of aortic arch from homozygous WHHL rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=4
▼ - 6 months. n=5
▲ - 9 months. n=6
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

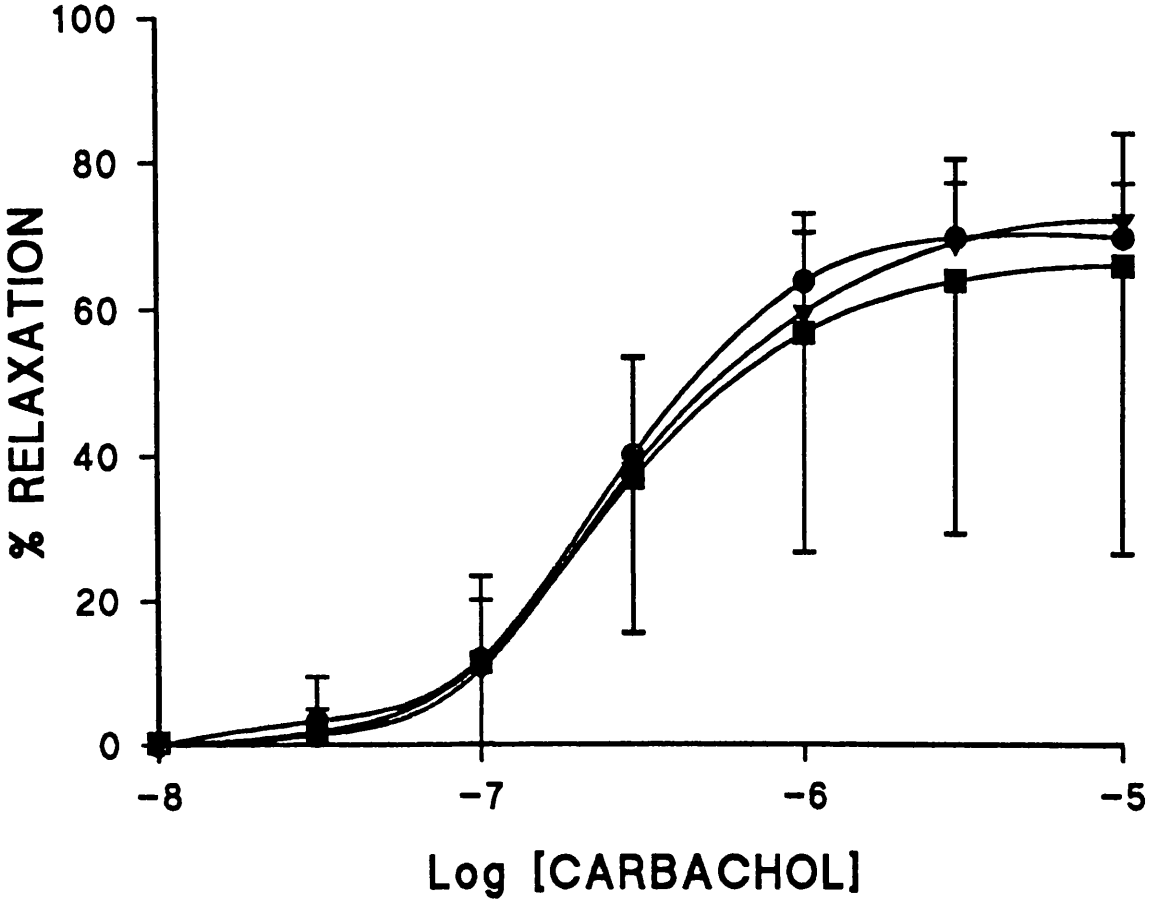


FIGURE 4.23. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of aortic arch from heterozygous WHHL rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

artery from both homozygous and heterozygous WHHL rabbits.

In carotid artery rings from both homozygous and heterozygous WHHL, there was a large variation in responsiveness. In carotid artery rings from homozygous WHHL rabbits, no statistically significant differences were observed in the phenylephrine induced contraction in the different groups (Figure 4.24).

In the carotid artery rings from the heterozygous WHHL rabbits the responses from the six month old animals were found to be significantly different from the responses of the 3 month old group from 3 x 10^{-7}M to 10^{-5}M phenylephrine (Figure 4.25). However, in contrast, there was no statistical difference between the responses of the 3 and 12 month old groups.

B. Endothelium Dependent Relaxation in the Carotid Artery of WHHL rabbits.

Carbachol induced endothelium dependent relaxation was examined in rings of carotid artery of both homozygous and heterozygous WHHL.

In both groups of animals there was little effect of increasing age on the ability of the tissues to relax. In the homozygous animals a slight downward trend is observed, however this was not found to be a statistically significant difference (Figure 4.26).

In the heterozygous animals, a similar small decrease is seen with increasing age, but as with the responses of the homozygote group, this was not found to be statistically significant (Figure 4.27).

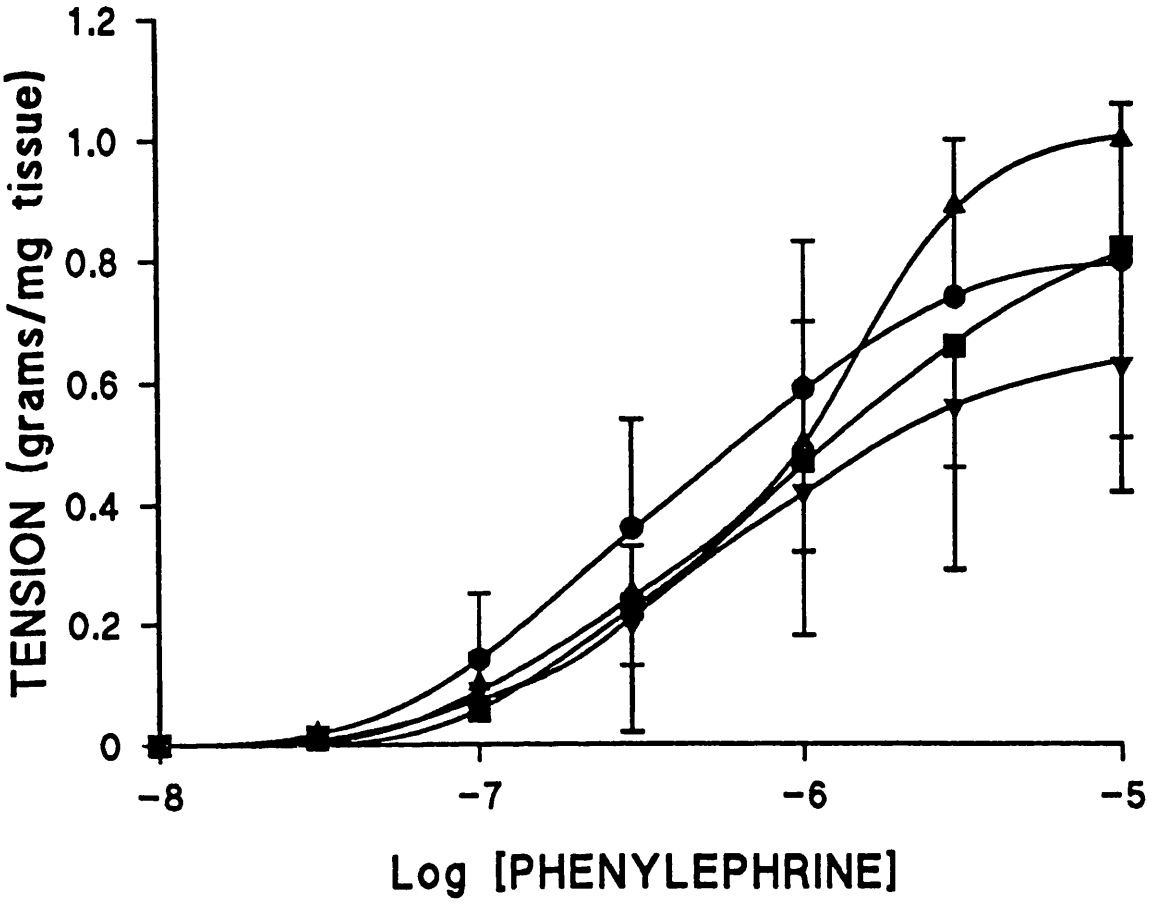


FIGURE 4.24. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of carotid artery from homozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months.	n=4
▼ - 6 months.	n=5
▲ - 9 months.	n=6
■ - 12 months.	n=7

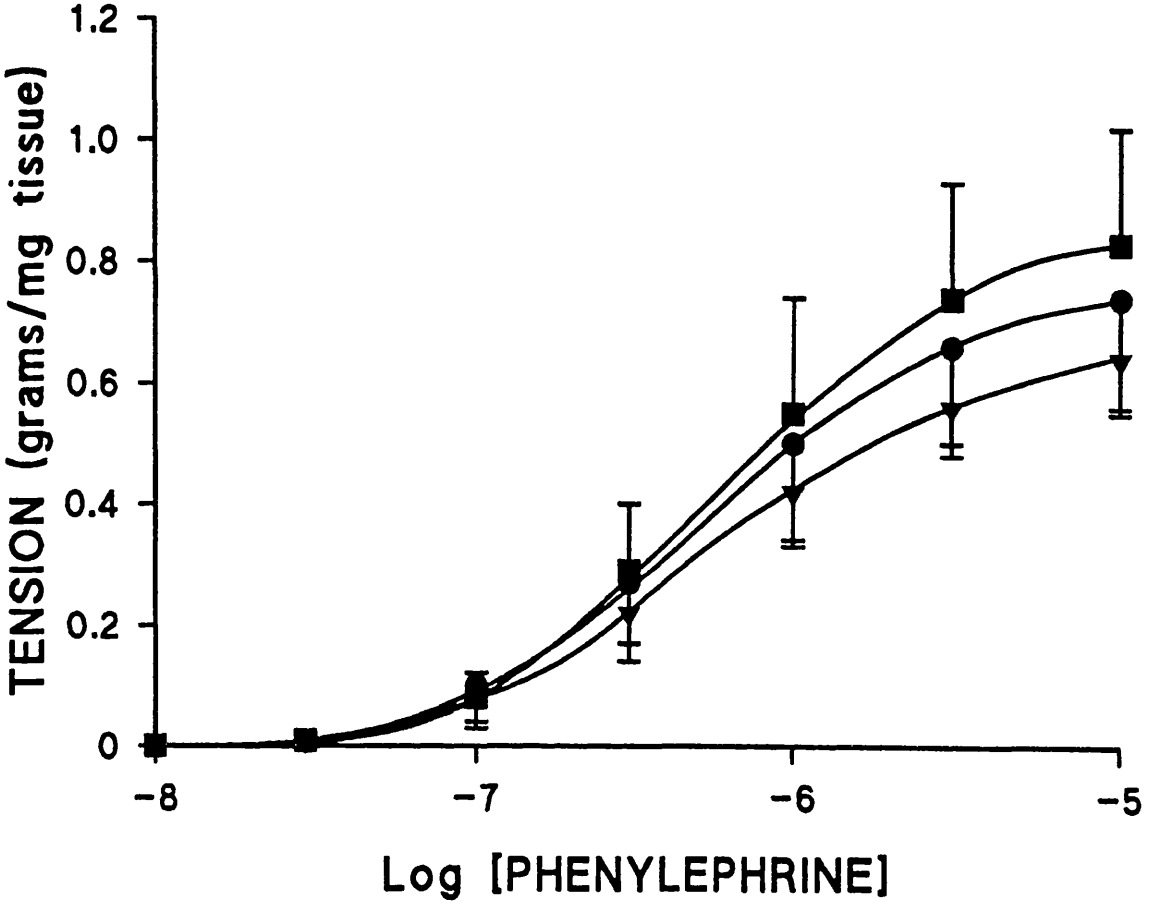


FIGURE 4.25. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of carotid artery from heterozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

* - $p < 0.05$ compared to 3 month group.

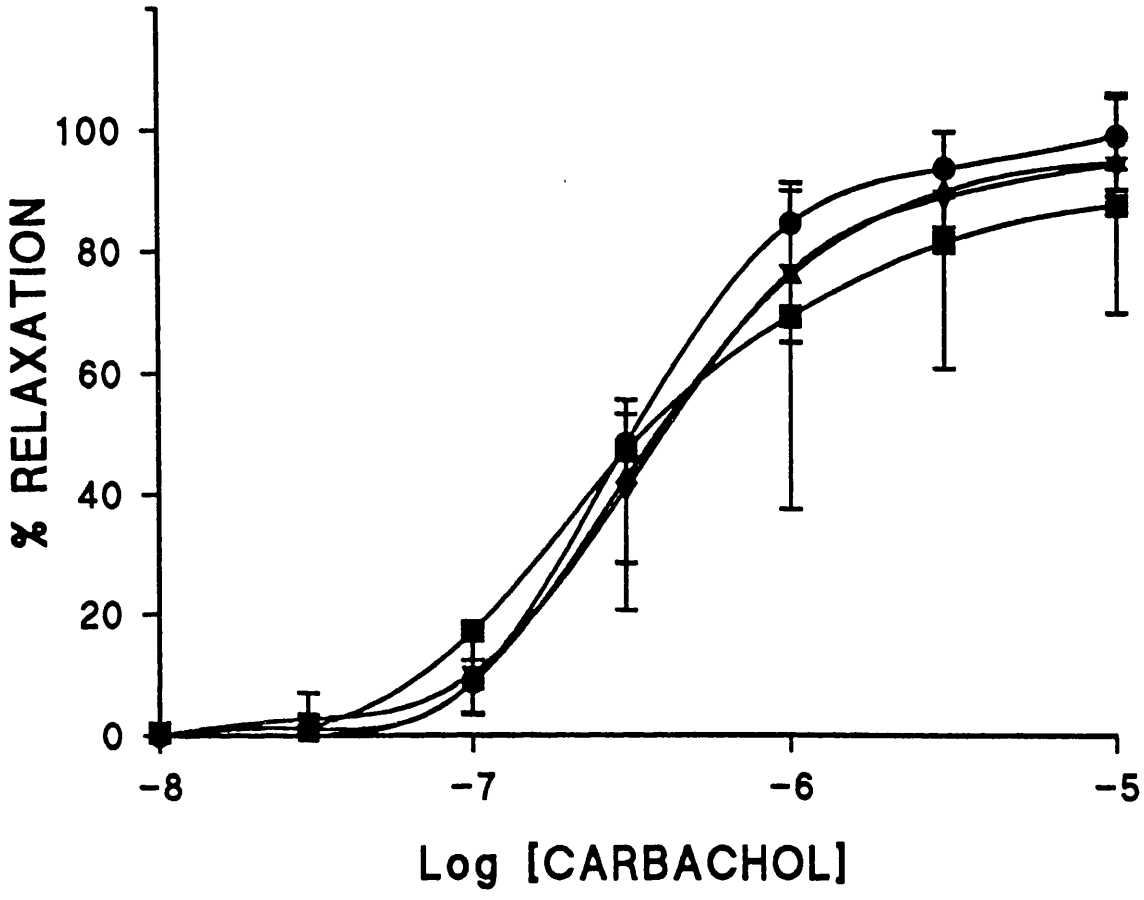


FIGURE 4.26. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of carotid artery from homozygous WHHL rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=4
▼ - 6 months. n=5
▲ - 9 months. n=6
■ - 12 months. n=7

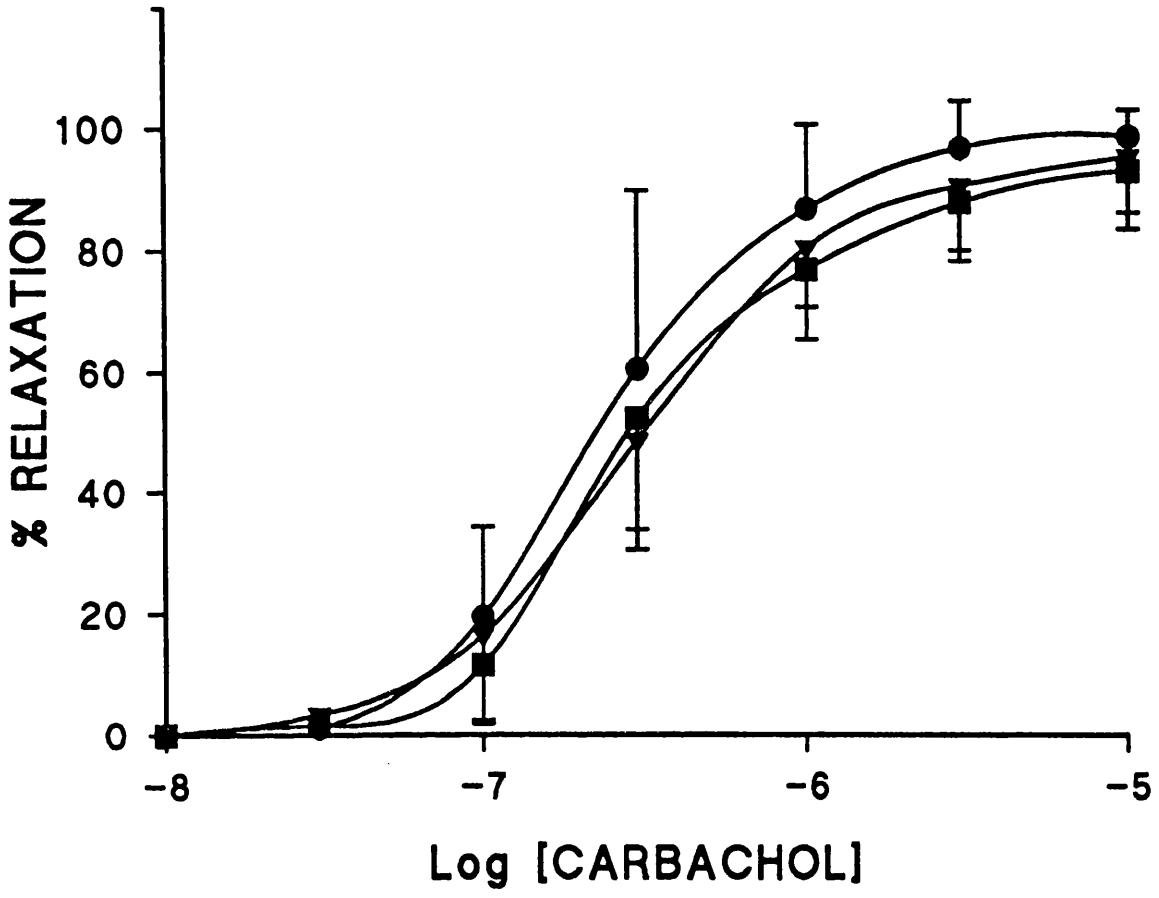


FIGURE 4.27. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of carotid artery from heterozygous WHHL rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=7
▼ - 6 months. n=7
■ - 12 months. n=7

4.4.5 STUDIES OF STRUCTURAL CHANGES IN WHHL RABBITS.

Three parameters were used to assess changes in the structure of the thoracic aorta in both homozygous and heterozygous WHHL rabbits.

A. Sudan Staining.

Sudan red is a lipophilic dye which differentially stains fatty streaks in the arterial intima. Percentage sudan staining was calculated using image analysis to quantify what proportion of the arterial intima is stained with sudan red.

In sections of thoracic aorta from homozygous WHHL rabbits sudan staining was found to increase with age (Figure 4.28a). There was no statistical difference between the amount of staining in the 3 and 6 month groups. By the age of 9 months, the level of staining was found to be significantly higher than in the 3 month group and there was a further increase by 12 months of age.

In the heterozygous group, much less staining was observed. Thoracic aorta from only one animal in the 3 month group showed a positive stain for sudan red. In the 6 month group there was no sudan staining in the sections of thoracic aorta. At the age of 12 months there was staining in the thoracic aorta from one animal (Figure 4.28b).

B. % Involved.

Using image analysis, the percentage of the internal circumference of thoracic aorta involved in atheroma was measured on histological sections. The pattern observed here is similar to that of the sudan staining.

In sections of homozygous rabbit aorta there was a progressive increase in the % Involved as the age of the animals increased (Figure 4.29a). In the 3 month homozygotes, there was no measurable signs of atheroma in the histological sections. By the age of 6 months, sections from 4 out of the 5 animals examined displayed quantifiable areas of atheromatous plaque. In all sections from 9 month old homozygotes there was detectable atheroma, and there was a statistically significant increase in the mean value. There was a slight further increase in the % involved by 12 months of age.

This parameter was zero in all sections from heterozygote rabbits except for one which was in the 12 month group (Figure 4.29b).

C. Ratio of Intima to Media.

The third parameter that was used to quantify structural changes in the WHHL rabbits was the percentage ratio of area of intima to area of media - using the internal elastic lamina as a dividing line. In sections from "normal" vessels with no detectable signs of atheroma, it is not possible to quantify the area of intima using our techniques. This is due to the fact that in normal vessels, the endothelium lies very close to the internal elastic lamina. Thus, in a section from a normal vessel this parameter would apparently be zero.

In sections of thoracic aorta from homozygous WHHL the ratio of intima to media increased progressively with increasing age (Figure 4.30a). The sections from the 3 month old group were normal i.e. the ratio was zero. By the age of 6 months there was a detectable thickening of the intimal layer in 4 out of the 5 rabbits studied. This had progressed by 9 months such that all the rabbits studied exhibited intimal

thickening, with the mean value significantly greater than that from 6 months. There was a further increase by the age of 12 months. The difference between the 6 month group and the 12 month group was not statistically significant due to the large variation in the data.

This parameter was zero in all sections from heterozygote rabbits except for one which was in the 12 month group (Figure 4.30b).

D. Visual Assessment of Sections of Thoracic Aorta.

The changes observed are presented in tabular form for both medial (Table 4.1) and intimal (Table 4.2) changes.

i. Normal Aorta (NZW).

Sections of thoracic aorta from the New Zealand White rabbit thoracic aorta were normal. The intima showed no signs of thickening or endothelial reactivity. The media exhibited a hint of elastic laminae separation in the innermost layers with minimal proteoglycan deposition.

ii. Homozygote WHHL Rabbit Aorta.

Sections of thoracic aorta from the homozygous animals revealed marked changes in vascular structure. Sections from 3 month animals appeared essentially normal (Plate 4.1).

Minimal proteoglycan deposition was observed in the media prior to the development of plaque. At 6 months, most sections demonstrated the begining of activity in the media - the inner 2-3 elastic laminae were disturbed, separated by proteoglycan deposition, small mononuclear cells, a few foam cells and sometimes

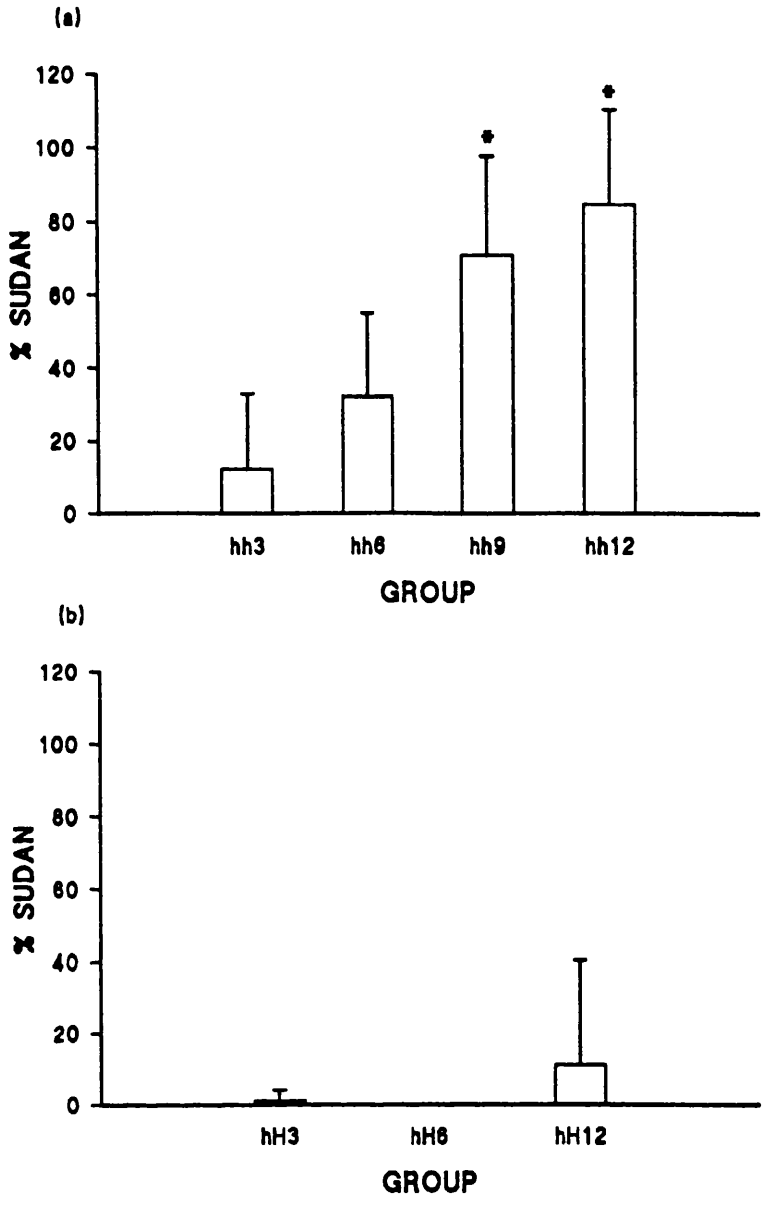


FIGURE 4.28. Percentage Sudan staining (%Sudan) in segments of thoracic aorta in (a) homozygous (hh) WHHL and (b) heterozygous (hH) WHHL. Data is mean ± sd. n=4-7.

* - $p < 0.05$ compared to 3 month group.

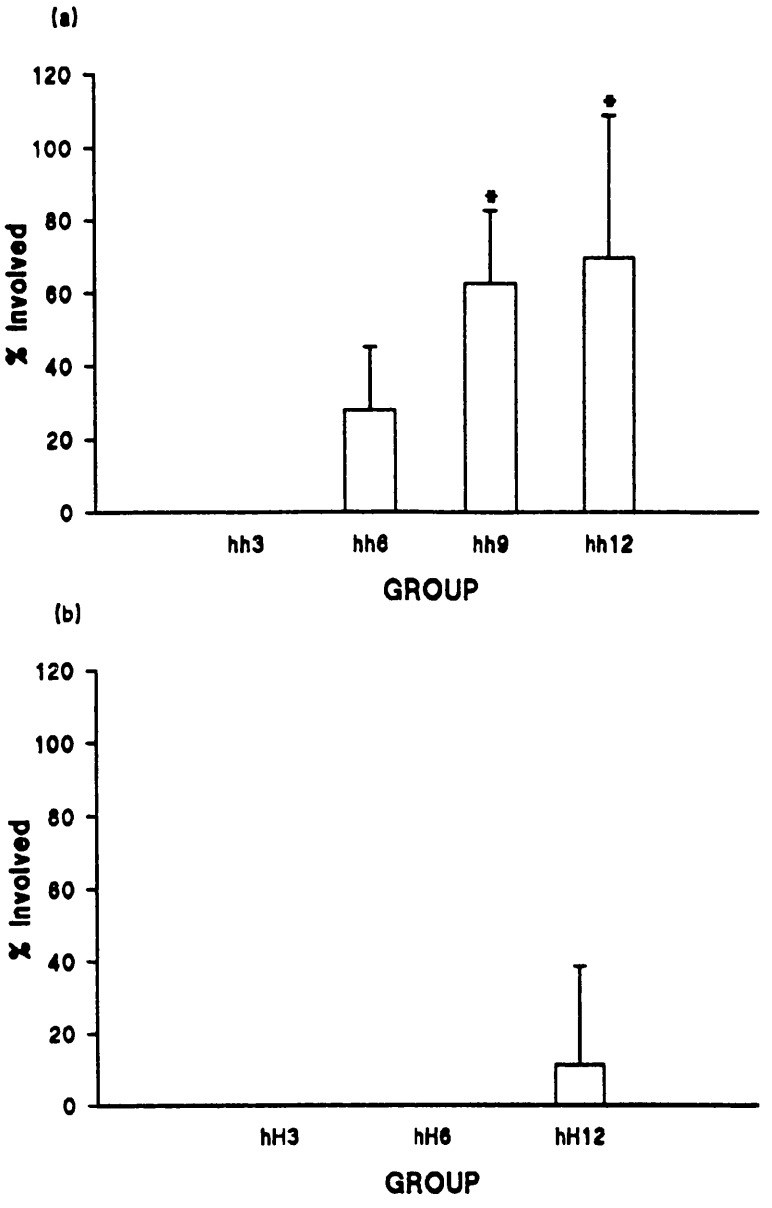


FIGURE 4.29. Percentage of the internal circumference of the vessel involved in atheroma (%Involved) in sections of thoracic aorta in (a) homozygous (hh) WHHL and (b) heterozygous (hH) WHHL. Data is mean ± sd. n=4-7.

* - $p < 0.05$ compared to 6 month group.

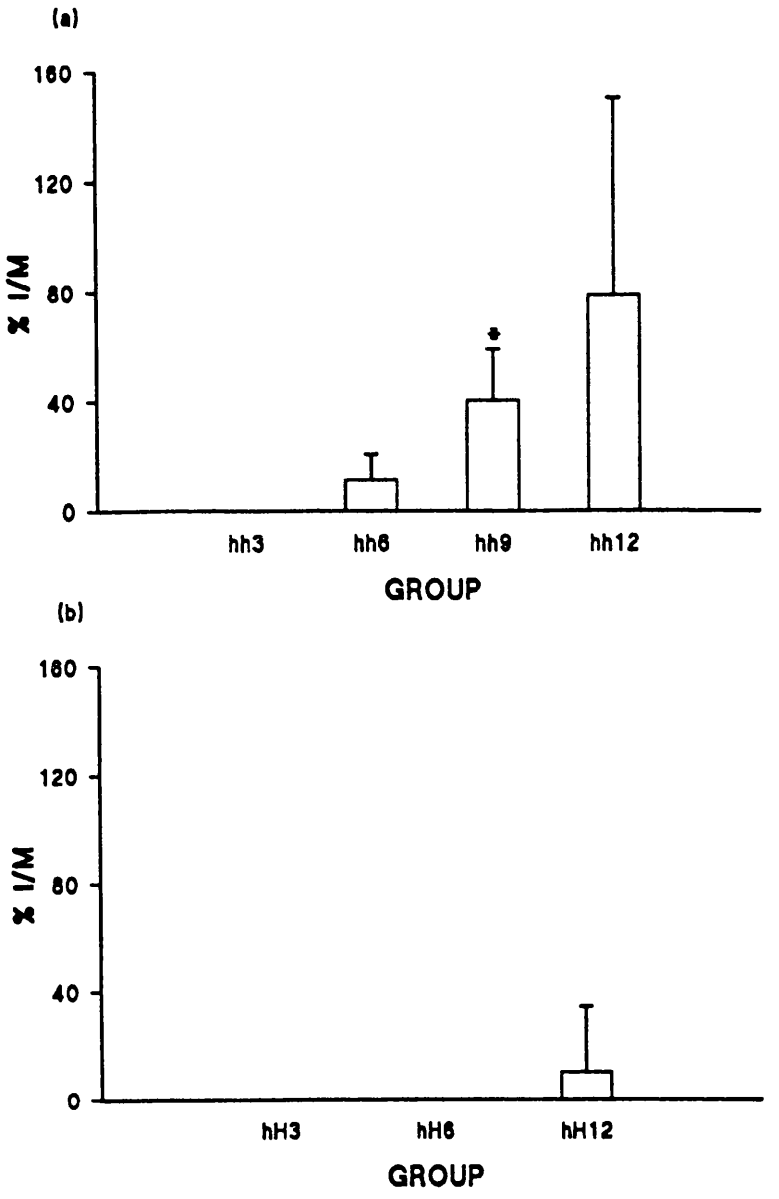


FIGURE 4.30. Percentage ratio of the area of intima to the area of media (%I/M) in sections of thoracic aorta in (a) homozygous (hh) WHHL and (b) heterozygous (hH) WHHL. Data is mean ± sd. n=4-7.

* - $p < 0.05$ compared to 6 month group.

GROUP	RAB	LIPID	SMC-OR	E-LAM	PG-PLAQ	PG-DIST	COLL
hh3	F60						
	F61					*/-	
	F74					*/-	
hh6	M47			*S	**	*	
	M50		*	*S	*	*	
	M51			*S		*	
	F42	*	*	*	**		*
	M54	**	*	**	**	*	
hh9	F29	*	*	***S	**	*	*
	F34	*	*	***SB	**	CIRC	*
	M41	*	*	*	*	*	*
	M40	*	*	*SB	*	*	*
	M43	*	*	***S	*	*	
	F38	*	*	**SB	**	*	*
hh12	M13	*	*	***SB	**	CIRC	**
	F16	***	*	***SB	**	CIRC	**
	M25		*	***S	**	*	*
	F25	*	*	***SBF	**	CIRC	**
	R031			*S	*	*	
	F37		*	*S	*	*	
	M44	*	*	***SB	*	CIRC	*
hH3	M61					*/-	
	M62						
	F52					*	
	M63						
	M66					*	
hH6	F43					*/-	
	M53			*		*	
	M55			*S	*		
	M56						
	M57					*	
	F45			*S		**	
	F46					*	
hH12	F12		*	**SB	**	**	*
	F13					*	
	M27			*		*	
	M15					*	
	F22					*	
	F49			*S		*	
NZW3	N73			[S]		*	
	N72			[S]		*	
NZW20	N3302			*S	**		
	N5245			*	**	*	**

TABLE 4.1. Tabular representation of the changes observed in the media by visual assessment of the histological sections of thoracic aorta. RAB represents rabbit identification; SMC-OR - alterations in the orientation of smooth muscle cells; E-LAM - elastic lamina, with [S] indicating beginning of separation, S - separation, B - broken, F- fragmented; PG-PLAQ - proteoglycan under area of plaque; PG-DIST - proteoglycan distant to plaque, with CIRC indicating that plaque is circumferential. The number of stars indicates the degree of change on a scale of 0-3.

GROUP	RAB	PLAQ	ENDO	FOAM	SMC	EC LIP	PG	COLL
hh3	F60							
	F61							
	F74							
hh6	M47	P	R	**	*			
	M50	P	R	**		*	**	*
	M51							
	F42	P	R	***		*	**	*
	M54	P	R	***			**	*
hh9	F29	P	R	**		**	**	**
	F34	P	R	***	*		**	**
	M41	P	R	**			*	**
	M40	P		*	*	**	*	*
	M43	P	P	***	**	*	**	**
	F38	P	R	***	*	**	**	**
hh12	M13	P	P	**	**	**	**	***
	F16	P		**	***	***	***	***
	M25	P		**			**	*
	F25	P	R	**	***	***	***	***
	R031	P	R	***			*	
	F37	P	R	***			**	*
	M44	P	R	**	***	***	**	***
hH3	M61							
	M62							
	F52		P					
	M63							
	M66							
hH6	F43							
	M53							
	M55							
	M56							
	M57		P					
	F45							
	F46							
hH12	F12	P	R	***	*	*	**	**
	F13							
	M27		R					
	M15							
	F22							
	F49							
NZW3	73							
	72							
NZW20	3302	P	P	**	*	**	**	
	5245	P		***	*	**	*	**

TABLE 4.2. Tabular representation of the changes observed in the intima by visual assessment of the histological sections of thoracic aorta. RAB represents rabbit identification; PLAQ - atherosclerotic plaque, with P indicating plaque present; ENDO - endothelium, with R indicating reactive and P - plump; FOAM - foam cells; SMC - smooth muscle cells; EC LIP - extracellular lipid; PG - proteoglycan; COLL - collagen. The number of stars indicates the degree of change on a scale of 0-3.

extracellular lipid. Proteoglycan was also observed in areas were no plaque was present (Plate 4.2a). Minimal smooth muscle cell reorientation was apparent. The intima showed early plaque development, with foam cells and extracellular lipid. Signs of proteoglycan and collagen deposition were observed. The endothelium showed signs of activation. By 9 months, these changes had progressed: in the media, further separation of the elastic laminae had occured, medial smooth muscle cells were changing orientation and foam cells, collagen and proteoglycans were present (Plate 4.2b). In the intima, smooth muscle cells, foam cells and proteoglycans were present. It was also possible to detect 'reduplication of the elastic laminae' by collagen in the intima. The endothelium appeared activated. By 12 months, the development of atheroma was marked in all samples. However, even within the 12 month age group, the extent of the disease ranged from relatively mild, focal lesions to complete circumferential involvement by atheroma. In these severe lesions the intima contained many foam cells, smooth muscle cells, proteoglycans (Plate 4.3) and collagen. In areas, the lipid was extracellular and cholesterol had formed crystals (Plate 4.4a). This was most prominent in the deepest layers of the intima where there was further splitting of the inner elastic laminae. In the most advanced lesions, the atheroma eroded into the inner media leading to fragmentation of the innermost elastic laminae. Calcification (Plate 4.4b) and macrophage-like cells were noted. In contrast to human atheroma there appears to be little or no inflammation of the vessel wall (Stratford et al, 1986; Van der Wal et al, 1989). There were many smooth muscle cells on the luminal aspect of the thickened intima. These varied in orientation: at the luminal surface, the smooth muscle cells were circumferential to the long axis of the vessel - as in the media; however, deeper in the intima smooth muscle cells ran longitudinally (Plate 4.3). There was also 'reduplication of the elastic laminae' by collagen in the deeper intima to a greater extent than in the 9 month group. In the subendothelial intima, the smooth muscle cells were also delineated by collagen.

iii. Heterozygote WHHL Rabbit Aorta.

The sections of thoracic aorta from heterozygote WHHL rabbits were also assessed in this manner. At 3 months the aorta was histologically normal. Sections from the 6 month animals were near normal, apart from early lipid deposition and the presence of a few foam cells causing the separation of the innermost elastic laminae. The intima appeared normal. At 12 months of age the appearances were essentially similar to the 6 month heterozygotes. In the sections from one animal, there was a clearly defined atherosclerotic plaque containing foam cells and collagen. However few differentiated smooth muscle cells were visible. The endothelial cells covering the area of plaque appeared prominent and clumped in groups. Proteoglycan deposition in the inner media was frequently observed in heterozygotes of all ages, prior to the development of atherosclerotic plaque.

Transmission electron micrographs were prepared from sections of thoracic aorta from two of the 12 month old heterozygote WHHL rabbits (N.B. These were not the same animal in which clear atheroma had been noted). The electron micrographs reveal smooth muscle cells which have migrated into to the intima, collagen and proteoglycan deposition in the intima (Plate 4.5). On both the transmission (Plate 4.6) and scanning (Plate 4.7) electron micrograph blebs can be seen on the luminal surface of the endothelial cells.

4.4.6 CORRELATION AND ANALYSIS OF STUDIES IN WHHL RABBITS.

In the study of the development and progression of atherosclerosis in these WHHL rabbits, three general aspects have been examined :-

i. Cholesterol levels.

ii. Functional characteristics.

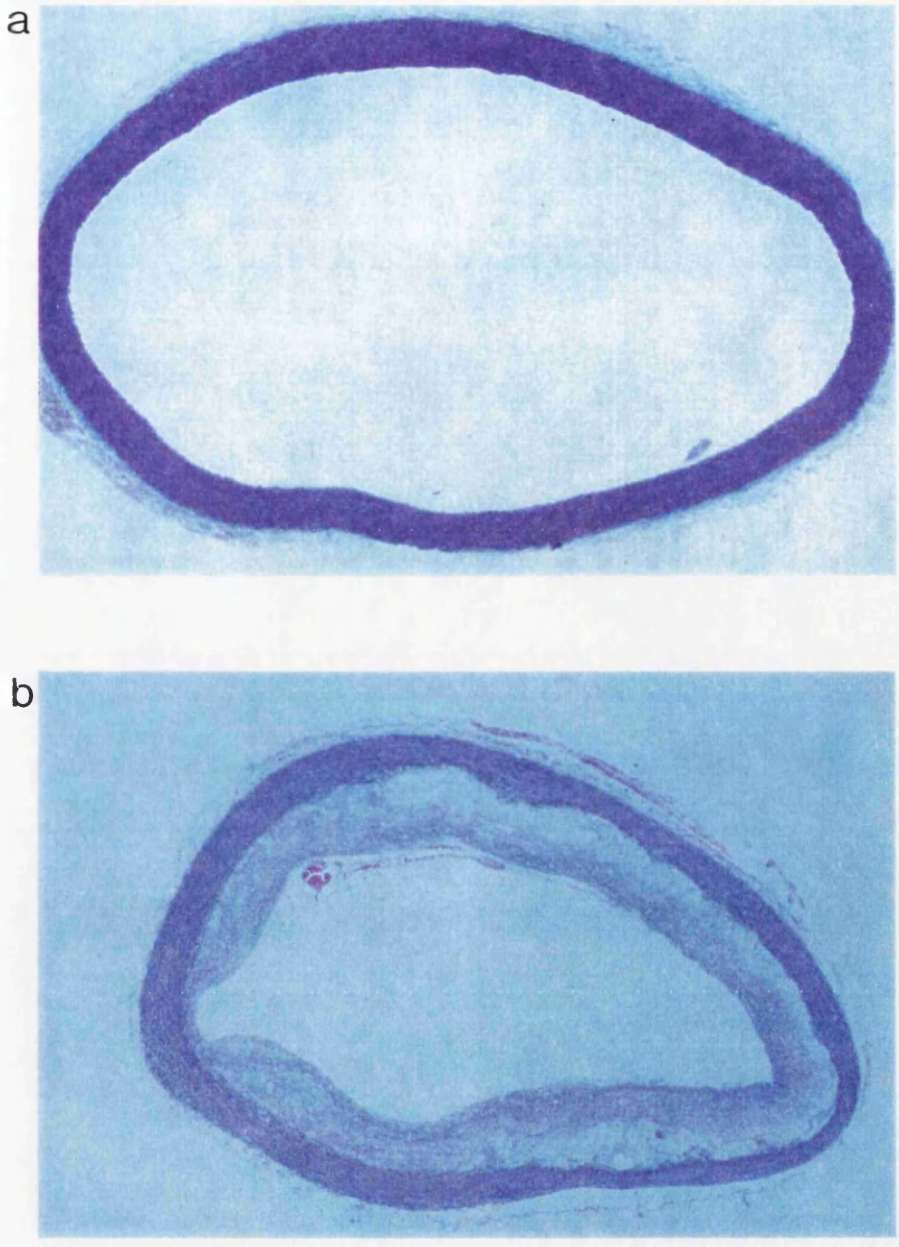


PLATE 4.1. Low power light micrographs of transverse sections of thoracic aorta a) from a 3 month old heterozygote WHHL rabbit showing normal morphology b) from a 12 month old homozygote WHHL rabbit demonstrating a grossly thickened intima.

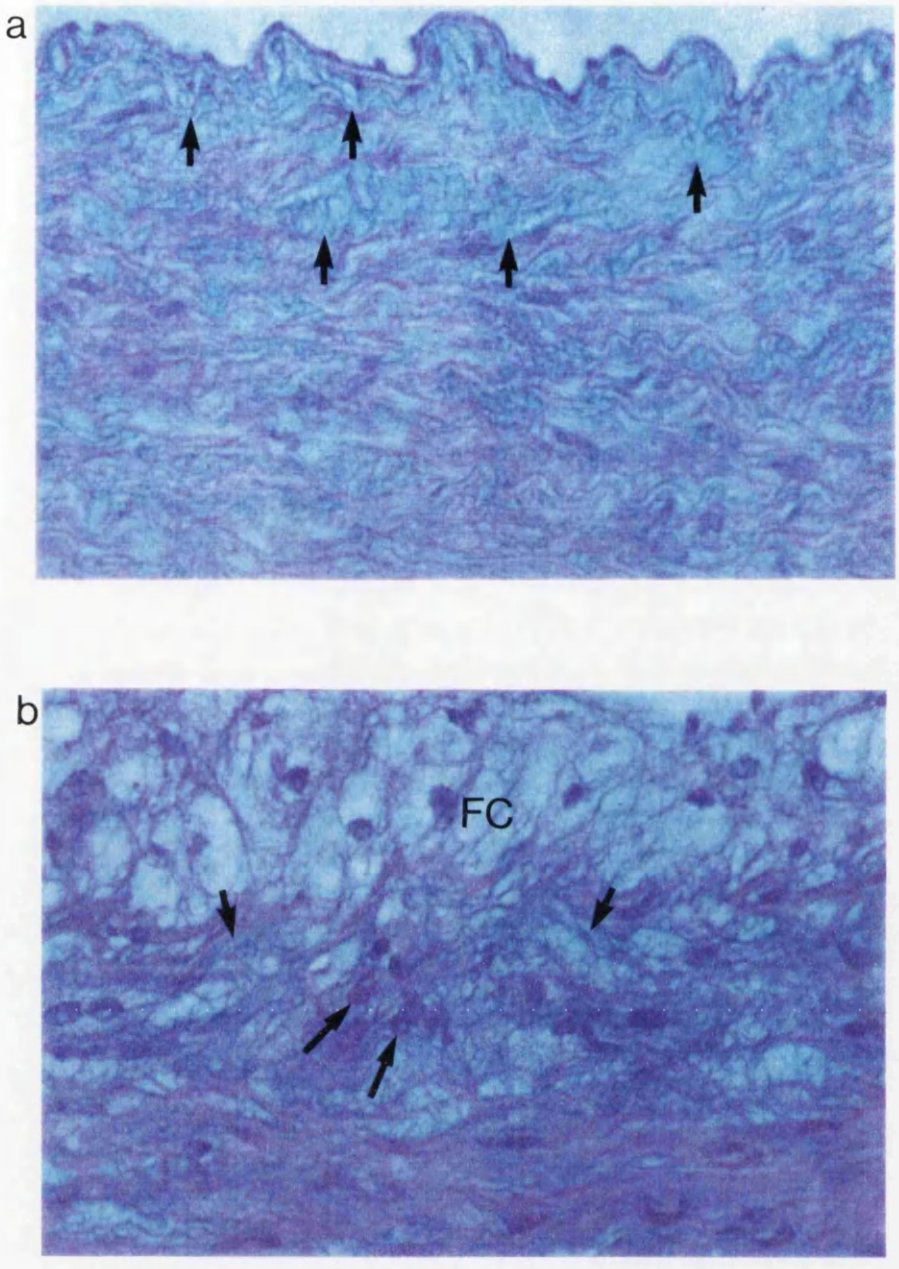


PLATE 4.2. Light micrographs of transverse sections of thoracic aorta a) showing proteoglycan deposition (arrows) in the inner media of a region distant to plaque development. b) showing proteoglycan deposition (short arrows) in the inner media underlying an early plaque, which is composed mainly of foam cells (FC). Altered smooth muscle cell orientation (long arrows) is also observed.

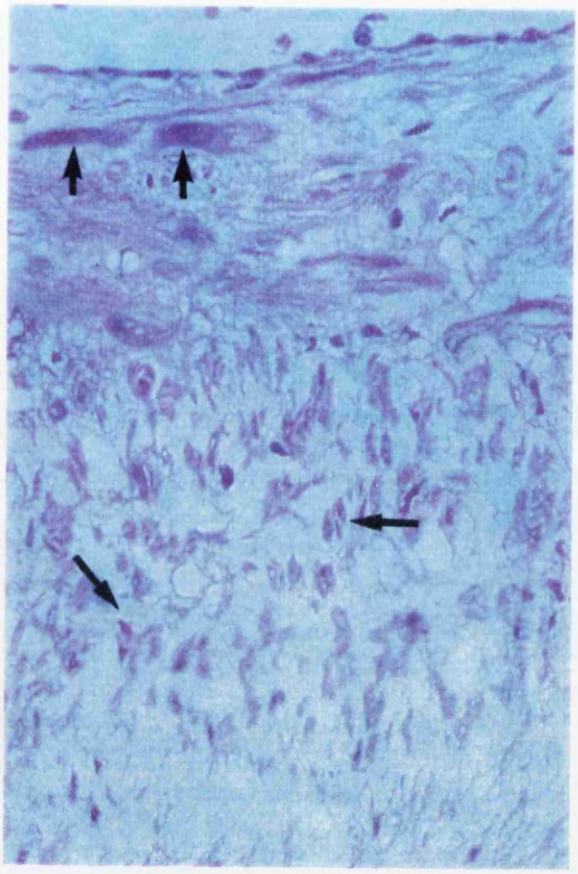


PLATE 4.3. Light micrograph of a transverse section of thoracic aorta showing the superficial part of the intimal plaque depicted in Plate 4.1. Smooth muscle cells in the innermost intima can be seen running circumferentially (short arrows), whereas those deeper in the intima are longitudinally orientated (long arrows).

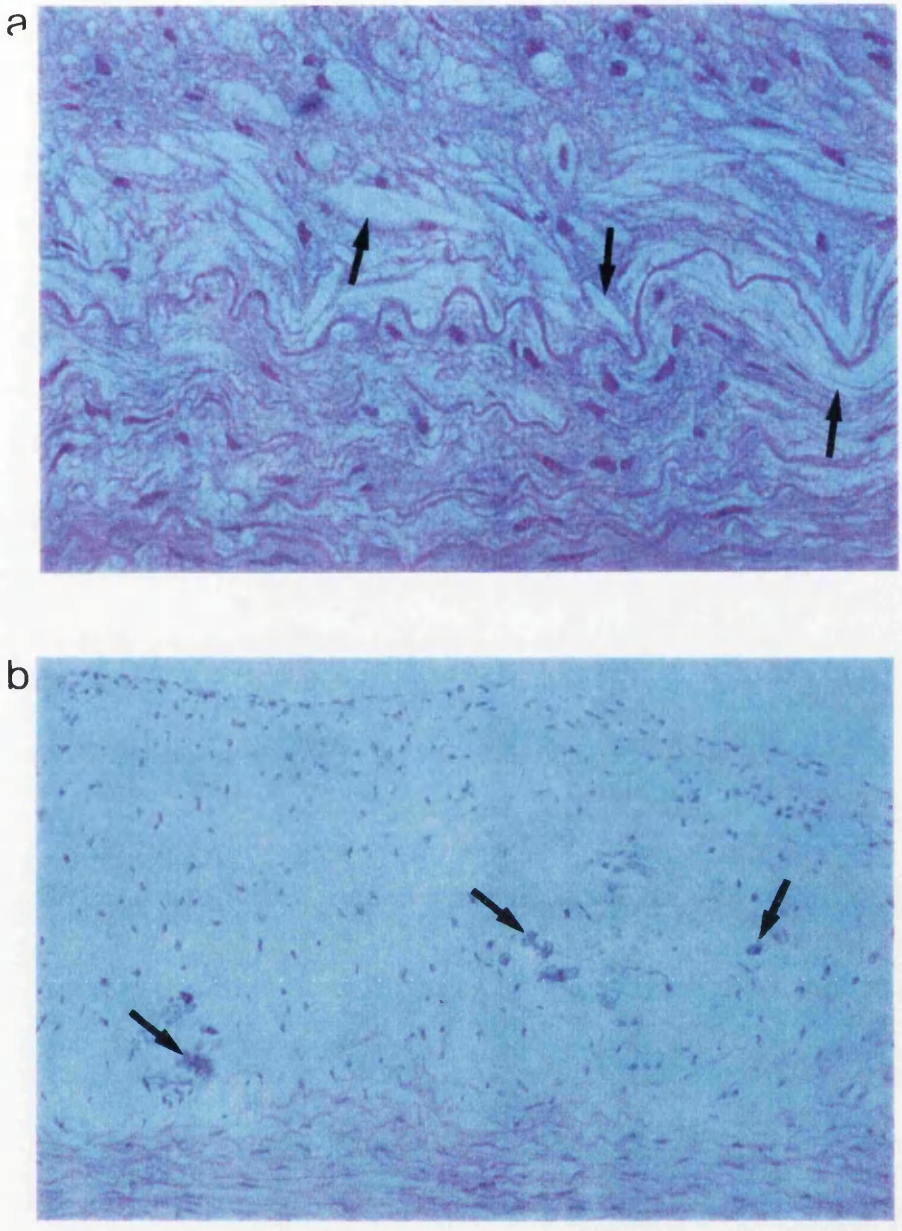
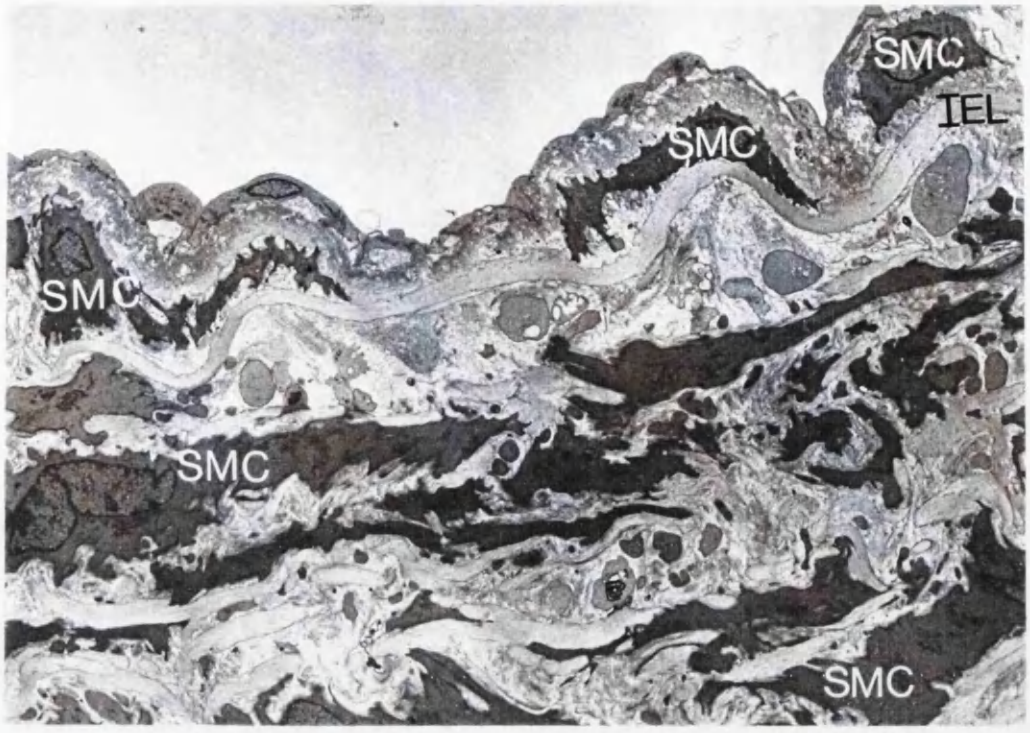


PLATE 4.4. Light micrographs of transverse sections of thoracic aorta showing plaques with a) extracellular lipid with cholesterol clefts (arrows) and b) calcification of extracellular lipid (arrows).

A



B

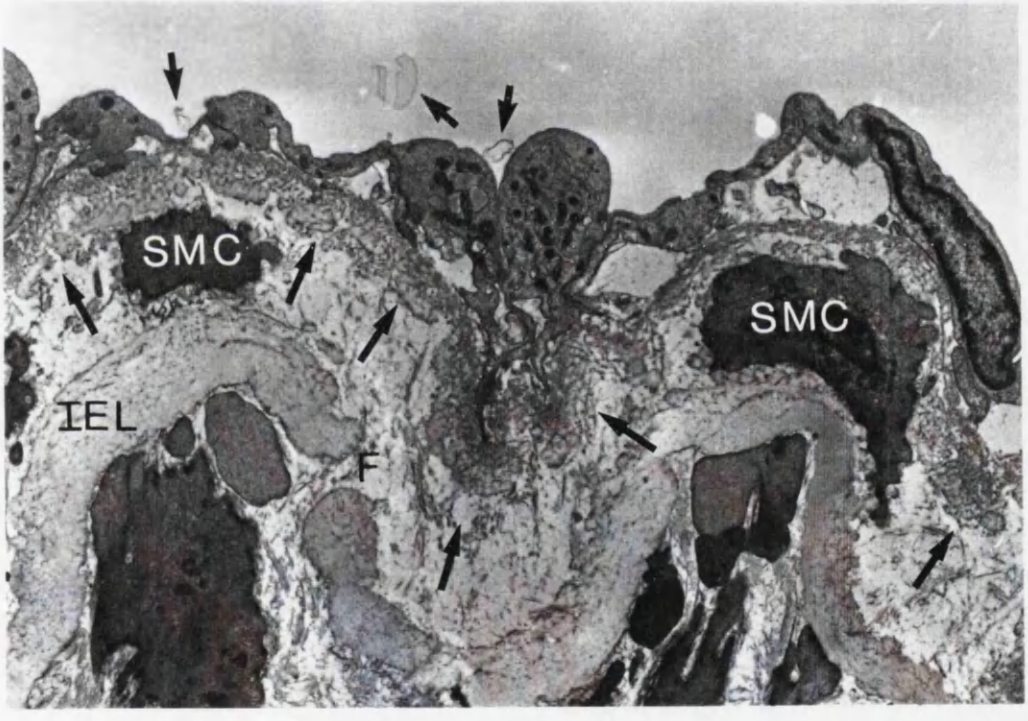
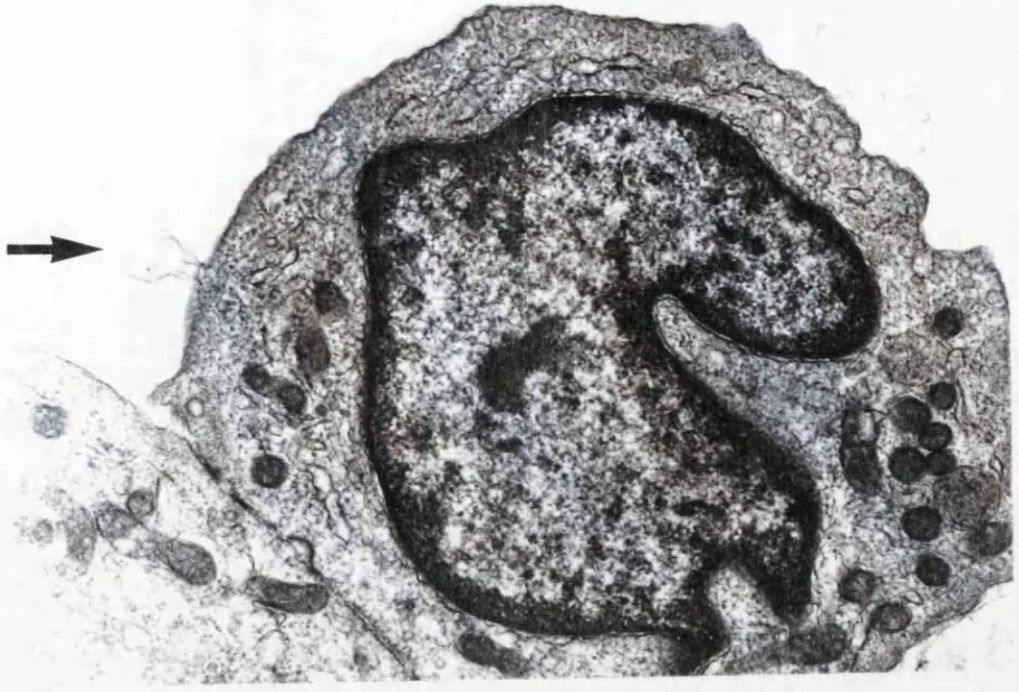


PLATE 4.5. Low power transmission electron micrographs of thoracic aorta from a 12 month heterozygous WHHL rabbit. a) showing the composition of the media. There are circumferential smooth muscle cells (SMC) with intervening elastic laminae and collagen. The intima is thickened due to the presence of smooth muscle cells and matrix elements. IEL denotes internal elastic laminae (x 2100). b) showing plump endothelial cells which have small blebs on their surfaces (short arrows). They overlie a thickened intima which contains a mixture of collagen and proteoglycans (long arrows) and smooth muscle cells (SMC), which have presumably migrated through fenestra (F) in the internal elastic lamina (IEL) (x 6000).

A



B

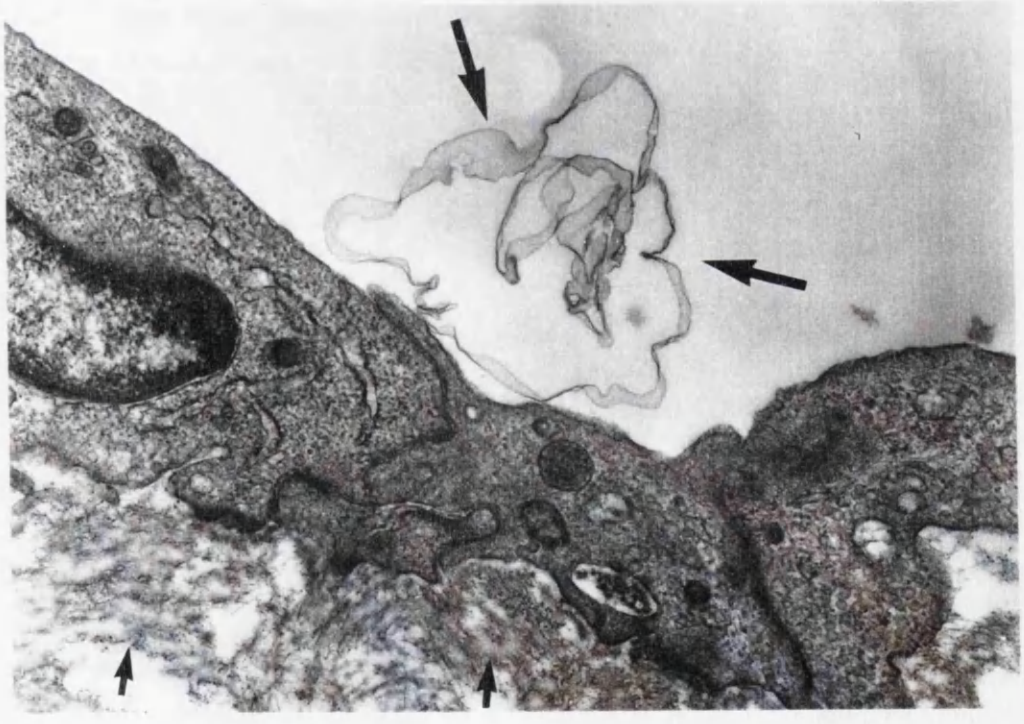


PLATE 4.6. High power transmission electron micrographs of thoracic aorta from a 12 month heterozygous WHHL rabbit. a) showing an endothelial cell with surface blebbing (arrow). (x 23500). b) showing a collapsed bleb (long arrows) on the surface of an endothelial cell, which overlies a heterogeneous mass of collagen fibres and amorphous material, probably proteoglycan (short arrows). (x 23500).

A

B

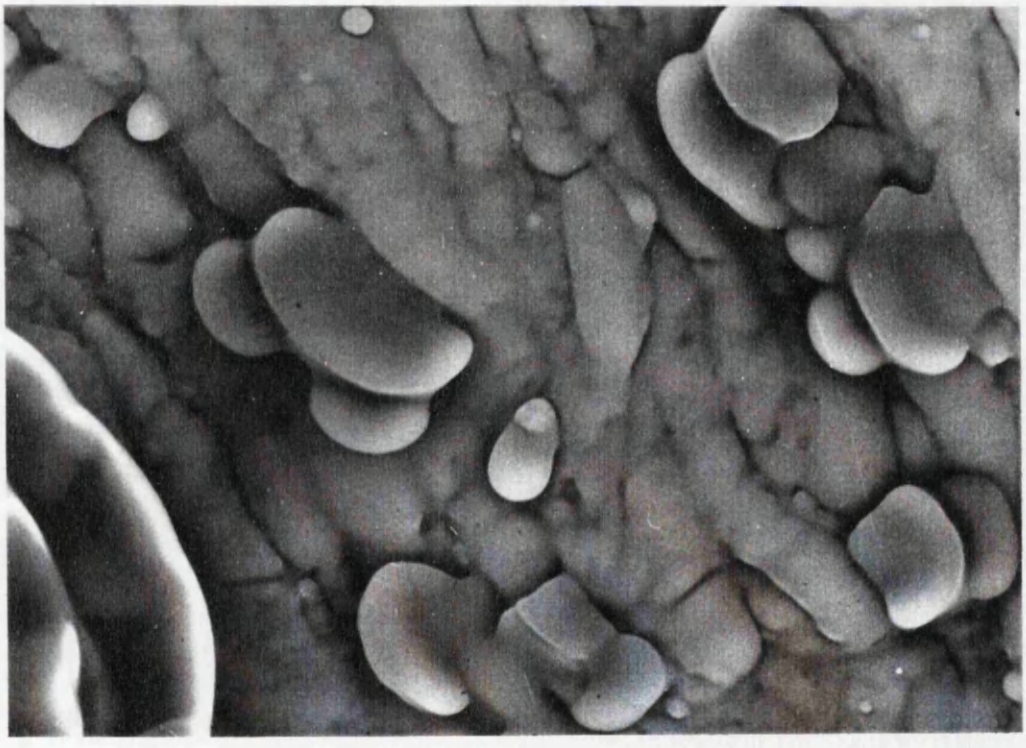


PLATE 4.7. Scanning electron micrograph showing endothelial aspects of the aorta from a 12 month old heterozygous WHHL rabbit showing abundant blebs on the cell surface. (a. x 561, b. x 3781).

iii. Structural characteristics.

In order to examine any possible relationship between the three aspects of the disease state, variables were plotted against one another and where appropriate, linear correlation and regression were analysed.

A. Relationship between serum cholesterol levels and function of the thoracic aorta.

The correlation of contractile function with serum cholesterol level at study was examined.

At the age of 3 and 6 months, there was no significant correlation between serum cholesterol level and the maximum response to phenylephrine (10^{-5}M) (Figure 4.31a & b). By the age of 12 months, there was a significant correlation between serum cholesterol level and contractile function (Figure 4.31c).

The correlation between serum cholesterol levels and maximum carbachol induced relaxation was also examined.

At the age of 3 months, no significant correlation was found. However, at both 6 and 12 months of age, a significant correlation was observed between serum cholesterol level and maximum relaxation induced by 10^{-5}M carbachol (Figure 4.32). At 6 months of age, the gradient of the line of regression was -1.85, by the age of 12 months this gradient had become much steeper with a value of -6.75. Thus, as the age of the animal increases the effect of the serum cholesterol on the ability of the tissue to relax becomes more dramatic.

B. Relationship Between Cholesterol Levels and Structural Parameters.

The correlation of serum cholesterol levels with sudan staining was examined (Figure 4.33). At the age of 3 months no significant relationship was found. At both 6 and 12 months of age, significant correlations were observed between serum cholesterol levels and sudan staining. At 6 months of age, the gradient of the line of regression was 2.73, by the age of 12 months this had increased to 8.74. Thus, as would be expected as the age of the animal increases, there is more of an effect of serum cholesterol on the level of sudan staining.

Similar patterns are observed with the % involved and the ratio of intima to media. At the age of 3 months, % involved was zero. At the age of 6 months there was a positive correlation between % involved and the serum cholesterol level, with the line of regression having a gradient of 2.09. At 12 months of age again there is a significant positive correlation and the gradient is relatively steeper than at 6 months at 7.61 (Figure 4.34).

At the age of 3 months the %I:M was zero in all animals, at 6 months there was a significant positive coorelation with a gradient of 0.734. At 12 months the gradient of the line of regression was 10.7 (Figure 4.35).

Thus, for all three parameters that were used for assessing changes in the structure of the thoracic aorta, it can be seen that as the age of the animal increases the effect of the serum cholesterol level on the structure becomes more apparent.

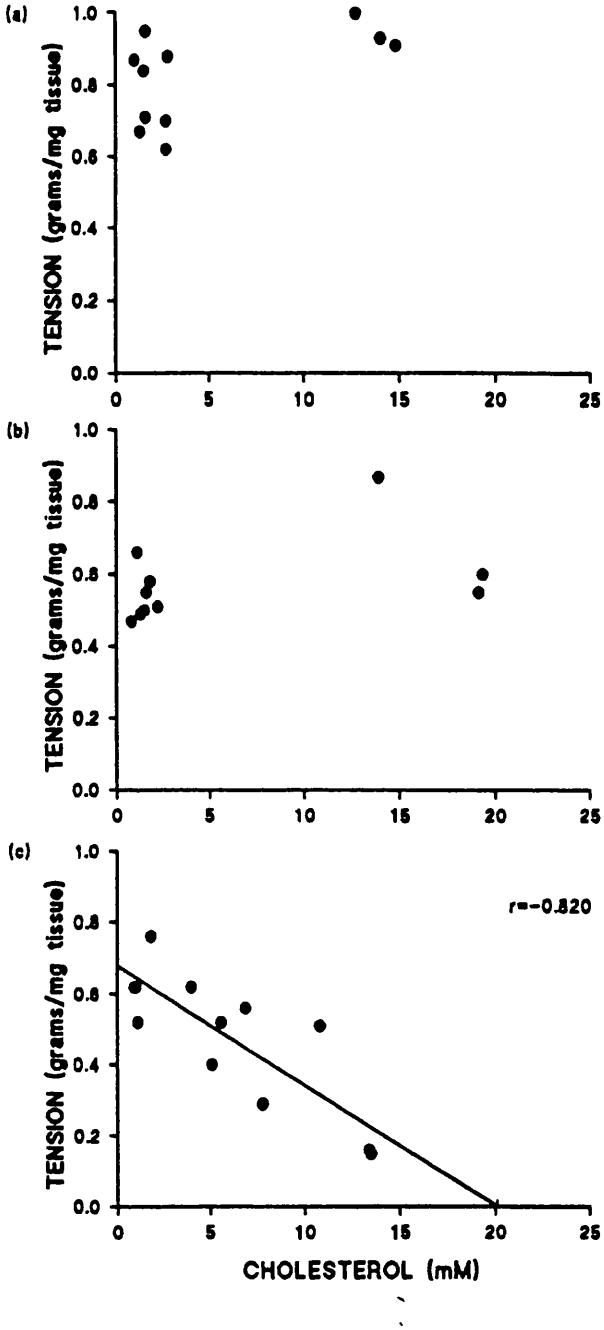


FIGURE 4.31. Bivariate plots of maximum phenylephrine induced contraction in rings of thoracic aorta (TENSION) versus serum cholesterol levels at study age - (a) 3 months, (b) 6 months and (c) 12 months. Where appropriate the correlation and fitted line are shown. n=11-14.

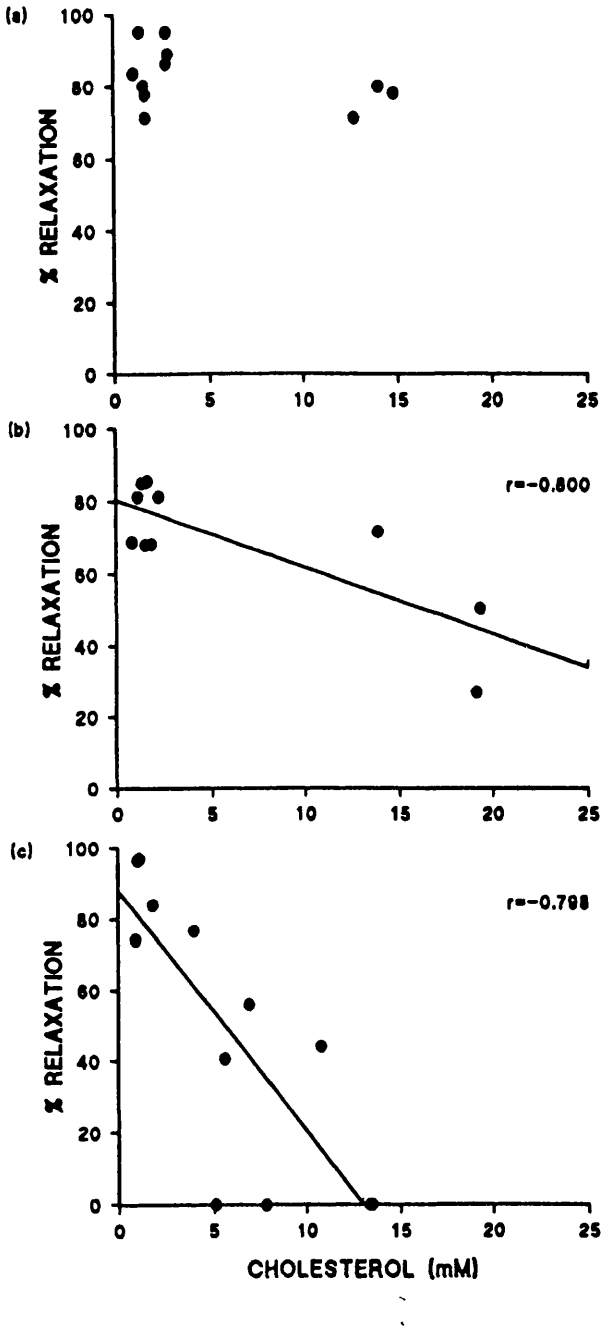


FIGURE 4.32. Bivariate plots of maximum carbachol induced relaxation in rings of thoracic aorta (% RELAXATION) versus serum cholesterol levels at study age - (a) 3 months, (b) 6 months and (c) 12 months. Where appropriate the correlation and fitted line are shown. n=11-14.

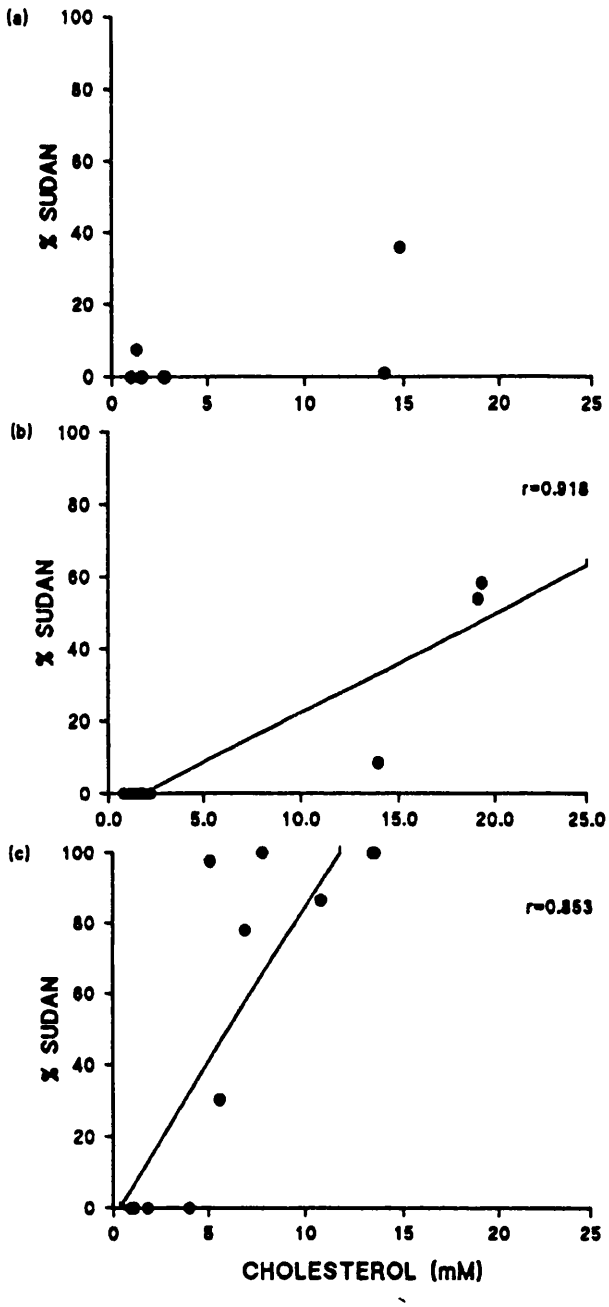


FIGURE 4.33. Bivariate plots of the percentage sudan staining (% SUDAN) in rings of thoracic aorta versus serum cholesterol levels at study age - (a) 3 months, (b) 6 months and (c) 12 months. Where appropriate the correlation and fitted line are shown. n=11-14.

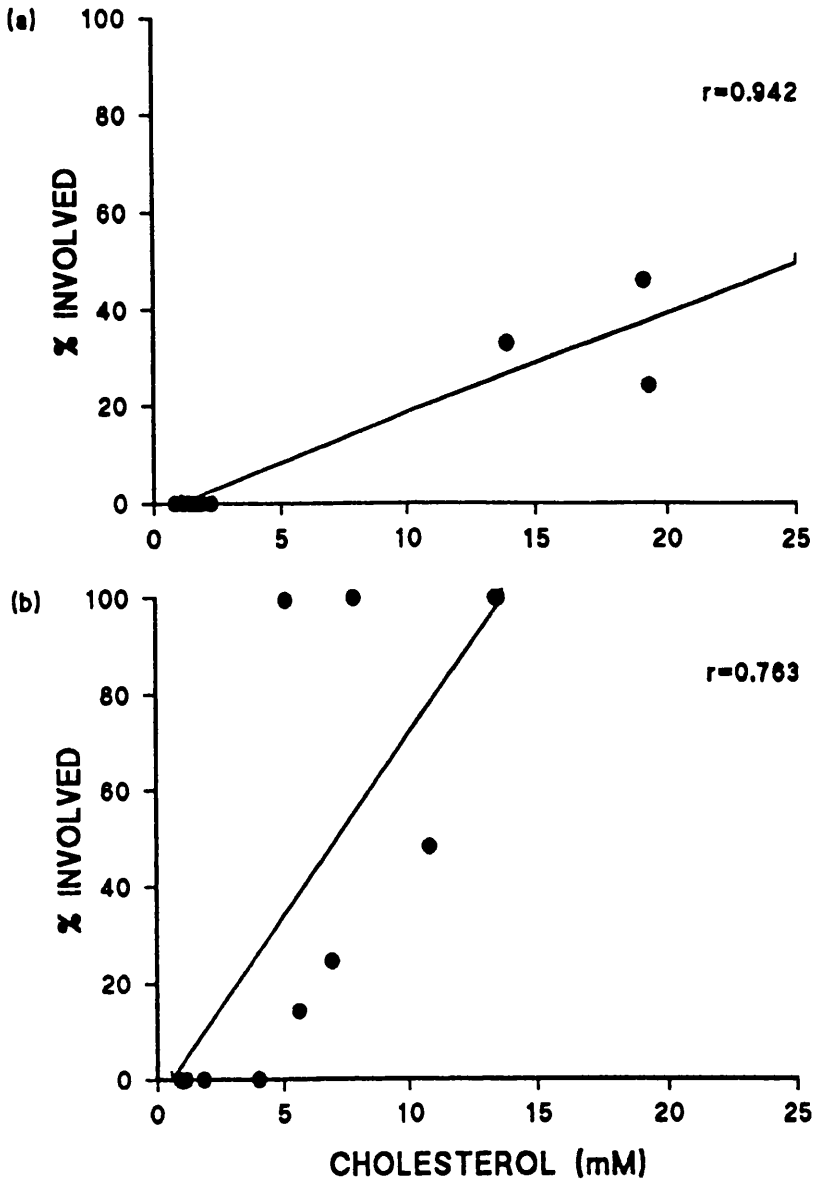


FIGURE 4.34. Bivariate plots of the percentage of the internal circumference involved in atheroma (% INVOLVED) in rings of thoracic aorta versus serum cholesterol levels at study age - (a) 6 months and (b) 12 months. Where appropriate the correlation and fitted line are shown. n=12-14.

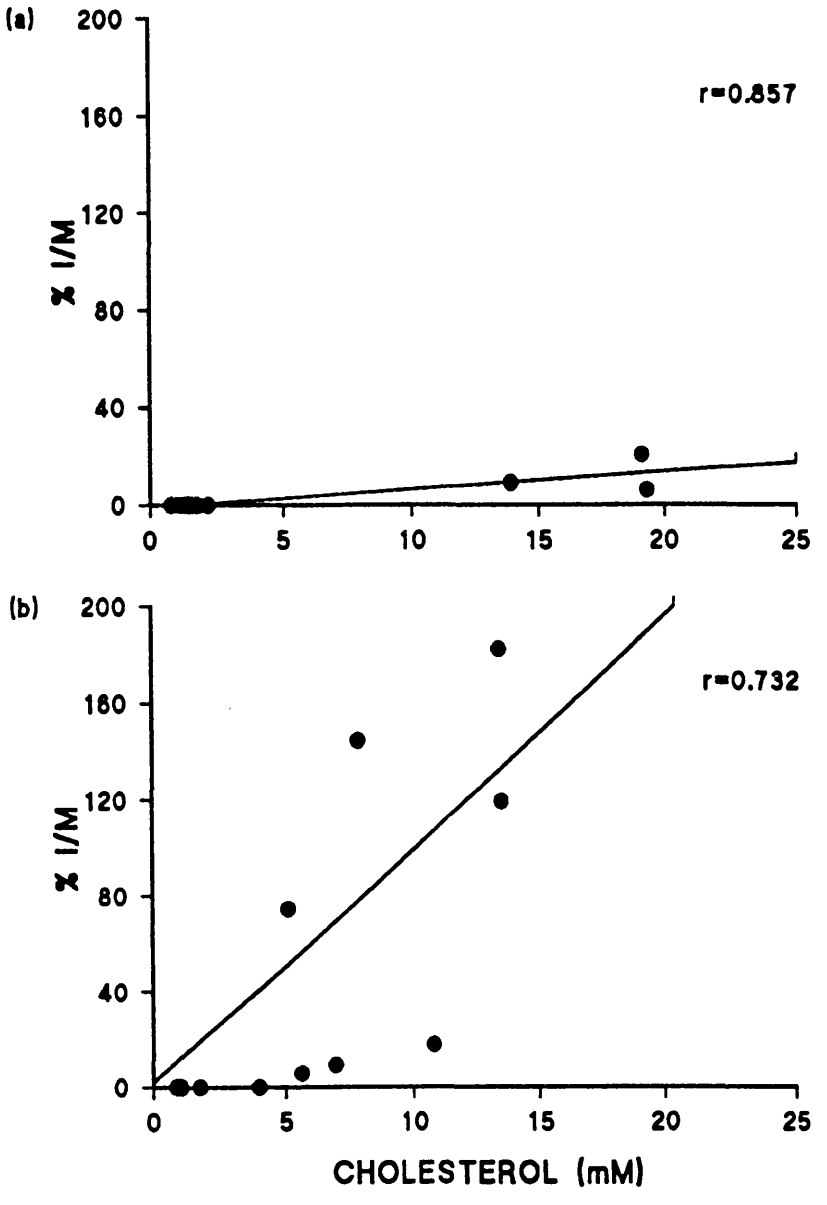


FIGURE 4.35. Bivariate plots of the percentage ratio of the area of intima to the area of media (%I/M) in rings of thoracic aorta versus serum cholesterol levels at study age - (a) 6 months and (b) 12 months. Where appropriate the correlation and fitted line are shown. n=12-14.

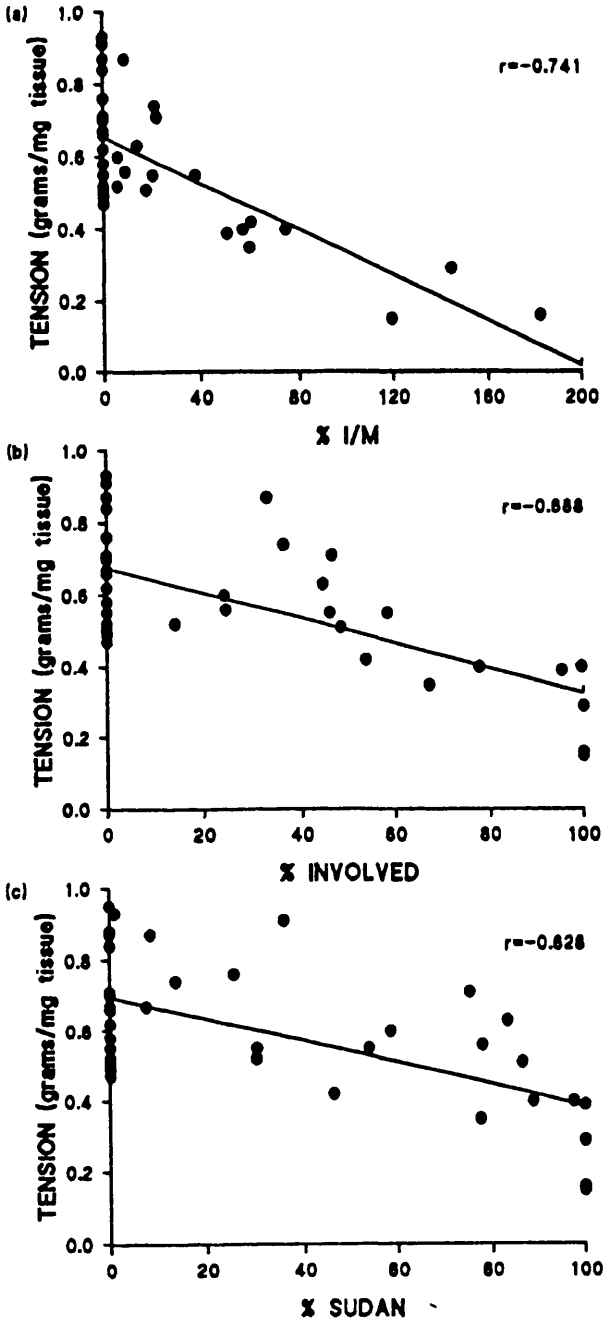


FIGURE 4.36. Bivariate plots of maximum phenylephrine induced contraction in rings of thoracic aorta (Tension) versus (a) the percentage ratio of area of intima to area of media (%I/M), (b) percentage of the internal circumference involved in atheroma and (c) the percentage sudan staining in thoracic aorta from WHHL rabbits. Where appropriate the correlation and fitted line are shown. n=43.

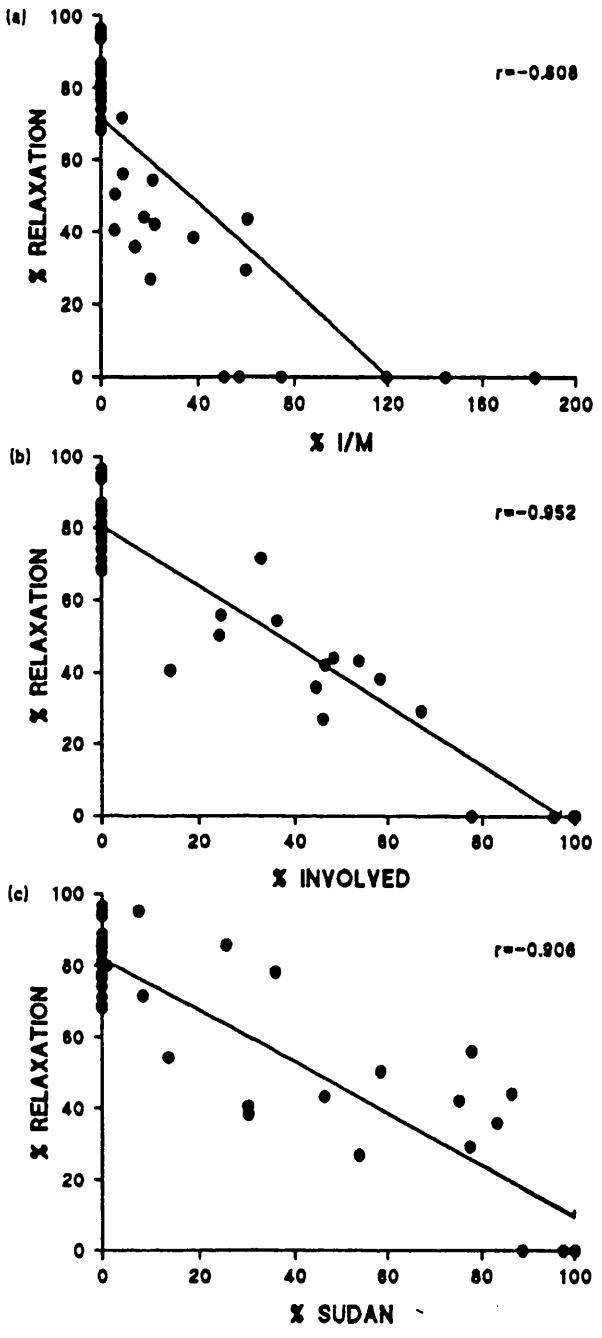


FIGURE 4.37. Bivariate plots of maximum carbachol induced relaxation in rings of thoracic aorta (% RELAXATION) versus (a) the percentage ratio of area of intima to area of media (%I/M), (b) percentage of the internal circumference involved in atheroma and (c) the percentage sudan staining in thoracic aorta from WHHL rabbits. Where appropriate the correlation and fitted line are shown. n=43.

C. Relationship Between Changes in Structure and Changes in Function.

The relationship between the three structural parameters and contractile function was examined. In all three cases, a significant correlation was found (Figure 4.36). Thus, the greater the changes in structure the less the vessel was able to contract.

The relationship between the three structural parameters and the relaxant properties of the vessel were also examined. For all three parameters, a significant correlation was found (Figure 4.37). For the correlations between relaxation and % sudan or % involved, the data was spread evenly around the line of regression. In the relationship between relaxation and % ratio of intima to media, the line of regression is displaced towards the right of the data. There are three data points whose % ratio of intima to media value exerts a large influence on the line of regression. Thus, if the line of regression was calculated without these points it would be much steeper. One interpretation of this fact is that once the %I:M gets to a certain value (~80%) the vessel is no longer able to relax, thus although the ratio is still able to increase it cannot exert any further influence on the relaxant properties of the vessel.

4.5 DISCUSSION.

The data presented in this chapter represent a study designed to provide an overview of changes associated with the development and progression of atherosclerotic disease in WHHL rabbits. The study is not confined to the homozygote rabbits of this strain but has encompassed the heterozygote rabbits as well. Initially, the heterozygote animals were studied in order to provide a control group for the homozygote rabbits. However the results have clearly demonstrated that there are pathological changes in both groups of animals. Thus, to enable a comparison to a normocholesterolaemic control, New Zealand White rabbits have also been studied.

There is very limited information in the literature regarding the heterozygote WHHL rabbits. However, the rationale for studying these animals is strong as, in the human condition of familial hypercholesterolaemia, the heterozygote state is a more common condition. Also, there is greater potential for clinical intervention as the disease does not produce symptomatic atherosclerosis until the fourth or fifth decade in man.

Serum cholesterol levels were used to categorise the WHHL rabbits at weaning. The homozygous animals have massive elevations in serum cholesterol due to their almost complete deficit of functional LDL receptors. As the homozygote animals increased in age there was a marked fall in serum cholesterol levels. This finding is consistent with other reports on changes in cholesterol levels during the maturation of WHHL (Chinellato et al, 1991; Fukuo et al, 1991; Giuirato et al, 1993; Kolodgie et al, 1990).

There is little information in the literature regarding cholesterol levels in heterozygous WHHL. One study by Esper et al in 1993 reports no change in serum cholesterol levels in heterozygote WHHL during the first eighteen months of life, by two years of age, they report a small rise in cholesterol levels. This is in accordance with the results of this study, in which cholesterol levels of the heterozygotes were found to remain constant throughout the twelve month study period.

One of the primary aspects of this study was to investigate changes in the vasoactive function in three arterial tissues :- the thoracic aorta, the aortic arch and the carotid artery.

In both the homozygous, heterozygous and New Zealand White rabbits it was observed that contractile function of the thoracic aorta, in response to phenylephrine

decreased with maturation. This trend was least apparent in the New Zealand White rabbits, and most dramatic in the homozygote rabbits. When the three groups of animals were compared at age matched points a slight hyper reactivity to phenylephrine was observed in the 3 month homozygous group. As the animals increased in age and the atherosclerotic disease progressed a significant decline in contractile function was observed in the homozygous group and to a lesser extent the heterozygous group when compared to the New Zealand White control.

Other studies examining adrenergic contractile function in WHHL rabbit thoracic aorta have produced a range of results. Kolodgie et al (1990) studied three homozygote age groups :- 3-5 months, 6-9 months and 12-14 months and compared these to two poorly age matched New Zealand White rabbit control groups of 5-6 months and 10-14 months. They observed that responses to phenylephrine were consistently less in the homozygous WHHL groups when compared to the New Zealand White controls. They also reported that their middle age group produced greater contractile responses than either the young or old groups. However the contractile responses in this report are expressed in grams tension, with no account taken for the change in tissue weight that occurs with age. As shown in Figures 4.5 and 4.6, this method of calculation can mask changes in response.

An earlier study using helical strips of thoracic aorta found no difference in response to phenylephrine when 8-12 month old homozygous WHHL rabbits were compared to age matched Japanese White rabbits (Yokoyama et al, 1983). The results of these experiments were expressed as a percentage of the maximum response obtained and thus is only examining the relative sensitivity to phenylephrine rather than the magnitude of response. Figure 4.16 shows, that in our study, there is no obvious shift to the left or right of the dose response curve and thus is in agreement with those of Yokoyama and colleagues.

Other studies have used noradrenaline as the adrenergic agonist. In 1989 Wines et al published a study using aortic rings from 1 and 6 month old homozygote WHHL and New Zealand White rabbits. No hyper-reactivity was observed when the responses of the homozygous WHHL were compared to the New Zealand White rabbits. However, as with our study, a decline in contractile function was observed from 1 to 6 months. The fact that no hyper-reactivity was observed may be due to the different age points studied. The hyper-reactivity seen in our study at 3 months may not have been developed at 1 month of age. The difference in observation may also be due to the fact that Wines and coworkers expressed their contraction as a percentage of a standard potassium chloride induced contraction. If there were differences in the potassium chloride induced contraction between groups this may affect the interpretation of the results. Similarly for the study by Chinellato and colleagues (1991), in this study of homozygous WHHL rabbits, three wide age groups were studied :- 4-6 months, 7-11 months and 12-14 months. They found little difference in noradrenaline induced contractile responses in thoracic aorta from the three groups. However, as above, the results were expressed as a percentage of the potassium chloride induced tone.

Finally, one further study examines adrenergic contraction in aortic rings (Ragazzi et al, 1989). This study found no difference between WHHL and New Zealand White rabbits at the age of 11-14 months. However the results are expressed in mN and thus no account was given to tissue weight.

These and other studies have examined other vessels and / or used other agonists. In 1992, Cirollo and coworkers reported no change in hind limb vascular resistance in response to phenylephrine or serotonin when comparing homozygote WHHL and

New Zealand White rabbits at 2 years of age. Brizzolara and coworkers (1992) reported an increased sensitivity to noradrenaline in rings of hepatic artery from homozygous WHHL compared to New Zealand White rabbits. Studies in rings of superior mesenteric artery have shown a hyper reactivity to noradrenaline in 4 month homozygous WHHL rabbits compared to New Zealand White rabbits and a decrease in reactivity to noradrenaline in 12 month WHHL rabbits compared to New Zealand White rabbits (Stewart-Lee et al, 1991b).

Another agonist in which there has been some interest is serotonin. Hyper-reactivity to serotonin has been reported in aortic rings from homozygous WHHL by three groups (Kolodgie et al, 1990; Wines et al, 1989; Yokoyama et al, 1983).

The contractile response of the aortic arch to phenylephrine was examined in our study and was found to reflect the changes observed in the thoracic aorta, this is also reported by Kolodgie and colleagues (1990).

The response of the carotid artery to phenylephrine was found to be virtually unaffected by either age or progression of the disease. This is in accordance with the study by Yokoyama and colleagues in 1983.

With regard to the heterozygous WHHL, there seems to be no comparable published work regarding the contractile function of the vasculature. Thus this study has provided information regarding the changes in contractile response to adrenergic agonists during the maturation of heterozygous WHHL rabbits up to one year of age.

Relaxation and the way in which it is affected by atherosclerosis has also been studied in WHHL rabbits. Though, as with the studies on contraction, there is virtually no data concerning the responses of the heterozygous animals.

Carbachol induced endothelium dependent relaxation was examined in homozygote and heterozygote WHHL and also in New Zealand White rabbit aorta. In the New Zealand White rabbits there was little change in the response up to 12 months of age. In the heterozygote WHHL a decline in the ability to relax was observed from 3 to 6 months of age, with no further change by 12 months. In the homozygotes there was a progressive loss of endothelium dependent relaxation from 3 through to 12 months of age. In all groups, at all ages a high dose of SNP was able to induce 100% non-endothelium dependent relaxation.

When the responses of the three groups of animals are compared at age matched points, it can be seen that even as early as 3 months of age the ability to relax in response to carbachol is impaired in the heterozygote WHHL aorta and to a greater extent in the homozygous WHHL aorta when compared to the control 3 month New Zealand White group. The difference between the groups becomes progressively greater by six months of age. By 12 months the functional relaxation in response to carbachol is almost completely lost in the homozygous WHHL rabbit aorta, however the heterozygotes maintain the same level of response that was observed at 6 months of age.

Other groups investigating the relaxant properties of the thoracic aorta have generally observed similar effects. Hirata and colleagues (1992) studied strips of aorta from homozygous WHHL and demonstrated a decrease in relaxation to acetylcholine from a 6-11 month group to a 12-18 month group. They also reported data for full dose response curves to SNP and found no significant difference between these two groups. Wines and coworkers (1989) used the muscarinic agonist methacholine to examine endothelium dependent relaxation in the aorta of

homozygous WHHL rabbits. They observed that, even as early as 3 months of age, there was a decrease in endothelium dependent relaxation in the homozygous WHHL compared to the New Zealand White control rabbits. Chinellato and colleagues (1991) also presented a study examining relaxation in the thoracic aorta from 3 age groups of homozygous WHHL. They found a decrease in the ability to relax from the 4-6 month group to the 7-11 month group, with little further change by 12-14 months. When compared to control responses from New Zealand White rabbit aorta, they observed the responses of the 4-6 month group were in fact greater in the WHHL group than the normocholesterolaemic control. However, with the older groups, this trend was dramatically reversed. Ragazzi and colleagues (1989) reported that acetylcholine induced endothelium dependent relaxation was impaired in homozygote WHHL rabbit thoracic aorta when compared to age matched New Zealand White control rabbits, but SNP induced non-endothelium dependent relaxation was unaffected. Koldgie and colleagues (1990) reported only a small decrease in aortic endothelium dependent relaxation with increasing age, using the agonists acetylcholine, ATP and the calcium ionophore A23187. They studied 3 groups of homozygous WHHL at 3-5 months, 6-9 months and 12-14 months of age.

Other groups have used other tissues to examine relaxation in WHHL rabbits. In 1992 Cirollo et al examined changes in hind limb blood flow in response to acetylcholine. They reported a non-significant decrease in blood flow in 2 year old WHHL when compared to age matched New Zealand White controls. No signifcant change was observed in the response to SNP.

Two studies have reported an increased reactivity to muscarinic endothelium dependent relaxation. The first involves the responses of the hepatic artery, where a decrease in acetylcholine induced relaxation was observed from 4 to 6 months, followed by an increase at 12 months. This effect was only observed in female and

not male WHHL rabbits (Brizzolara et al, 1992). The second study was in the basilar artery, in this instance responses were greater at six months than either at 4 or 12 months of age (Stewart-Lee et al, 1991a). Both authors suggest the role of a compensatory vasodilatory mechanism.

The responses of the aortic arch were also examined. In the homozygous animals the effects are similar to those in the thoracic aorta, but the extent of impairment is greater. This trend was also demonstrated by Kolodgie and colleagues (1990). In segments of aortic arch from the heterozygous WHHL, although there is less relaxation than in the thoracic aorta, there is no clear difference between the three age groups studied.

The reponses of the carotid artery did not seem to be affected either by age or progression of disease. There is no comparative data in the literature.

In order to allow interpretation of the changes in the vasoactive function that have been observed in this study, it is necessary to examine and quantify the parallel changes that are occuring in the structure of the vasculature. This is also necessary to allow a valid comparison with other reports from the literature. Although all WHHL rabbits descend from a single original colony, there will now be a wide variation between the various colonies around the world. This is due to the fact that, in order to improve breeding characteristics and general health of these animals, the original strain has been cross-bred by individual groups. Thus there will now be several variations of the "WHHL rabbit", these different colonies may develop atherosclerosis at different rates depending on the influence of other genetic characteristics.

Structural changes were assessed in four different ways :- quantification of sudan

staining, quantification of the percentage of the internal circumference of the thoracic aorta involved in atheroma, measurement of the ratio of the area of intima to the area of media and a visual assessment of the pathology of the sections.

The sudan staining technique gives information about fatty lesion development at a gross level. The percentage sudanophilia of samples of thoracic aorta was quantified using image analysis. In heterozygous animals sudan staining was minimal. In fact only two animals, one 3 month and one 12 month produced any positive stain. In homozygotes, even as early as 3 months 50% of the animals studied had some degree of sudan staining and it was found that sudan staining progressively increased with age.

The level of sudan staining in thoracic aorta from homozygous WHHL varies widely in the literature. To allow comparison, if the 9 month age point is used :- the lowest level of staining reported is 5% at 9 months (Kolodgie et al, 1990), other reports include 24% (Fukuo et al, 1991) and 71% (Daugherty et al, 1991). Thus the value in our study also 71% at nine months is broadly comparable.

The next two parameters used to assess structural changes in sections of thoracic aorta have little comparable data in the literature. The percentage of the internal circumference of thoracic aorta involved in atheroma, not suprisingly, demonstrated a similar pattern to that of the sudan staining. In sections from the heterozygote WHHL rabbits only one, from the 12 month old group, showed any detectable lesion. In the homozygote WHHL rabbits, as with the sudan staining, there was a progressive increase in the percentage involved from 3 months through to 12 months.

These two parameters, percentage sudan staining and percentage involved, reflect how the disease is progressively increasing to encompass more of the luminal surface

of the thoracic aorta.

The third parameter measured was the percentage ratio of area of intima to area of media. This parameter reflects how, once atheroma is initiated, the intima thickens as the plaque develops. Once the initial lesion has developed, more cholesterol is deposited and the endothelium is progressively separated from the smooth muscle. In the heterozygote WHHL rabbits this parameter was zero in all but one, 12 month old, animal i.e. in the majority of heterozygote sections, no intimal thickening was observed, this was also true of the 3 month old homozygote group. From 6 months upwards, in the homozygote WHHL rabbits, there was a progressive thickening of the area of intima in the thoracic aorta, thus an increase in the ratio of intima to media was observed. By the age of 12 months, the area of intima was on average 80% of the area of media. In some sections, the intima was nearly twice as thick as the media, representing massive cholesterol deposition and plaque development.

One other report in the literature uses the same parameter (Kolodgie et al, 1990). In this paper much less intimal thickening was observed in the thoracic aorta of homozygote WHHL at comparable age points, for example, only 10% by 12-14 months of age. However, in this study other structural parameters are comparably less, as discussed earlier.

In some of the more severe example of atheroma, the intima may be thicker than the media. The endothelium is, by this stage, widely separated from the medial smooth muscle cells and thus, even if it were functional and able to release vasoactive factors, the site of action for these factors is now far removed.

Sections of thoracic aorta from WHHL rabbits have been histologically assessed

previously, though as with the other aspects of the work on WHHL rabbits, the data presented in the literature is almost exclusively confined to the homozygote animals. Previous reports have discussed the similarities between atherosclerosis in WHHL rabbits and in the human condition of familial hypercholesterolaemia. Nolte and colleagues (1990) described atheroma in six WHHL rabbits between the ages of 5 and 15 months. The description of atheroma is comparable to that of the 12 month homozygote group in our study - they describe the presence of foam cells, connective tissue and cholesterol clefts in the intima extending through the internal elastic lamina into the media.

One study examined both homozygote and heterozygote WHHL rabbits. This colony is described as a "modified strain of WHHL rabbits" due to cross breeding with another commercial colony (Gallagher et al, 1988). However, as discussed, earlier this is probably true of most WHHL rabbit colonies. The study describes rabbits from 2 to 12 months. All homozygous animals develop aortic atherosclerosis by 4 months of age, early lesions have the histological appearance of fatty streaks and progress to complicated disease at 6-12 months. In the description of heterozygote WHHL rabbits, Gallagher and colleagues report no significant histological changes up to 6 months of age, with a distinctive pattern of calcific arteriosclerosis in most heterozygote WHHL rabbit aorta by 18 months of age.

Visual assessment of the sections of thoracic aorta revealed the development of classic atheroma - very similar in nature to atheroma found in humans. In a comparison of the pathobiology of WHHL rabbits and the human condition of familial hypercholesterolaemia, Buja and coworkers (1990) describe atherosclerosis in homozygous WHHL rabbits. They report initiation of atheroma in 3 month old animals - progressively developing to large raised lesions in older (6-15 months) animals. These lesions have central cores of extracellular lipid, peripheral regions of

foam cells and superficial fibrous capsules. As in our study, the endothelium of the lesions was generally intact, with electron microscopy revealing undulation of the endothelium and variation in endothelial cell shape. This study by Buja and colleagues also reports that the pathology of disease seen in the WHHL rabbits closely resembles that seen in human familial hypercholesterolaemia.

Hence, changes associated with the development and progression of atherosclerosis in both homozygous and heterozygous WHHL rabbits have been examined from three distinct angles, at four time points over a one year period :- cholesterol levels, vascular function in response to both constrictor and dilator agents and also changes in vascular structure at both macro- and microscopic levels. It is now possible to examine the inter-relationship between these three aspects of disease. As both heterozygote and homozygote WHHL rabbits have been studied there is a wide range of cholesterol levels and stages of disease to allow a valid analysis.

The relationship between serum cholesterol levels and vascular function was examined. At the age of 3 months, no significant relationship between cholesterol and phenylephrine induced contraction of the thoracic aorta was found. However, the slight hyper-reactivity to phenylephrine in homozygotes is reflected in Figure 28a. By the age of 12 months, a negative relationship is observed between cholesterol and the maximum phenylephrine response i.e. as the cholesterol level increases, the maximum responses to phenylephrine decreases.

Similar effects are seen when the relationship between serum cholesterol levels and the maximum carbachol induced relaxation is examined. No significant relationship is seen at 3 months, but by 6 months, a negative correlation is observed. By 12 months of age, a stronger relationship is found. Thus as cholesterol increases the maximum relaxation is found to decrease. It can also be seen that, as the animals get

older and the exposure time to cholesterol increases, the effect on relaxation becomes more pronounced.

From this data it can be seen that the changes observed in vascular function in WHHL rabbits are related to the elevated serum cholesterol levels which result from the defecit of functional LDL receptors.

Comparable trends were observed when the relationship between serum cholesterol levels and the three numerical histological parameters were examined. From the age of 6 months, there was a positive correlation between serum cholesterol levels and (i) percentage sudan staining , (ii) percentage involved and (iii) the percentage ratio intima to media. When the relationship was examined at 12 months an increase in gradient was seen in all three parameters. Thus it can be sugested that the changes in vascular structure in WHHL rabbits are related to the elevated serum cholesterol levels.

The relationship between vascular function and vascular structure was also examined. There was a negative relationship between maximum response to phenylephrine and both percentage sudan staining and percentage involved. However the slope of the line of regression is shallow, thus, even when 100% of the intima is covered with atheroma, the vessel still has the ability to constrict. However the relationship between maximum phenylephrine induced contraction and the ratio of intima to media has a much steeper line of regression. Thus the thickening of the plaque has a much greater effect on the ability of the vessel to contract - as the intima thickens vessel constriction is impaired. There was also a negative correlation between maximum carbachol induced relaxation and all three structural parameters. Thus the greater the development of atheroma - both in proportion of intima covered in atheroma and the relative thickness of the developed atheroma - the greater the

impairment of relaxation in response to carbachol.

The mechanisms by which the vascular responses are impaired has not been directly examined by this study. The effects on the contractile responses are complex. Initially, a hyper-reactivity to phenylephrine was observed in the homozygous group. Hyper-reactivity to other contractile agonists has been reported to occur under atherosclerotic conditions (Kolodgie et al, 1990; Yokoyama et al, 1983; Henry and Yokoyama, 1980). These studies reported hyper-reactivity to histamine and serotonin in WHHL rabbits, yet normal responses to the receptor independent constrictor KCl, indicating a receptor mediated enhancement of response. These authors suggest an increase in receptor density as the mechanism of increased responsiveness. Nanda and Henry (1982) published an abstract reporting an increase in the number of both serotonergic and α-adrenergic receptors in aortae from rabbits fed a high cholesterol diet.

As the animals increased in age, the hyper-reactivity appears to be overcome by the progression of the disease as the structural changes start to occur in the vessel. As the atheroma progressively intrudes into the media, causing disruption of the elastic laminae the ability of the vessel to constrict is impaired. This is seen most dramatically in the homozygote WHHL rabbits and to a lesser extent in the heterozygote WHHL rabbits, where the atheroma is minimal.

Migration of smooth muscle cells from the media to intima may also contribute to the observed loss of contractile function. It has been observed that smooth muscle cells, under certain conditions including atheroma, will alter their phenotype from the contractile status to the secretory status - thus the overall contractile ability of the vessel segment as a whole may be impaired. In our studies proteoglycan deposition was observed in the inner media, before the development of plaque. Proteoglycan

inhibits cell migration and can induce cell differentiation.

The effects of the disease on the relaxant properties of the vessel are also dramatic. Even in the initial stages of the disease, the ability of the vessel to relax in response to carbachol is impaired. This is illustrated in both the heterozygote and homozygote WHHL rabbits, where carbachol induced endothelium dependent relaxation is impaired even at 3 months - a stage at which structurally the vessels are virtually normal. Changes in function, prior to the manifestation of structural modification, were also observed in aorta from homozygote WHHL rabbits by Wines and colleagues (1989). Hence this indicates that some modification of the EDRF / NO pathway is responsible for the impairment of endothelium dependent relaxation. This modification is probably localised to the endothelium, as the experiments with SNP in this, and other studies (Kolodgie et al, 1990; Ragazzi et al, 1989), showed no change in responses to directly acting nitrovasodilators in the early stages of atherosclerosis. In 1991, Tagawa and coworkers used a cascade system to demonstrate that acetylcholine induced endothelium dependent relaxation is decreased in atheromatous aortic strips from homozygous WHHL rabbits. Furthermore, it has been shown in cholesterol fed rabbits that decreases in endothelium dependent relaxation can be reversed with the NO precursor L-nitro arginine (Girerd et al, 1990).

As the animals age and the disease progresses, the increased development of atheroma leads to a further impairment of the EDRF response. Two mechanisms could be responsible for this further loss in function. Firstly, the modification of the EDRF / NO pathway, discussed above, may develop further leading to a progressive loss in function. Secondly, as the atheroma develops the site of synthesis and the site of action of EDRF become spatially separated. Thus, as EDRF has a very short half-life, a progressively greater proportion of this labile compound will fail to reach

its site of action in its active form, and thus will fail to induce relaxation.

The mechanisms of the alterations in vasoactive responses could be further investigated. Ligand binding studies could be performed to investigate the role of α-adrenoceptor density in the hyper-reactivity to phenylephrine. In order to investigate the alterations in the EDRF / NO pathway studies could be carried out using cascade systems to determine if there is a decrease in the availability of EDRF. L-arginine, the precursor of nitric oxide and NO-synthase inhibitors such as L-N^G-nitroarginine (Moore et al, 1990) could be utilised to investigate the involvement of alterations in the synthetic pathway of NO.

So, in summary, it can be seen that as atherosclerosis develops in WHHL rabbits, a range of structural and functional changes take place in both the homozygote and heterozygote rabbits. The changes in function are related to the changes in structure. However, it should be noted that the vasoactive function of the aorta is modified before any gross morphological changes have occured. This is most clearly demonstrated in the heterozygote WHHL rabbits. In these animals changes in both phenylephrine induced contraction and carbachol induced endothelium dependent relaxation are seen, in rings of thoracic aorta which are considered to be histologically normal.

This study has provided novel information on the disease development in WHHL rabbits. One of the most interesting aspects is the studies in the heterozygote WHHL rabbits which, until now, have been virtually excluded from the research in this field.

CHAPTER FIVE.

EFFECTS OF SIMVASTATIN TREATMENT ON THE DEVELOPMENT AND PROGRESSION OF ATHEROSCLEROSIS IN WHHL RABBITS.

5.1 INTRODUCTION.

Population studies have shown that lipid lowering strategies are able to reduce the risk of coronary heart disease and myocardial infarction (Lipid Research Clinics Coronary Primary Prevention Trial, 1984a & 1984b). Clinically, dietary modification is often the first approach, however, as the greater proportion of cholesterol in the body is synthesised endogenously, this approach often fails to result in adequate cholesterol lowering. Thus pharmacological intervention is necessary. Several agents are currently available. These agents include bile acid binding resins (colestipol and cholestyramine), fibric acids (gemfibrozil, chlofibrate and fenofibrate), probucol, nicotinic acid and neomycin. All of these agents are capable of lowering serum cholesterol levels (Hoeg et al, 1986). However, a range of adverse effects are associated with these agents, which has limited their wide spread, long term use in the general population.

One possible therapeutic strategy to effectively lower cholesterol is the inhibition of endogenous cholesterol synthesis. Two papers in the 1960's reported details of two compounds :- Triparanol (MER-29) and AY-9944 which inhibited the later stages of cholesterol synthesis (Avigan et al, 1960; Dvornik et al, 1963). However, data were rapidly reported demonstrating that these compounds led to the accumulation of sterol intermediates which resulted in ichthyosis and posterior lenticular cataracts (Anchor et al, 1961; Laughlin and Carey, 1962). Hence, the development of these compounds was curtailed.

Subsequently, in 1976 Endo and his colleagues in Japan reported the development of a compound which was able to reduce blood cholesterol levels in hens and dogs (Endo et al, 1976b). This compound, mevastatin (compactin), had been isolated from the fungus Penicillium Citrinum. It was also demonstrated that mevastatin and its

analogues acted near the beginning of the biosynthetic pathway of cholesterol to inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase - a rate limiting enzyme in the biosynthetic pathway for cholesterol (Figure 1.1 and figure 5.1) (Endo et al, 1976a). A structurally related compound - lovastatin was later isolated from cultures of Aspergillus and Monascus species (Endo, 1979; Alberts et al, 1980). Many structural analogues of the two initial compounds have since been synthesised and evaluated, two of these compounds - pravastatin and simvastatin have been developed for clinical use along with mevastatin and lovastatin.

Structurally, the HMG CoA reductase inhibitors are composed of a hexahydroaphthalene ring system with two appendages:- a methylbutyrate ester and a hydroxyacid that can form a six membered lactone ring. All these compounds, except pravastatin, are administered as prodrugs in the lactone form. They are absorbed from the gastrointestinal tract and in the liver undergo chemical and / or enzymatic hydrolysis to the active β-hydroxyacid metabolite (Figure 5.1). (Pravastatin is administered as the acid and does not require activation). In this open, acid form it can be seen that there is a close structural analogy to the structure of HMG CoA itself. These compounds are then able to act as potent, reversible, competitive inhibitors of HMG CoA reductase.

The minor structural differences between the four compounds affect their individual potency and efficacy. The ability to inhibit HMG CoA reductase activity is approximately ten times higher for simvastatin (Ki Value = 1.2×10^{-10}M) than pravastatin (Ki Value = 2.3×10^{-9}M) (Hoeg and Brewer, 1987).

Clinically, all the HMG CoA reductase inhibitors are reported to have similar effects. For the purpose of this discussion, the focus will be on simvastatin as this is the compound which has been used experimentally in this study. The HMG CoA

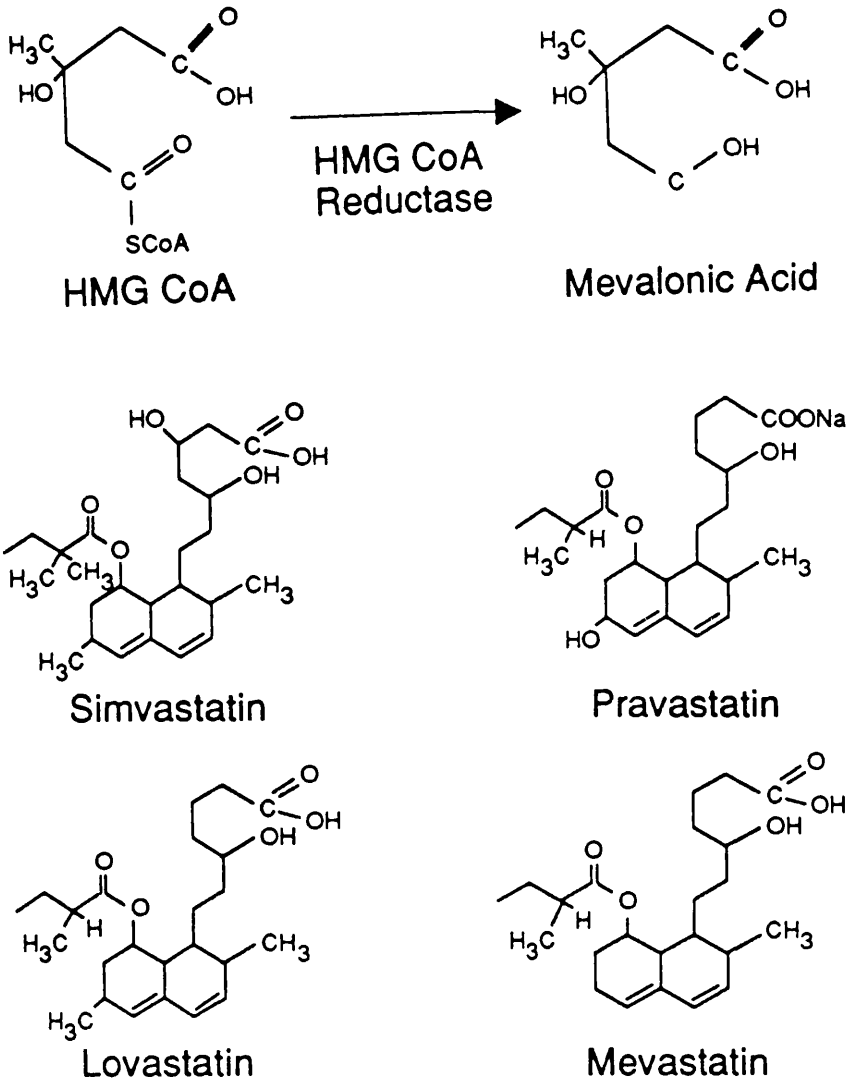


FIGURE 5.1. Chemical structures of HMG CoA and its' derivative mevalonic acid. Also shown chemical structures of the HMG CoA reductase inhibitors simvastatin, pravastatin, lovastatin and mevastatin in the active β-hydroxyacid form.

reductase inhibitors have been shown to be effective lipid lowering agents in patients with hyperlipidaemia. Typical falls in LDL-cholesterol following treatment with simvastatin range from approximately 20% at 2.5 mg/day to around 40% ar 20 mg/day (Todd and Goa, 1990).

Despite the fact that these compounds are known to potently inhibit HMG CoA reductase, their precise mechanism of action remains unclear. The enzyme HMG CoA reductase is controlled by a multivalent feed back pathway. The end products of the mevalonate pathway :- cholesterol, dolichol, ubiquinone and isopentenyl tRNA act to suppress HMG CoA reductase through negative feedback on the enzyme (Figure 1.1). Thus, when the HMG CoA reductase inhibitors lead to a decrease in synthesis of cholesterol and the other end products of the pathway, this negative feedback is removed. This leads to an increase in the synthesis and the activity of the enzyme (Henwood and Heel, 1988). This has been shown experimentally in cultured human fibroblasts, where inhibition of HMG CoA reductase leads to increased amounts of the enzyme due to an increase in the rate of transcription and translation (Brown et al, 1978). Through these compensatory mechanisms, these cultured cells are able to restore the rate of cholesterol synthesis almost to normal even in the presence of relatively high concentrations of the inhibitor. In humans, it has been shown that there is no correlation between the magnitude of change in the rate of cholesterol synthesis and the decrease in plasma LDL (Grundy and Bilheimer, 1984). This suggests that some other mechanism must be responsible for the observed decreases in plasma cholesterol.

It has been proposed that the fall in LDL cholesterol results predominantly from an increase in the receptor mediated clearance of LDL and IDL from the plasma and that this increase in clearance results from an increase in the number of hepatic LDL receptors. Reductase inhibitors induce an increase in LDL receptor number by

decreasing the cholesterol mediated feedback on LDL receptor synthesis. A short DNA sequence, designated the sterol regulatory element occurs in the promoter regions of the genes for HMG CoA synthase, HMG CoA reductase and the LDL receptor. Normally when sterol accumulates within the cell it acts upon the sterol regulatory element to decrease the transcription of all three genes (Smith et al, 1988). This repression appears to protect the cell from excessive cholesterol accumulation by limiting uptake and decreasing synthesis of LDL when appropriate. Thus in the presence of HMG CoA reductase inhibition, this regulatory control is removed due to the decrease in cellular cholesterol levels and thus the rate of transcription of all three genes will be increased. Hence there will be an increase in activity of HMG CoA synthase and reductase - restoring cholesterol synthesis to normal, and there will be a concurrent increase in the number of LDL receptors thus enhancing IDL and LDL clearance from the plasma (Goldstein and Brown, 1984).

Simvastatin has also been shown to inhibit the absorption of [^{3}H]-cholesterol from the gastrointestinal tract of rabbits fed a high cholesterol diet (Ishida et al, 1990). This is due to a decrease in the activity of intestinal acyl coenzyme A : cholesterol acyl transferase which participates in the absorption of exogenous cholesterol. Similarly in humans, pravastatin has been shown to inhibit cholesterol absorption in subjects with familial hypercholesterolaemia (Miettinen, 1991).

Clinical trials with the HMG CoA reductase inhibitors have shown beneficial effects on plasma LDL concentrations. Dose dependent decreases in plasma levels of total and LDL cholesterol have been reported in patients with heterozygous familial hypercholesterolaemia (Illingworth and Sexton, 1984; Havel et al, 1987; Quincey et al, 1992). In patients with homozygous familial hypercholesterolaemia the effectiveness of HMG CoA reductase inhibitors is dependent on whether the patient is receptor negative, in which case the inhibitors are ineffective (Uauy et al, 1988), or

receptor defective, where moderate reduction in LDL-cholesterol levels are found (Thompson et al, 1986; Laue et al, 1987).

To date, these studies have only examined the effect of these compounds on cholesterol levels, with no data available on the long term efficacy of these compounds on reducing the development of atherosclerosis. Studies in WHHL rabbits would provide information on the potential benefits of the HMG CoA reductase inhibitors in LDL receptor deficient disease states.

Several groups have conducted studies with HMG CoA reductase inhibitors in homozygous WHHL rabbits. Primarily, these studies have examined the effects of pravastatin on cholesterol levels (Tsujita et al, 1986; Watanabe et al, 1988; Khachadurian et al, 1991; Kishida et al, 1991; Kuroda et al, 1992). Two of these studies also examined the effect of pravastatin on the development of atheroma (Watanabe et al, 1988; Khachadurian et al, 1991). Fukuo and colleagues (1991) studied the effects of simvastatin on cholesterol levels and aortic atheroma.

Other groups have investigated the effects of other lipid lowering / anti-atherogenic strategies in homozygous WHHL rabbits. The effects of probucol have been examined by several groups (Kita et al, 1988; Steinberg et al, 1988; Daugherty et al, 1991; Mao et al, 1991; Nagano et al, 1992). Each of these reports demonstrated that probucol was able to significantly retard the progression of atheroma. However it has been proposed that this is, at least in part, due to the antioxidant properties of probucol rather than its hypolipidaemic effects (Mao et al, 1991). Several other agents have also been shown to decrease atheroma in homozygous WHHL rabbits including the ACE inhibitors captopril and trandolapril (Chobanian et al, 1990; Chobanian et al, 1992), Vitamin E (Williams et al, 1992), and fish oil (Lichtenstein and Chobanian, 1990).

However none of these studies have examined vascular reactivity following lipid lowering therapy. Furthermore, no published studies have examined the effect of lipid lowering or anti-atherogenic therapy in the heterozygote WHHL rabbits. This type of study would be of utmost clinical relevance, as it is subjects with heterozygous familial hypercholesterolaemia who could most clearly benefit from an effective lipid lowering and anti-atherogenic therapy with HMG CoA reductase inhibitors in order to prevent or minimise the inevitable development of atherosclerosis and coronary heart disease.

The potential use of these agents in homozygous familial hypercholesterolaemia is also of importance. An effective lipid lowering strategy in these subjects may help to retard the onset and / or impede the development of the severe atherosclerosis observed as a result of this disease state.

5.2 AIMS.

The aims of the experiments described in this chapter were to assess the effects of the HMG CoA reductase inhibitor simvastatin on the development and progression of atherosclerotic disease in WHHL rabbits. Simvastatin was chosen due to its high *in vitro* efficacy and on the basis of preliminary studies in the department (Hassan, 1990).

5.3 METHODS.

The effects of treatment with simvastatin were examined in young rabbits treated from the age of 3 months until they were studied at 6 months of age and also in an

older group of rabbits, treated from the age of 9 months until they were studied at the age of 12 months. These treatment groups were studied in both homozygous and heterozygous WHHL rabbit groups.

The parameters used to assess the effects of the treatment were the same as in the control studies discussed in Chapter four. Serum cholesterol levels were measured. Vascular reactivity was assessed in the thoracic aorta, examining both phenylephrine induced contraction and carbachol induced endothelium dependent relaxation. Pathological changes in vascular structure were quantified using percentage sudan staining, percentage of the internal circumference involved in atheroma and the percentage ratio of the area of intima to the area of media.

The treatment protocol for the study with simvastatin is described below :-

Rabbits were randomly allocated to treatment or control groups. Four groups of rabbits were treated with simvastatin :-

Group 1 - homozygous WHHL treated from the age of 3 to 6 months.
Group 2 - heterozygous WHHL treated from the age of 3 to 6 months.
Group 3 - homozygous WHHL treated from the age of 9 to 12 months.
Group 4 - heterozygous WHHL treated from the age of 9 to 12 months.

The simvastatin was administered orally at a dosage of 10 mg / Kg / day. The drug was dissolved in ether and applied to cabbage leaves, the ether subsequently evaporated and the drug was left coating the cabbage leaf. The cabbage was fed to the rabbit and was normally eaten immediately.

5.4 RESULTS.

5.4.1 Effects of Simvastatin Treatment on Cholesterol Levels in Homozygous WHHL.

Treatment with simvastatin (10 mg/Kg/day) had no significant effect on serum cholesterol levels in homozygous WHHL rabbits (Figure 5.2). In the group treated from 3 to 6 months of age there was a slight, non-significant fall in the treated group compared to the untreated group. In the older group treated with simvastatin from 9 to 12 months of age there was little difference in the cholesterol levels at the time of study.

5.4.2 Effects of Simvastatin Treatment on Cholesterol Levels in Heterozygous WHHL.

Treatment with simvastatin (10 mg/Kg/day) lead to a significant decrease in serum cholesterol levels in heterozygous WHHL rabbits treated from 3 to 6 months of age when compared to the untreated 6 month old group. In the older heterozygous WHHL rabbits there was a non significant decrease in cholesterol levels when compared to the untreated group (Figure 5.3).

5.4.3 Effects of Simvastatin Treatment on Functional Responses in Thoracic Aorta from WHHL.

A. Phenylephrine Induced Contraction.

Four groups of WHHL rabbits were treated with simvastatin. In homozygous WHHL

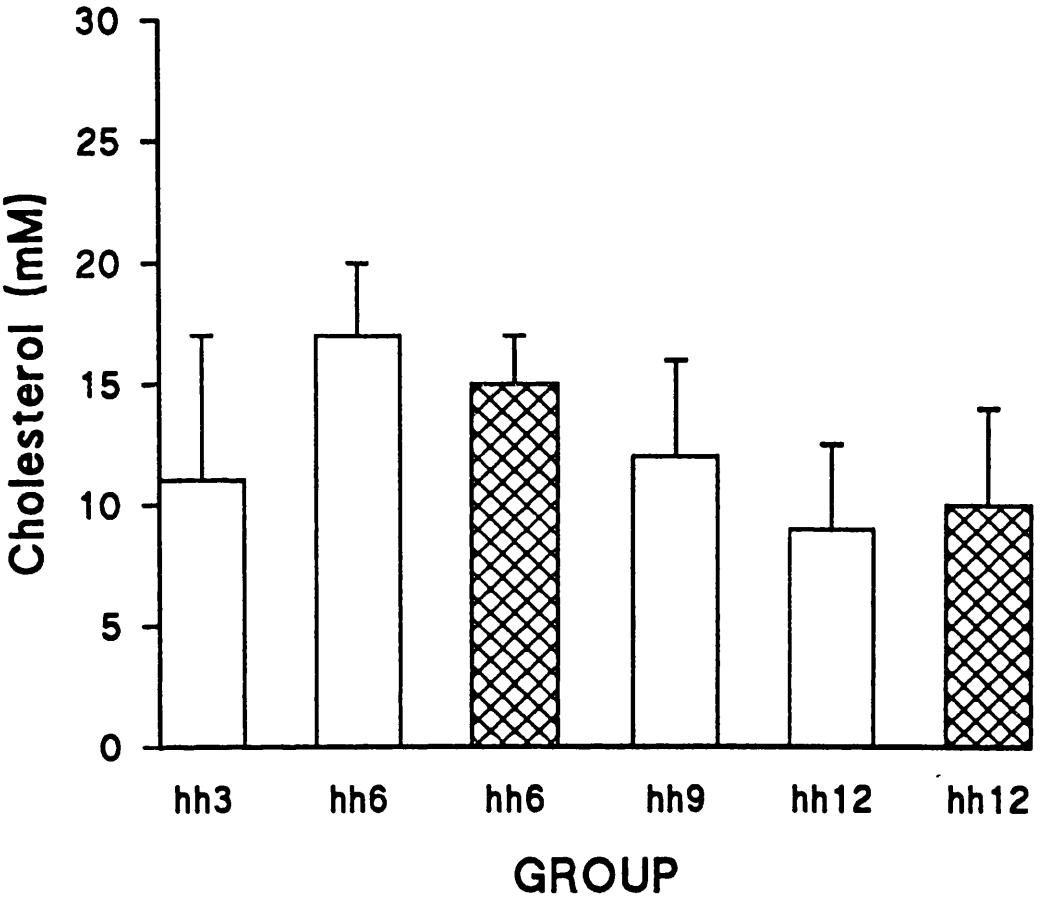


FIGURE 5.2. Serum cholesterol levels in homozygous WHHL rabbits at the age of study. Open bars are control rabbits, hatched bars are rabbits treated with Simvastatin. Data is mean ± s.d. n=4-7.

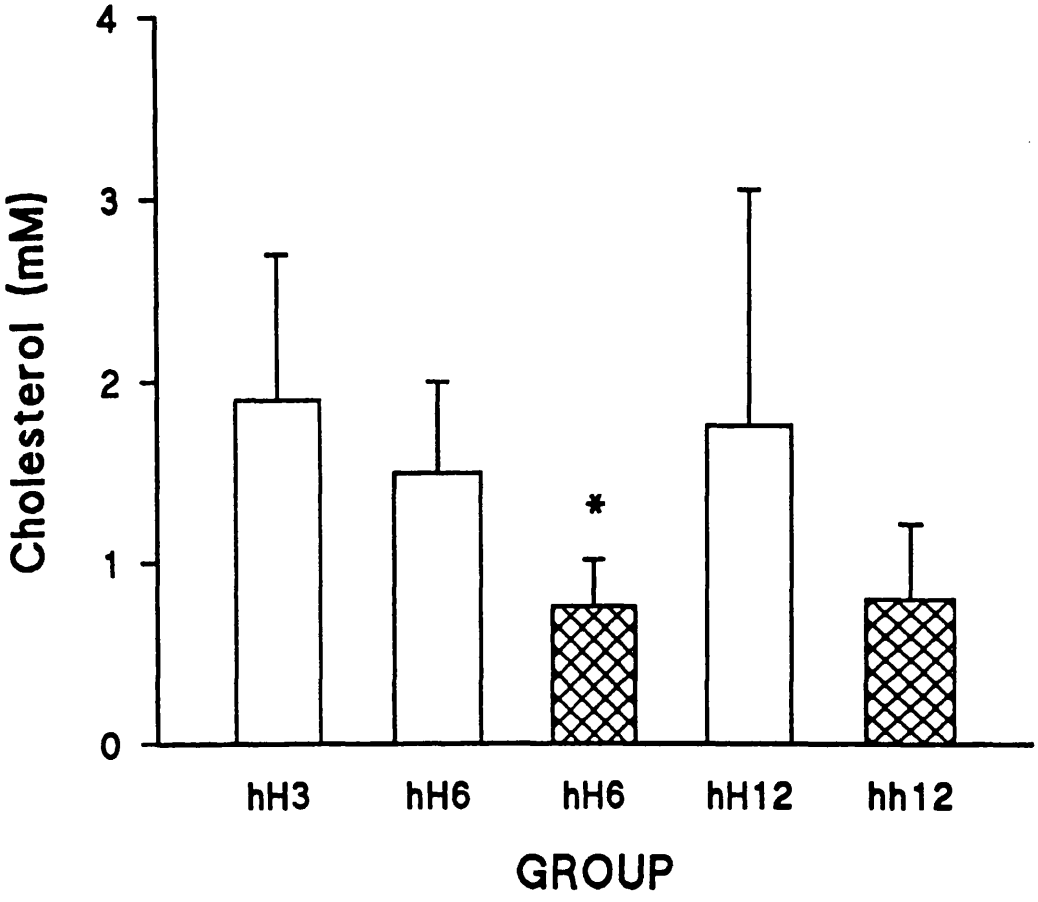


FIGURE 5.3. Serum cholesterol levels in heterozygous WHHL rabbits at the age of study. Open bars are control rabbits, hatched bars are rabbits treated with Simvastatin. Data is mean ± s.d. n=5-7.

* - $p < 0.05$ compared to age matched control group.

rabbits which were treated from with simvastatin from the age of 3 to 6 months there was a small, non-significant, retardation of the loss of phenylephrine induced contraction that had been observed in rings of thoracic aorta between the ages of 3 and 6 months (Figure 5.4).

In homozygous WHHL rabbits which had been treated with simvastatin from the age of 9 to 12 months, there was no difference in the response of the aorta to phenylephrine when compared to the 12 month untreated group (Figure 5.5).

In heterozygous WHHL rabbits, which were treated with simvastatin from the age of 3 to 6 months, there was a significant retardation of the loss of phenylephrine induced contraction which had been observed in thoracic aorta rings from the age of 3 to 6 months (Figure 5.6). The difference between the 6 month untreated group and the 3 month group was marginally non-significant at 10^{-7}M phenylephrine, 95% C.I. (-0.199,0.024) and more clearly significantly different from 3 x 10^{-7}M to 10^{-5}M phenylephrine. This is in contrast with the statistical analysis in Chapter 4 (Figure 4.8), where the analysis indicated that there was a marginally significant difference at 10^{-7}M. These analyses do not actually contradict each other, if the confidence intervals are compared it can be seen that they are in agreement with each other. The difference arises simply from the influence of the whole data set under analysis at each time. The whole set of data is used to estimate the variance, and thus calculate confidence intervals. Hence, the same data analysed within two separate data sets may produce slightly different results. However, as stated earlier, the trend towards significance at this point is clear.

The 6 month heterozygous group was not significantly different from the 3 month group, but was significantly different from the 6 month untreated group from 3 x 10^{-7}M to 10^{-5}M phenylephrine.

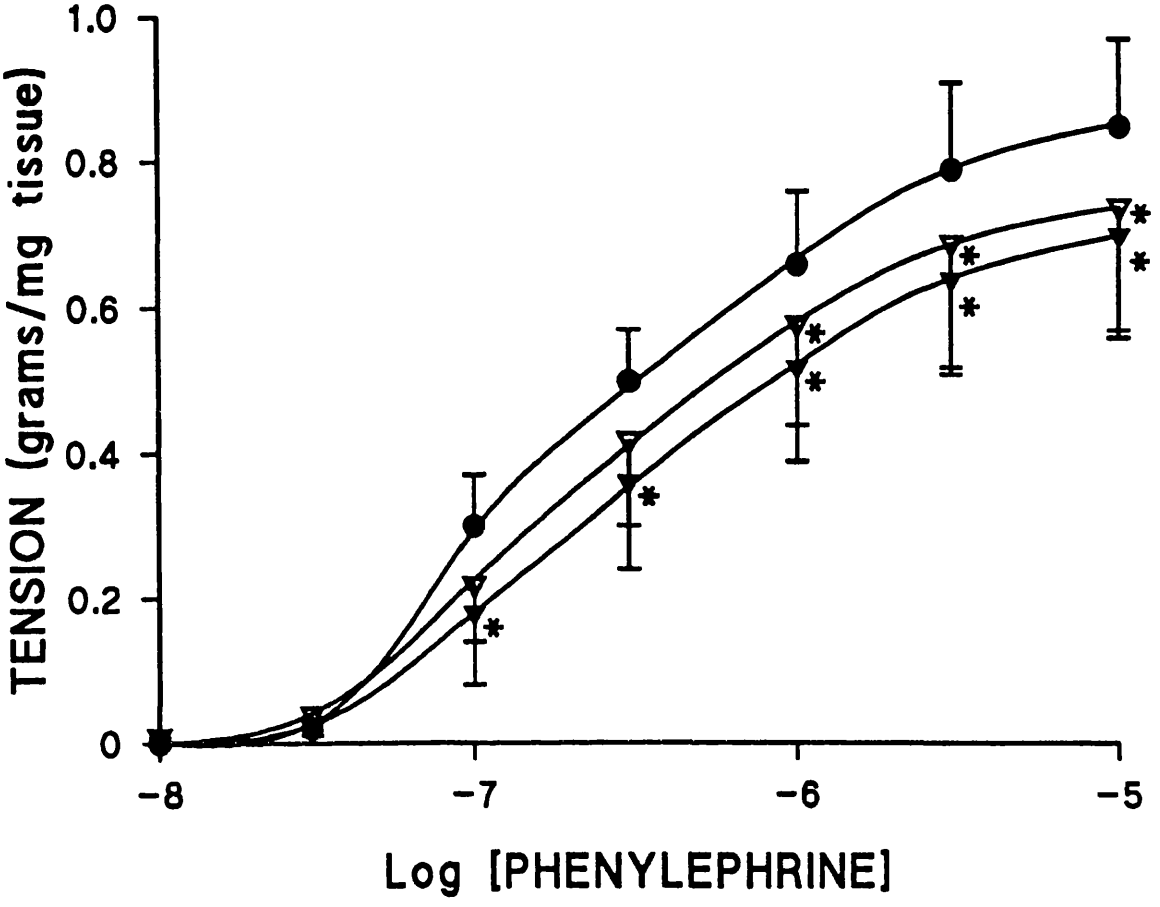


FIGURE 5.4. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months.	n=4.
▼ - 6 months.	n=5.
∇ - 6 months treated with Simvastatin.	n=5.

* - $p < 0.05$ compared to 3 month control.

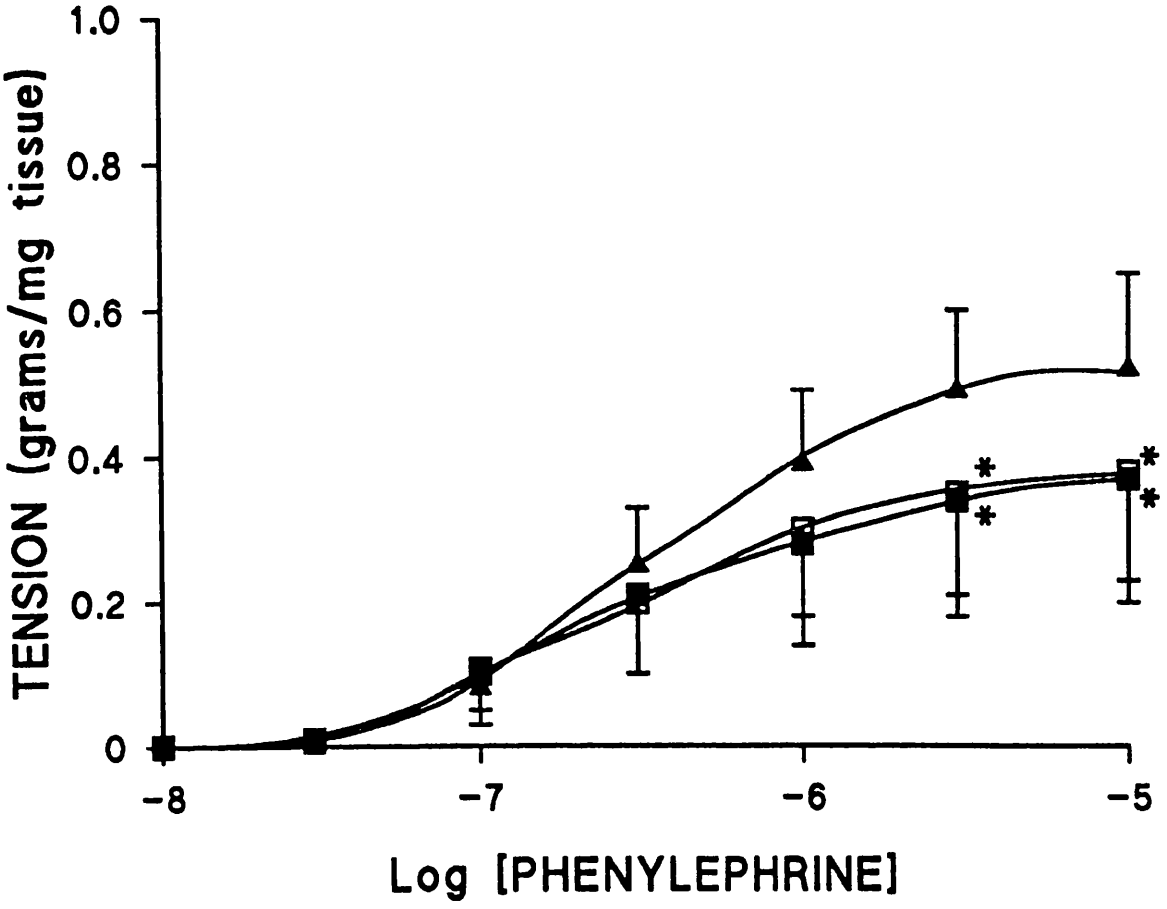


FIGURE 5.5. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

▲ - 9 months. n=6.
■ - 12 months. n=7.
□ - 12 months treated with Simvastatin. n=6.

* - $p < 0.05$ compared to 9 month control.

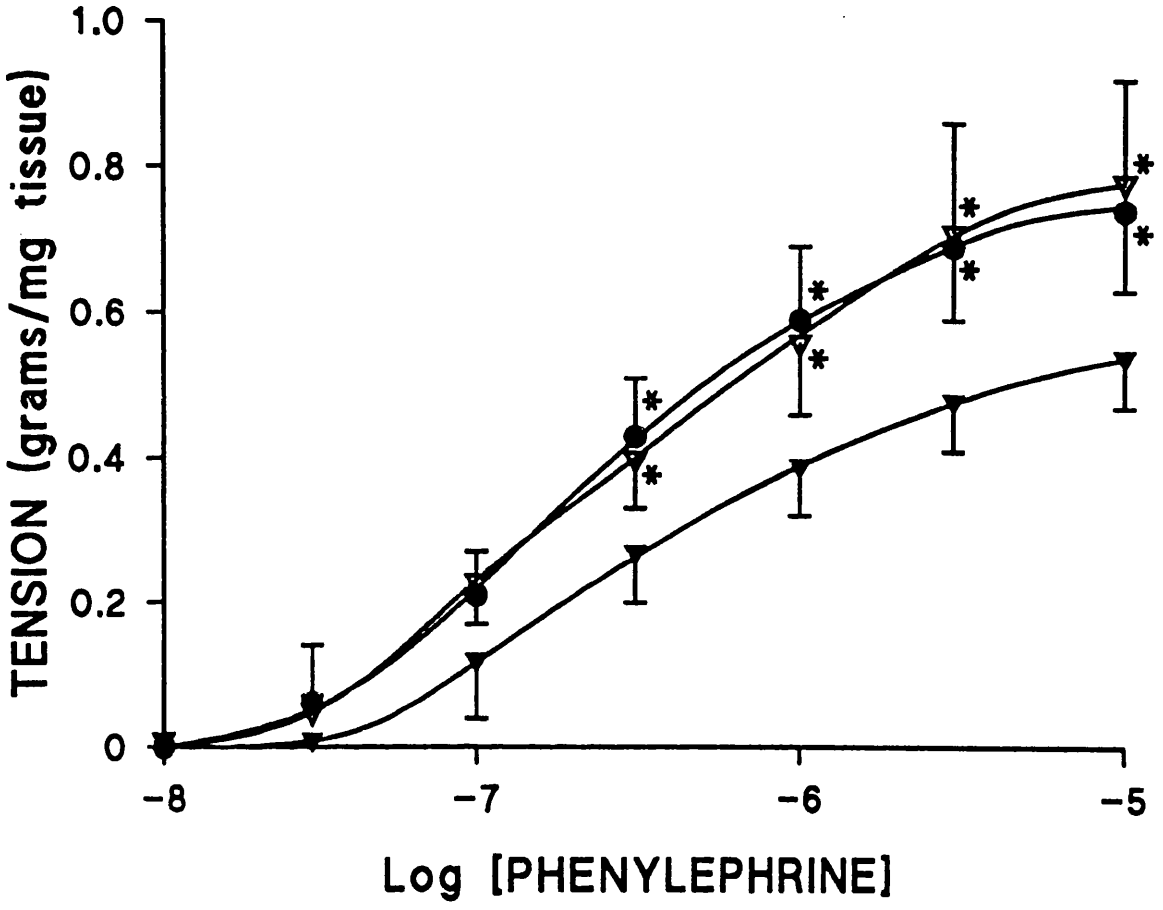


FIGURE 5.6. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

● - 3 months. n=7.
▼ - 6 months. n=7.
∇ - 6 months treated with Simvastatin. n=5.

* - $p < 0.05$ compared to 6 month control.

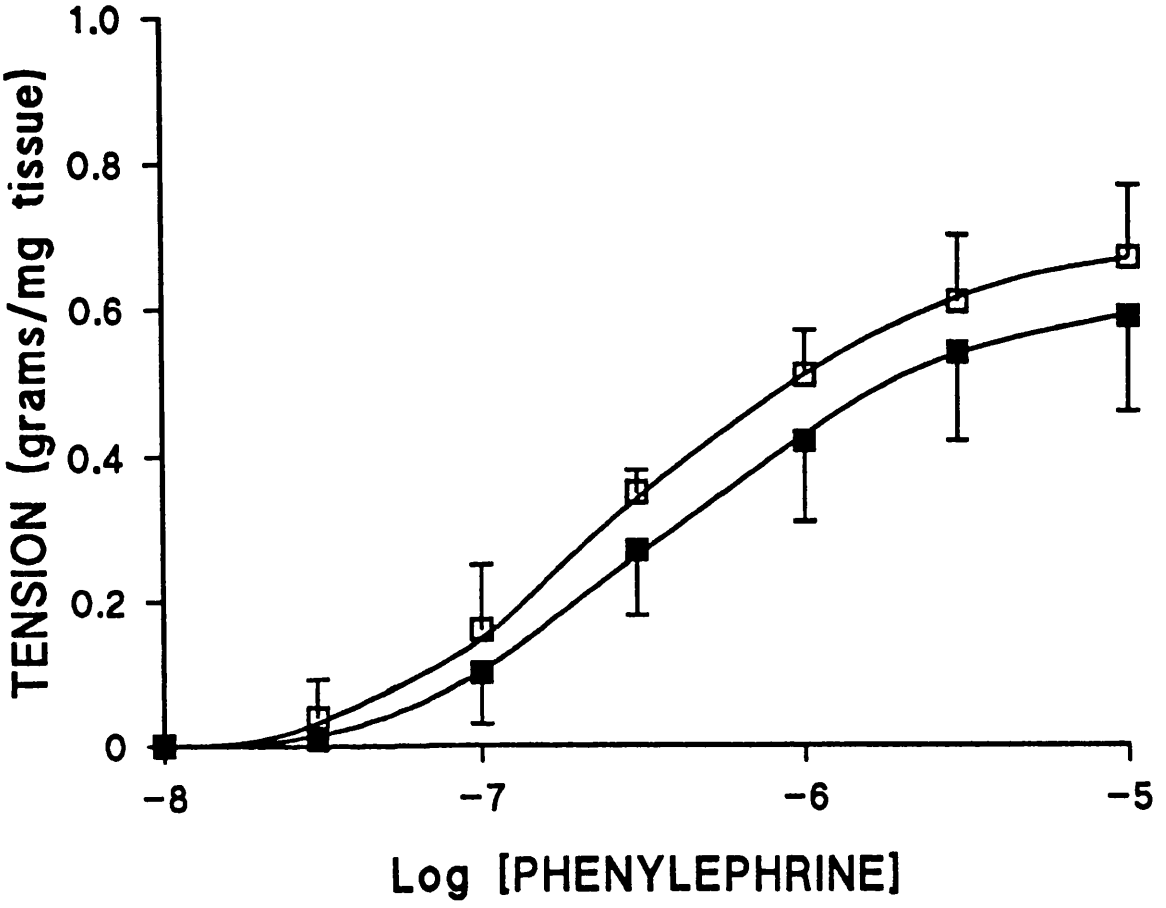


FIGURE 5.7. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed in grams tension / mg tissue. Data is mean ± s.d.

■ - 12 months. n=7.
□ - 12 months treated with Simvastatin. n=5.

In heterozygous WHHL rabbits which were treated from the age of 9 to 12 months there was a small non-significant increase in contractile function (Figure 5.7).

B. Carbachol Induced Relaxation.

The effects of simvastatin treatment on carbachol induced endothelium dependent relaxation was examined in the four groups of rabbits.

In homozygous WHHL rabbits which were treated with simvastatin from the age of 3 to 6 months there was an improvement in function ie. an increase in the level of relaxation in rings of thoracic aorta (Figure 5.8). There was no significant difference between the 3 month homozygote group and the 6 month untreated group. This is in contrast to the statistical analysis in Chapter Four (Figure 4.11). Previous analysis had indicated that there was a marginally significant difference between the 3 and 6 month homozygous groups at 10^{-6}M and 3 x 10^{-6}M carbachol. The analysis carried out with the simvastatin data indicated that the difference at these points is non-significant, 95% C.I. (-46.982,6.183 and -47.825,5.340 respectively). As discussed above, the two separate analyses do not contradict each other, the difference is simply due to the influence of the entire data sets in each analysis.

Thus, it can be seen that there is a trend towards a decrease in response when the 6 month group is compared to the 3 month group. However, the 6 month treated group was not significantly different from either the 3 or the 6 month group.

In the homozygous rabbits that were treated with simvastatin from the age of 9 to 12 months there was no effect on carbachol induced relaxation of thoracic aorta rings when compared to the 12 month homozygous untreated group (Figure 5.9).

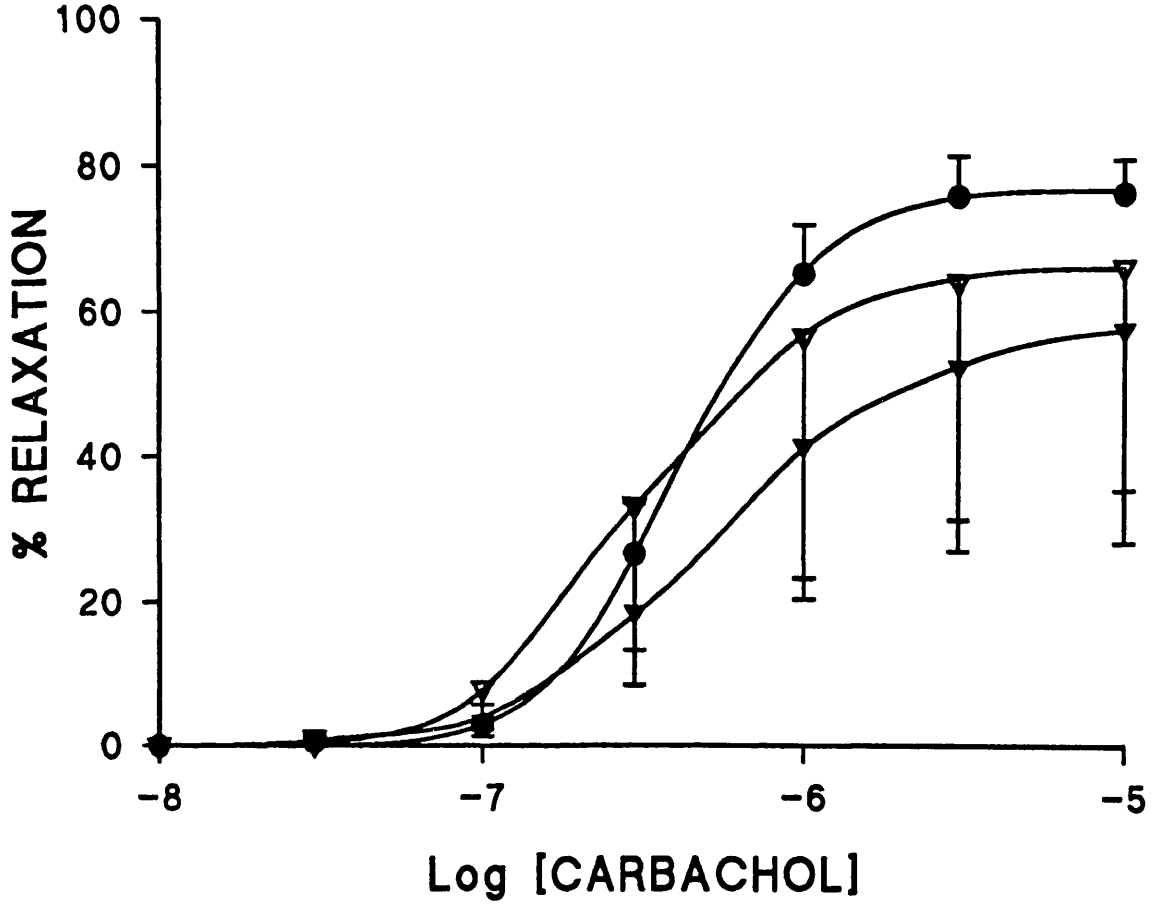


FIGURE 5.8. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone. Data is mean ± s.d.

● - 3 months.	n=4.
▼ - 6 months.	n=5.
∇ - 6 months treated with Simvastatin.	n=5.

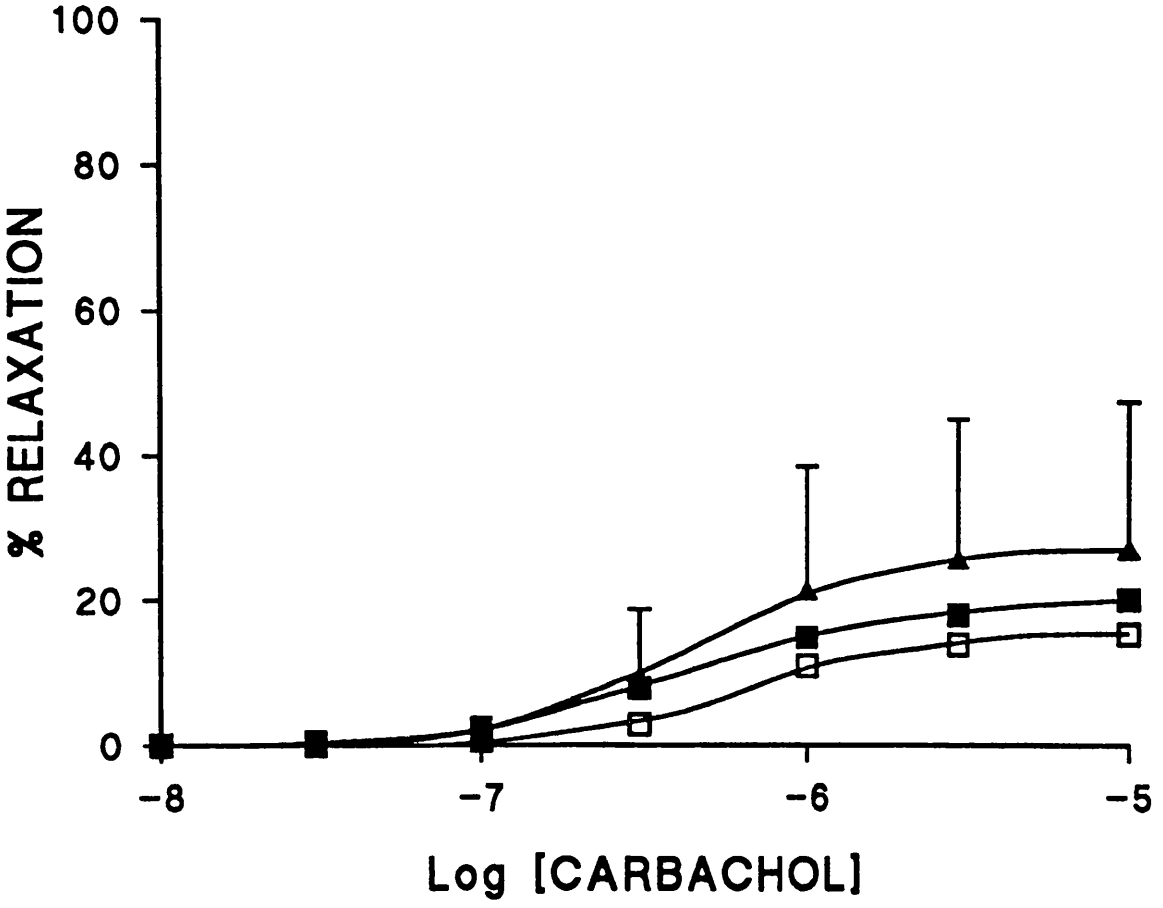


FIGURE 5.9. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from homozygous WHHL rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone. Data is mean ± s.d.

▲ - 9 months. n=6.
■ - 12 months. n=7.
□ - 12 months treated with Simvastatin. n=6.

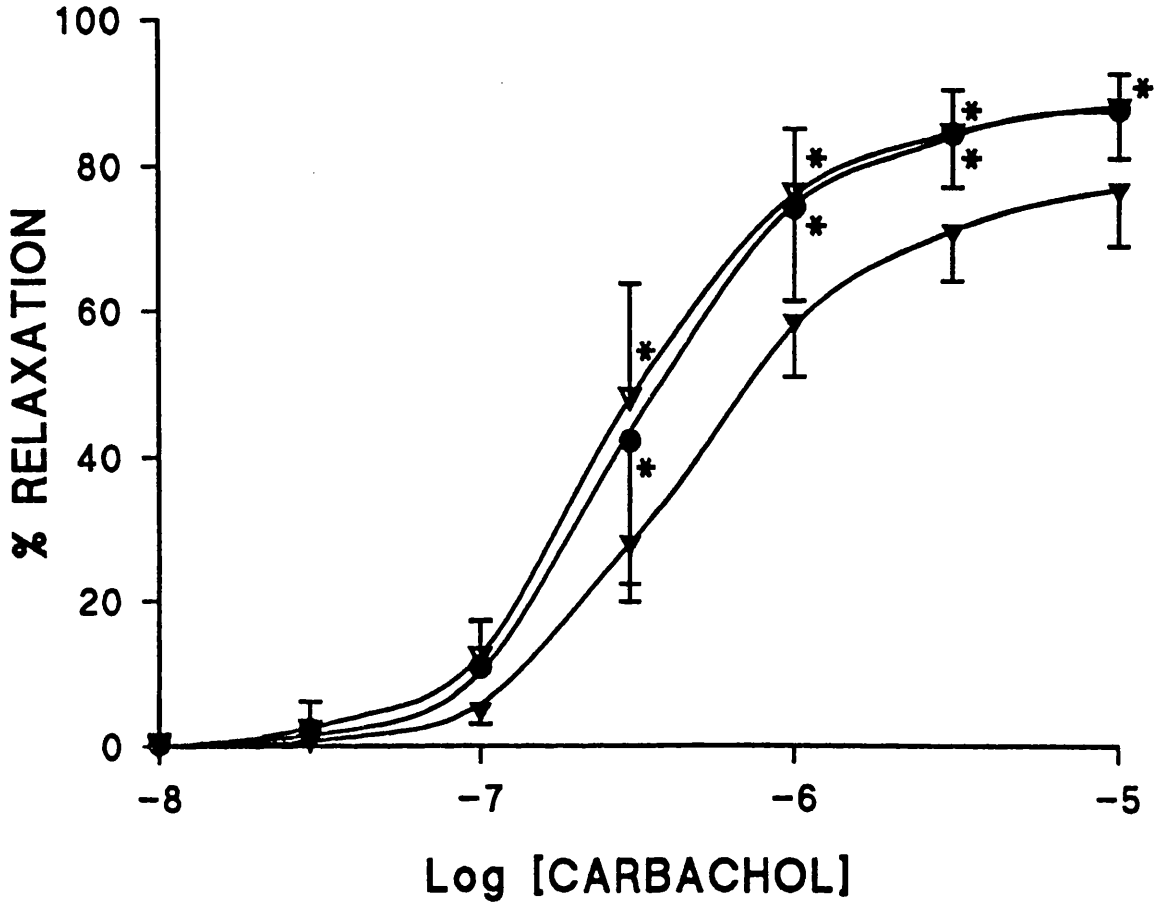


FIGURE 5.10. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone. Data is mean ± s.d.

● - 3 months. n=7.
▼ - 6 months. n=7.
∇ - 6 months treated with Simvastatin. n=5.

* - $p < 0.05$ compared to 6 month control.

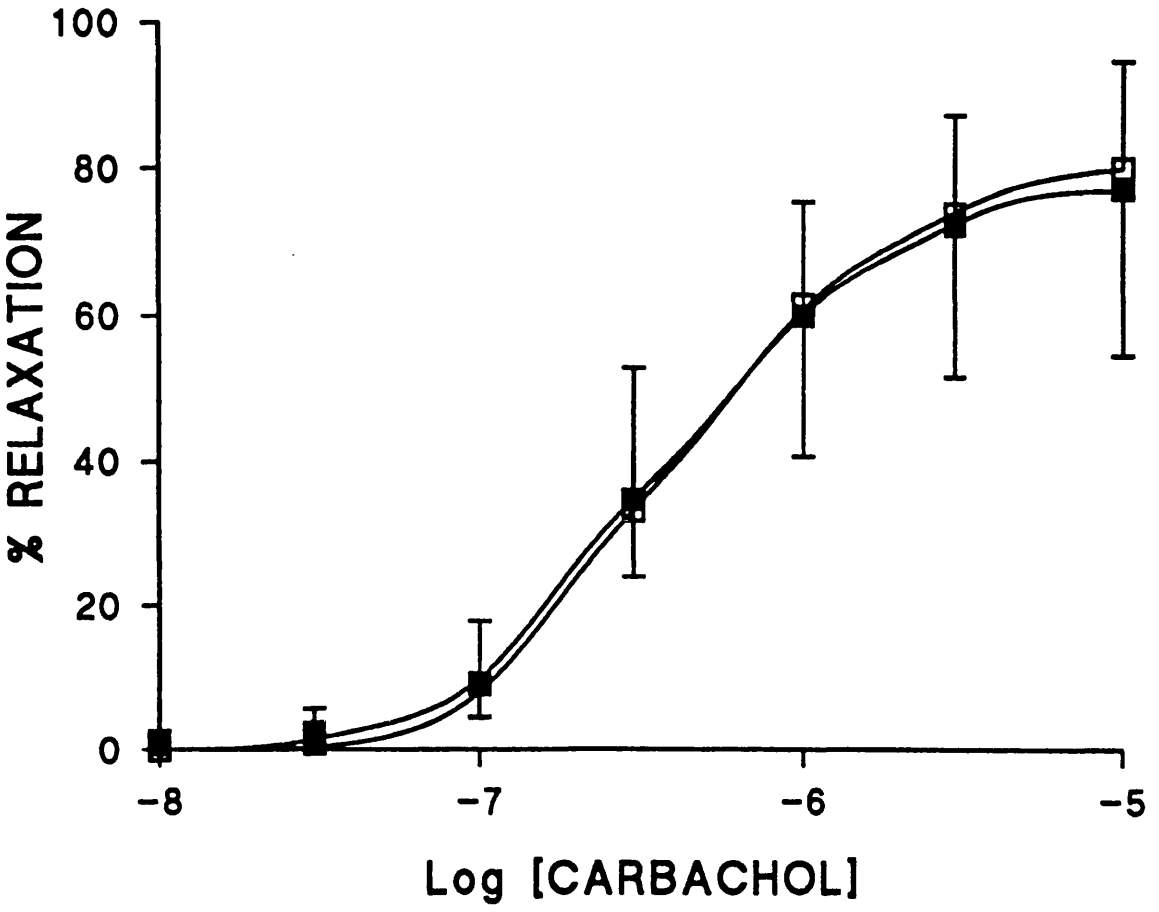


FIGURE 5.11. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from heterozygous WHHL rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone. Data is mean ± s.d.

■ - 12 months. n=7.
□ - 12 months treated with Simvastatin. n=5.

In the heterozygous WHHL rabbits which were treated with simvastatin from the age of 3 to 6 months of age there was a significant increase in carbachol induced relaxation of the rings of thoracic aorta when compared to the responses of the 6 month untreated group. The 6 month treated group was not found to be significantly different from the 3 month group, but was significantly different from the 6 month untreated group from 3 x 10^{-7}M to 10^{-5}M carbachol (Figure 5.10). The 6 month group was found to be significantly different from the 3 month group from 3 x 10^{-7}M to 3 x 10^{-6}M carbachol. As with the analysis of the homozygote responses there is a difference in points of significance when compared to the earlier analysis. The difference between the 3 and 6 month groups at 3 x 10^{-6}M carbachol was not found to be significant in the analysis in Chapter four (Figure 4.12), however the trend in the confidence intervals is comparable for both analyses, 95% C.I. (-24.808,-2.468).

In the heterozygous WHHL rabbits that were treated with simvastatin from the age of 9 to 12 months, there was no significant difference when compared with the 12 month untreated group (Figure 5.11).

Effect of Simvastatin Treatment on Structural Parameters in the Thoracic Aorta.

Simvastatin treatment (10mg/Kg/day) was found to have no effect on Sudan staining (Figure 5.12), % involved (Figure 5.13) or % ratio of intima to media (Figure 5.14) in any of the groups studied.

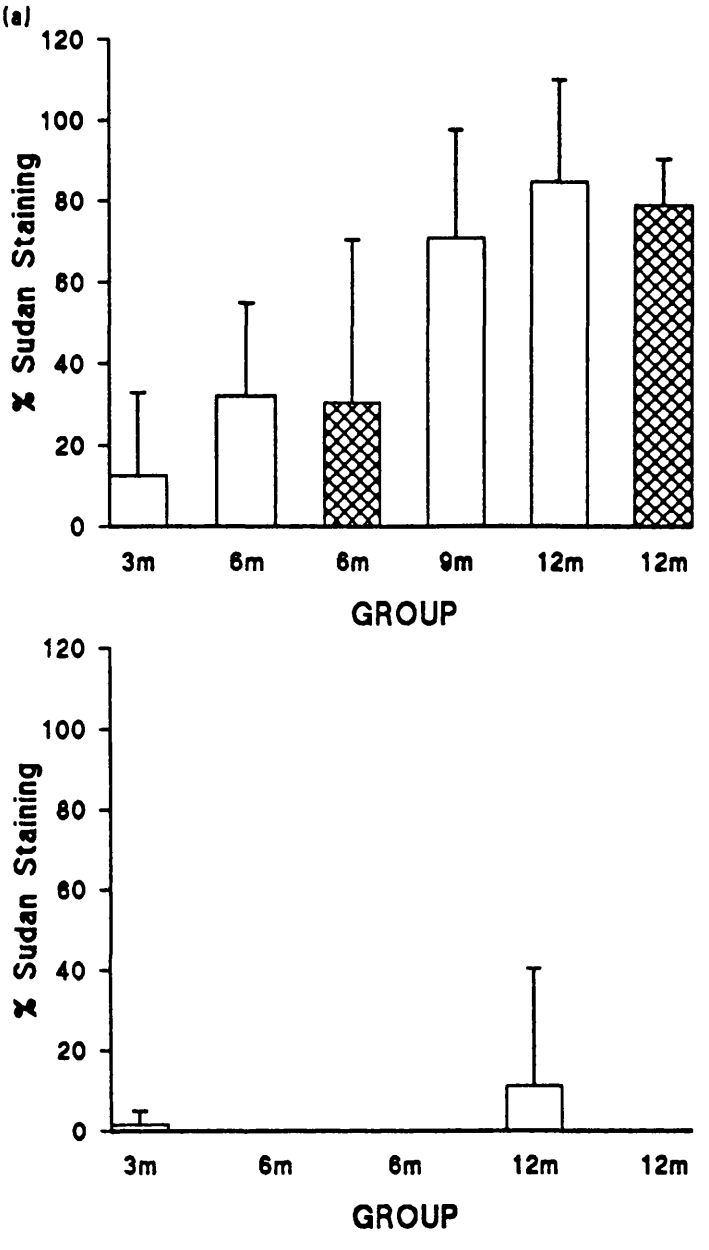


FIGURE 5.12. Percentage Sudan staining (%Sudan) in segments of thoracic aorta in (a) homoygous (hh) WHHL and (b) heterozygous (hH) WHHL. Hatched bars represent groups treated with simvastatin. Data is mean ± sd. n=4-7.

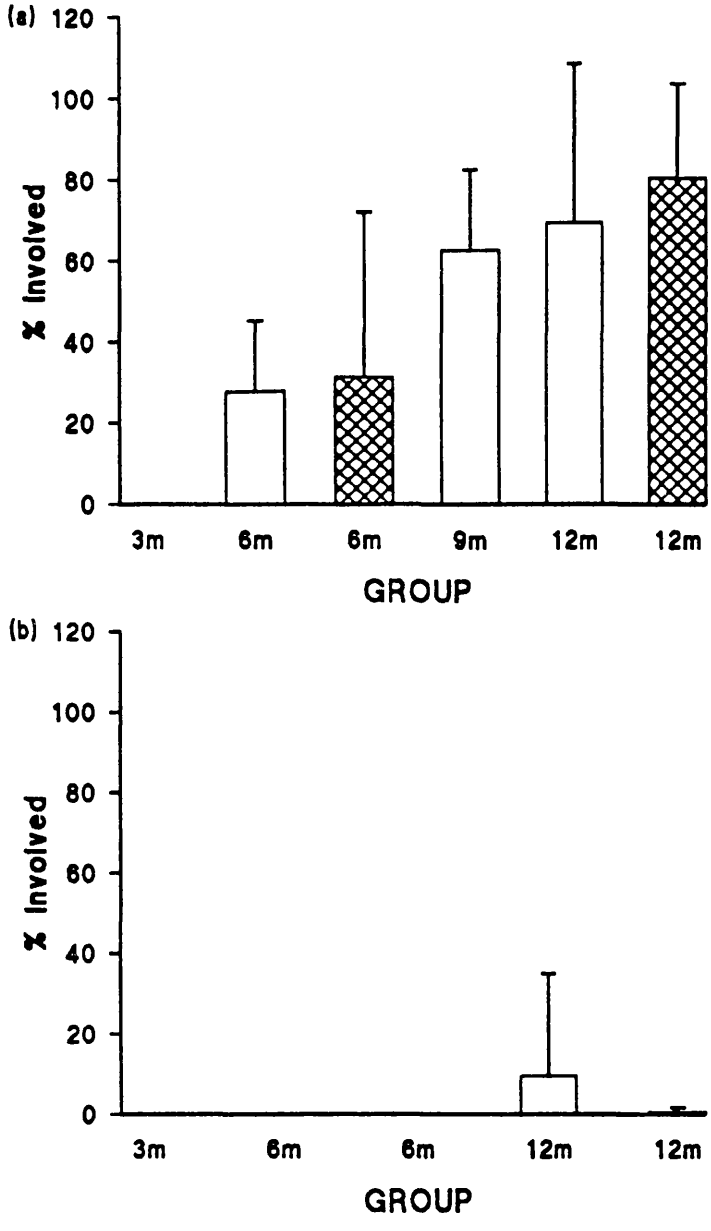


FIGURE 5.13. Percentage of the internal circumference of the vessel involved in atheroma (%Involved) in sections of thoracic aorta in (a) homoygous (hh) WHHL and (b) heterozygous (hH) WHHL. Hatched bars represent groups treated with simvastatin. Data is mean ± sd. n=4-7.

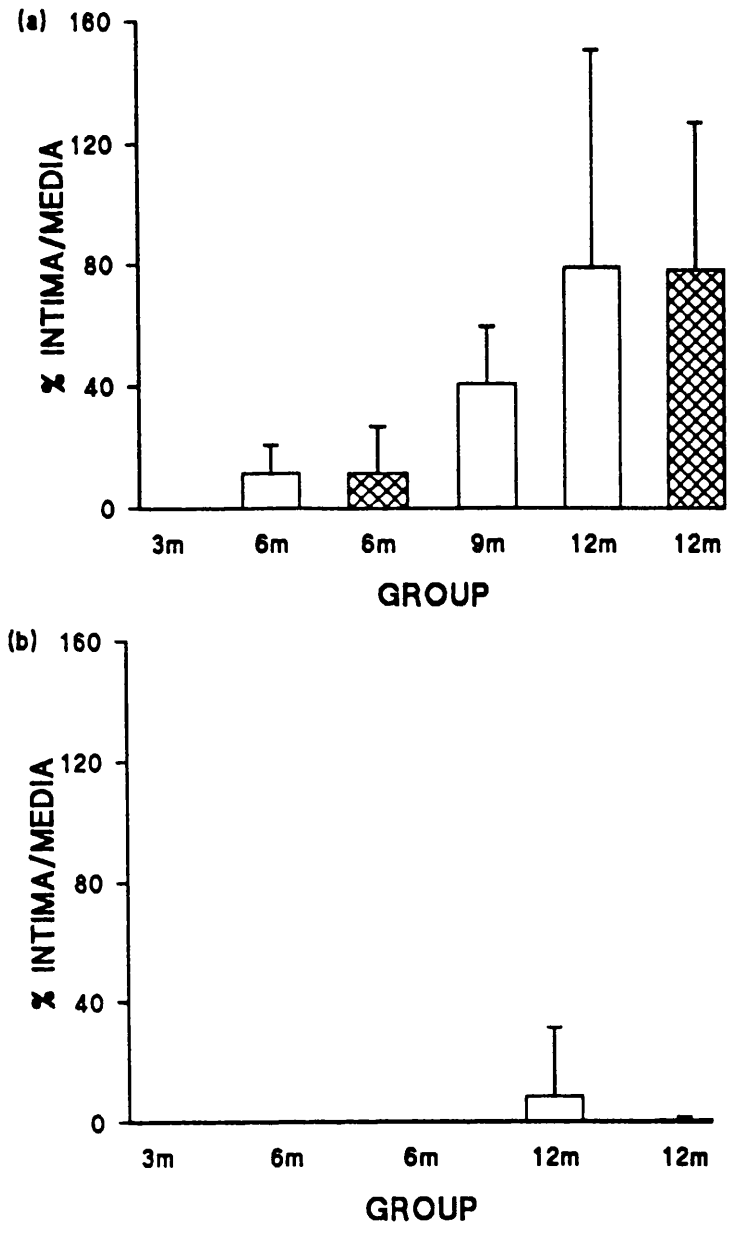


FIGURE 5.14. Percentage ratio of the area of intima to the area of media (%I/M) in sections of thoracic aorta in (a) homoygous (hh) WHHL and (b) heterozygous (hH) WHHL. Hatched bars represent groups treated with simvastatin. Data is mean ± sd. n=4-7.

5.5 DISCUSSION.

The experiments described in this chapter examine the effects of the HMG CoA reductase inhibitor simvastatin on the development and progression of atherosclerosis in both young and old WHHL rabbits. The study encompasses the effects of simvastatin in both homozygous and heterozygous WHHL rabbits. These studies were designed to examine the potential beneficial effects of this relatively new class of hypolipidaemic agents not only on serum cholesterol levels, but also on the vasoactive properties of the vasculature in the LDL receptor defective disease state found in both WHHL rabbits and the human condition of familial hypercholesterolaemia.

The dosage of simvastatin used in this study is relatively high when compared to the dosage used clinically in humans. This is consistent with other studies in rabbits Watanabe et al, 1988; Khachadurian et al, 1991). It may simply reflect species differences between rabbits and humans with regard to the absorption and / or metabolism of simvastatin. However one point to consider is that the elevations in plasma cholesterol concentrations observed in the homozygous WHHL rabbits (15-20mM) are relatively large even when compared to their human homozygous familial hypercholesterolaemia counterparts (typical plasma chol 9.1mM).

The hypolipidaemic effects of simvastatin were examined. In homozygous WHHL rabbits treatment with simvastatin was found to have a small, non-significant effect in the young (3-6 month) group, but no effect in the older (9-12 month) group. Fukuo and colleagues (1991) also examined the hypolipidaemic effects of this compound in homozygous WHHL rabbits. In their study, 3 month old rabbits were treated with the same dose as in our study (10 mg/Kg/day) for a total of 24 weeks. After 8 weeks of treatment, a non-significant fall in total cholesterol was observed. Following 16

weeks treatment, this fall had become statistically significant and remained so at 24 weeks treatment. Fukuo and colleagues also examined the effect of treatment in older (10 month) homozygous WHHL rabbits. In this group, no significant effect was observed even after 24 weeks treatment in accordance with the results of our study.

Other studies of the hypolipidaemic effects of HMG CoA reductase inhibitors in homozygous WHHL rabbits have used pravastatin. These studies have used a higher dose of pravastatin, 50 mg/Kg/day. In these studies, significant falls in serum cholesterol levels following treatment periods of 12 days (Tsujita et al, 1986), 4 weeks (Kuroda et al, 1992) and 6 months (Watanabe et al, 1988; Khachadurian et al, 1991) were observed. The fact that pravastatin appears to have a greater hypolipidaemic effect may reflect the difference in dosage.

In heterozygous WHHL rabbits, treatment with simvastatin was found to have a significant hypolipidaemic effect in the young (3-6 month) group only. There is no comparable data in the literature for our studies in the heterozygote WHHL rabbits.

The fact that simvastatin was more effective in the heterozygote WHHL rabbits supports the suggestion that HMG CoA reductase inhibitors act, at least in part, by increasing LDL receptor synthesis - heterozygote WHHL rabbits have only one defective gene, thus any therapy promoting LDL receptor expression would be able to act effectively on the normal gene. In homozygous rabbits, where both genes are defective, increased transcription of a defective receptor will not produce any beneficial effect.

The effects of treatment with simvastatin were also examined on the vasoactive properties of the vasculature. For simplicity, only the responses of the thoracic aorta are presented and discussed. The responses of this tissue demonstrated most clearly

the progressive changes associated with the development and progression of atheroma in the control studies on vasoactive function presented in Chapter four.

The effects of simvastatin treatment on the phenylephrine induced contraction parallelled the changes in serum cholesterol levels. In the homozygous WHHL rabbits, no significant protective effects were observed. However, in the young (3-6 month) homozygous group, a slight retardation of the loss of contractile function was observed. In the heterozygous WHHL rabbits beneficial effects of treatment were observed:- in the young (3-6 month) group simvastatin was able to completely prevent the loss of contractile function which had been observed from 3 to 6 months of age in the control studies; in the older (9-12 month) heterozygous, group a slight increase in contractile response was observed. In the control studies no change in vasoactive function was observed from the age of 6 to 12 months in the heterozygous WHHL rabbits and, for this reason, and problems associated with the breeding of sufficient animals, no 9 month control group was studied. As a consequence of this, valid statistical analysis cannot be carried out for the older (9-12 month) treatment group. However, the effect is slight compared with the fall in cholesterol in this group.

As with the contractile responses, the effects of simvastatin treatment on the carbachol induced endothelium dependent relaxation were found to generally parallel the changes in serum cholesterol levels. In homozygous WHHL rabbits the loss of carbachol induced relaxation from the age of 3 to 6 months was partially prevented by treatment with simvastatin. Statistical analysis of this treatment group did not demonstrate any statistical differences, however the 3 month control group is relatively small and this may mask the difference. In the older (9-12 month) homozygous group simvastatin had no beneficial effect. In the heterozygote WHHL rabbit groups, simvastatin was able to completely prevent the decrease in carbachol

induced endothelium dependent relaxation in the young (3-6 month) treated group. However in the older (9-12 month) heterozygous group no beneficial effect was seen.

As stated earlier there seem to be no comparable studies on the effects of HMG CoA reductase inhibitors on vasoactive function in WHHL rabbits for discussion.

Sections of thoracic aorta from the treated rabbits were also examined histologically. In the homozygous WHHL rabbit group no change in any of the three parameters - % sudan, % involved or % ratio of intima to media - was observed. Hence, the small retardation of the loss of phenylephrine induced contraction and carbachol induced relaxation was not associated with a decrease in atheroma. In the heterozygous WHHL rabbits, there was little gross pathological change in the control groups, thus treatment with simvastatin could not have any quantifiable effect in this instance.

Previous studies with pravastatin were also unable to demonstrate prevention or regression of aortic atherosclerosis (Watanabe et al, 1988, Khachadurian et al, 1991). However, Watanabe and colleagues (1988) did show a decrease in coronary atheroma following 6 months treatment with 50 mg/Kg/day pravastatin. The study by Khachadurian and co-workers (1991) was able to demonstrate a decrease in aortic cholesterol content.

Thus the changes in vasoactive function that were observed in the WHHL rabbits can be reduced by cholesterol lowering therapy. In the heterozygous, rabbits functional changes are observed before any gross morphological changes (see Chapter four). The fact that these functional changes can be prevented by a lipid lowering therapy indicates that this modification in function is, at least in part, induced directly by the elevated cholesterol levels.

In the homozygous animals, some retardation of the loss of function was observed. However, this was not parallelled by any decrease in the development of atheroma. Hence, the altered contraction and relaxation observed in homozygous relaxation is not simply due to the presence of atheroma, but as with the heterozygous animals, the elevation of serum cholesterol is able to affect the vasoactive properties of the thoracic aorta.

Studies have been carried out to assess the direct effects of lipoproteins on vascular responsiveness. Several groups have shown that endothelium dependent relaxation in rabbit thoracic aorta is impaired in the presence of oxidised LDL (Chin et al, 1992; Simon et al, 1990; Galle et al, 1991; Galle et al, 1992). This effect is presumably due to the direct inactivation of EDRF / NO by reaction with the oxidised LDL. However, some research groups have examined the responsiveness of the rabbit aorta after exposure to and removal of native and oxidised LDL. In these studies, a decrease in endothelium dependent relaxation to agonists such as acetylcholine and ATP has been observed following incubation with oxidised but not native LDL (Andrews et al, 1987; Jacobs et al, 1990; Kugiyama et al, 1990; Mangin et al, 1993). It has been proposed that the lipid is transfered to the endothelial cell membrane and that there it interferes with an intramembrane regulatory pathway shared by the receptor-effector pathways - possibly involving the G-protein mediating EDRF / NO release (Kugiyama et al, 1990, Mangin et al, 1993). Thus exposure to elevated oxidised LDL *in vivo*, as in the WHHL rabbits, could impair endothelium dependent relaxation prior to the actual development of atheroma.

In 1990, Galle and coworkers studied the contractile properties of the rabbit femoral artery in the presence and absence of native and oxidised LDL. This work

demonstrated that oxidised but not native LDL could cause a small constriction of the artery. Furthermore, it was demonstrated that oxidised but not native LDL could enhance the subsequent contractile response to noradrenaline, phenylephrine, serotonin or K^+. This enhancement was further amplified by the removal of the endothelium. Preincubation with calcium channel blockers such as verapamil, diltazem and nitrendipine was able to inhibit this enhancement. Thus Galle and colleagues suggest that oxidised LDL may induce an increase in transmembrane calcium ion flux in the vascular smooth muscle cells. This work is supported by a study in rat vascular smooth muscle cells (Sachinidis et al, 1990) in which it was demonstrated that LDL induced a dose dependent increase in the concentration of free intracellular calcium. The following year work was published demonstrating that incubation of vascular smooth muscle cells with LDL and free cholesterol-rich phospholipid liposomes increased the free cholesterol content of the smooth muscle cell plasma membrane and subsequently increased cytosolic calcium levels (Gleason et al, 1991).

In our studies with the WHHL rabbits, an early hyper-reactivity to phenylephrine was observed in the homozygous WHHL rabbits. The above studies suugests that this could be a direct effect of the increased LDL levels in the plasma. However this effect is overcome when the longterm effects of elevated plasma LDL induces the development of atherosclerosis and the associated degenerative effects on the vasculature.

Hence in summary, this study has demonstrated that the HMG CoA reductase inhibitor simvastatin is able to significantly retard the loss of vasoactive function associated with the development and progression of atherosclerotic disease in young heterozygous WHHL rabbits. Similar effects have been observed in young homozygous WHHL rabbits to a lesser extent, but not in the older animals. These

effects were not parallelled by a decrease in the development of atheroma.

Longer treatment schedules or a higher dosage may prove more effective in homozygous and older heterozygous WHHL rabbits.

This study indicates that simvastatin and probably other HMG CoA reductase inhibitors may prove an effective therapy for the reduction of the risk of atherosclerosis and coronary heart disease in human familial hypercholesterolaemia.

CHAPTER SIX.

EFFECT OF A XANTHINE OXIDASE / HYPOXANTHINE FREE RADICAL / REACTIVE OXYGEN SPECIES GENERATING SYSTEM ON THE FUNCTION OF ISOLATED AORTAE FROM NEW ZEALAND WHITE RABBITS.

6.1 INTRODUCTION.

As discussed in the general introduction (Chapter 1) free radicals / reactive oxygen species are highly reactive particles which are often produced in biological systems. Under normal cellular conditions, an array of protective enzymes and compounds are present in the cell designed to minimise free radical attack. These protective agents include enzymes such as SOD - responsible for the dismutation of the superoxide anion to hydrogen peroxide, and catalase - which is present to catalyse the degredation of hydrogen peroxide to molecular oxygen and water.

The potential toxicity of free radicals is exacerbated by their ability to initiate chain reactions. Due to their chemical nature when a free radical reacts with a non-radical species, by definition another free radical is formed. Thus the biological control of free radicals is essential.

As discussed earlier a role for free radicals / reactive oxygen species has been proposed in several aspects of the development of cardiovascular disease :- oxidation of LDL, ischaemia reperfusion injury and the initiation / development of atherosclerosis. It is possible that free radicals / reactive oxygen species may directly or indirectly affect the vasoactive function of vascular tissue.

Several groups have conducted studies using a variety of free radical and reactive oxygen species generating systems. In 1989, Pieper and Gross used a xanthine / hypoxanthine system to show that acetylcholine induced endothelium dependent relaxation was impaired in rat aortic rings following exposure to their system and that nitroglycerine induced endothelium independent relaxation was unaffected. Rubanyi and Vanhoutte (1986b) studied the effects of a xanthine / xanthine oxidase system on the responsiveness of the canine coronary artery. They also reported a

decrease in acetylcholine induced relaxation . However, in this and other studies (Lawson et al, 1990), the responses of the vessel to the various vasoactive compounds were examined in the presence of the free radical / reactive oxygen species generating system. Although this could produce some interesting information on possible events during exposure, only acute effects of the free radicals can be determined. One other problem with these studies is in the interpretation of results - effects observed may be due to intracellular changes or simply due to chemical modification of the agonists being used.

6.2 AIMS.

The aims of the experiments detailed in this chapter were, therefore, to examine the effects of a xanthine oxidase / hypoxanthine free radical and reactive oxygen species generating system on vascular smooth muscle and endothelial function in thoracic aorta from New Zealand White rabbits, after the source of free radicals / reactive oxygen species had been removed.

6.3 METHODS.

We examined contractile responses to the α_1-adrenoceptor agonist phenylephrine, which stimulates contraction of vascular smooth muscle through the phosphatidylinositol cycle. The vasorelaxant properties of the thoracic aorta were also examined using carbachol as a muscarinic agonist to stimulate EDRF production. The effects of SNP as a directly acting nitrovasodilator were studied to assess non-endothelium dependant relaxation.

The effects of these agonists were examined before and after exposure of rings of thoracic aorta to the xanthine oxidase and hypoxanthine free radical and reactive

oxygen species generating system. After rings had been exposed to the xanthine oxidase / hypoxanthine system, they were washed and allowed to re-equilibrate thus allowing us to examine the responses of the vessel after the source of free radicals / reactive species have been removed. A detailed protocol is given in the methods chapter - Chapter Two.

6.4 RESULTS.

6.4.1. Effect of Vehicle or Hypoxanthine / Xanthine Oxidase on Baseline Tension.

Over the thirty minute vehicle incubation period there was a small but significant fall in the baseline tension in the aortic rings (Figure 6.1). In the presence of the xanthine oxidase / hypoxanthine free radical generating system, there was a slowly developing rise in baseline tension (Figure 6.1). This rise in tension plateaued within the thirty minute period. After the baths were washed out, tension returned to the previous level, in both group. These effects were not modified following endothelial denudation (Figure 6.1).

6.4.2 Effect of Time / Vehicle on Responses to Phenylephrine.

Preliminary experiments showed that the addition of vehicle alone (0.5ml Krebs) to the organ bath for a thirty minute period had a small but significant effect on the cumulative concentration response curve to phenylephrine (Figure 6.2). As this effect was statistically significant the vehicle control data was used in all subsequent analysis as the standard control.

A cumulative concentration response curve to phenylephrine was still carried out before exposure of the ring to any agent to ensure that each individual ring was able to respond normally.

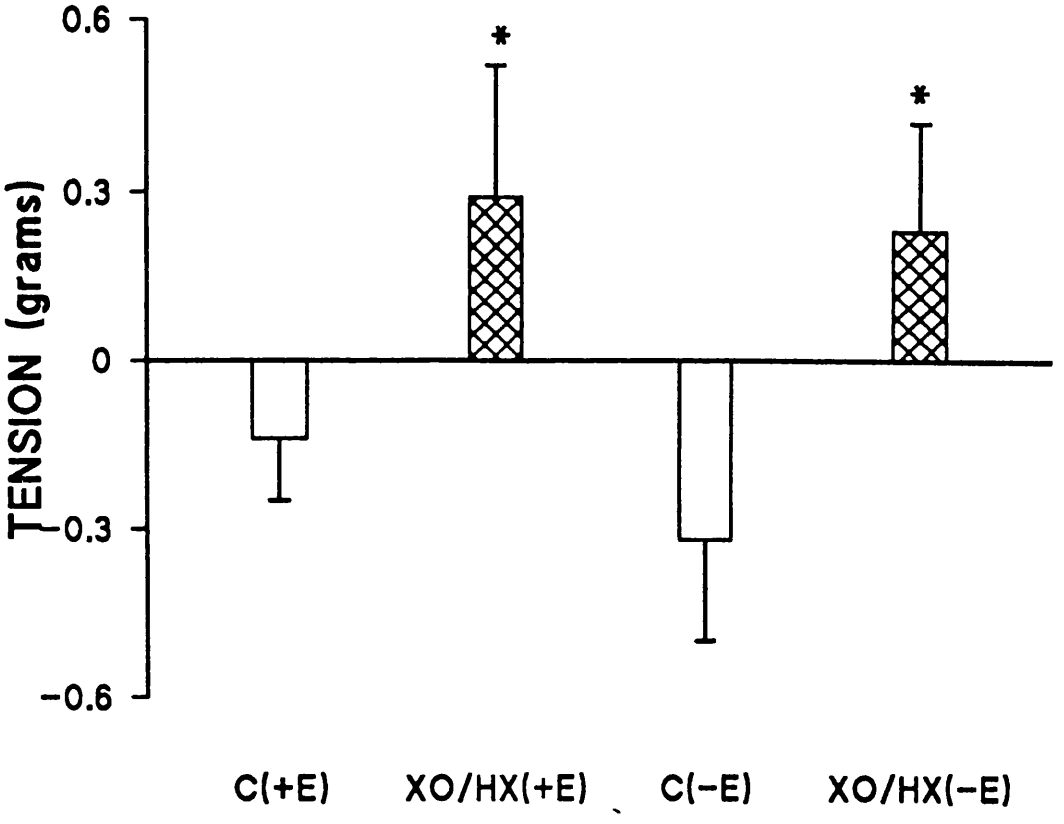


FIGURE 6.1. Change in baseline tension observed in rings of thoracic aorta during exposure to the xanthine oxidase / hypoxanthine system. Open bars represent control, hatched bars represent xanthine oxidase / hypoxanthine. Data is mean ± s.d. n=9.

* - $p < 0.05$ compared to control.

6.4.3 Effect of the Xanthine Oxidase / Hypoxanthine System on the Responses to Phenylephrine.

Exposure of the rings to the xanthine oxidase / hypoxanthine system lead to a significant impairment of the ability of the ring to constrict to phenylephrine. This effect was significant at all but the two lowest concentrations of phenylephrine (Figure 6.3).

6.4.4 Effect of the Xanthine Oxidase / Hypoxanthine System in the Absence of Endothelium.

It is well recognised that the endothelium acts *in vivo* as a regulatory layer within the vasculature. Therefore, we examined the effect of endothelial denudation on the responses to phenylephrine in the presence and absence of the xanthine oxidase / hypoxanthine system.

After endothelium removal, exposure of the ring to vehicle (0.5ml Krebs) alone for a thirty minute period had a significant effect on the responses to phenylephrine (Figure 6.4). Thus, as previously, we used this vehicle control data in all subsequent analysis as the standard control.

The effects of the xanthine oxidase / hypoxanthine system in the absence of endothelium are shown in Figure 6.5. There is a significant loss of responsiveness to phenylephrine from 10^{-7}M to $3x10^{-6}$M. At 10^{-5}M the effect is marginally non-significant, 95% C.I. (-0.191,0.003).

To examine the barrier function of the endothelium in this experimental model, the responses in the presence and absence of the endothelium were compared. Figure 6.6

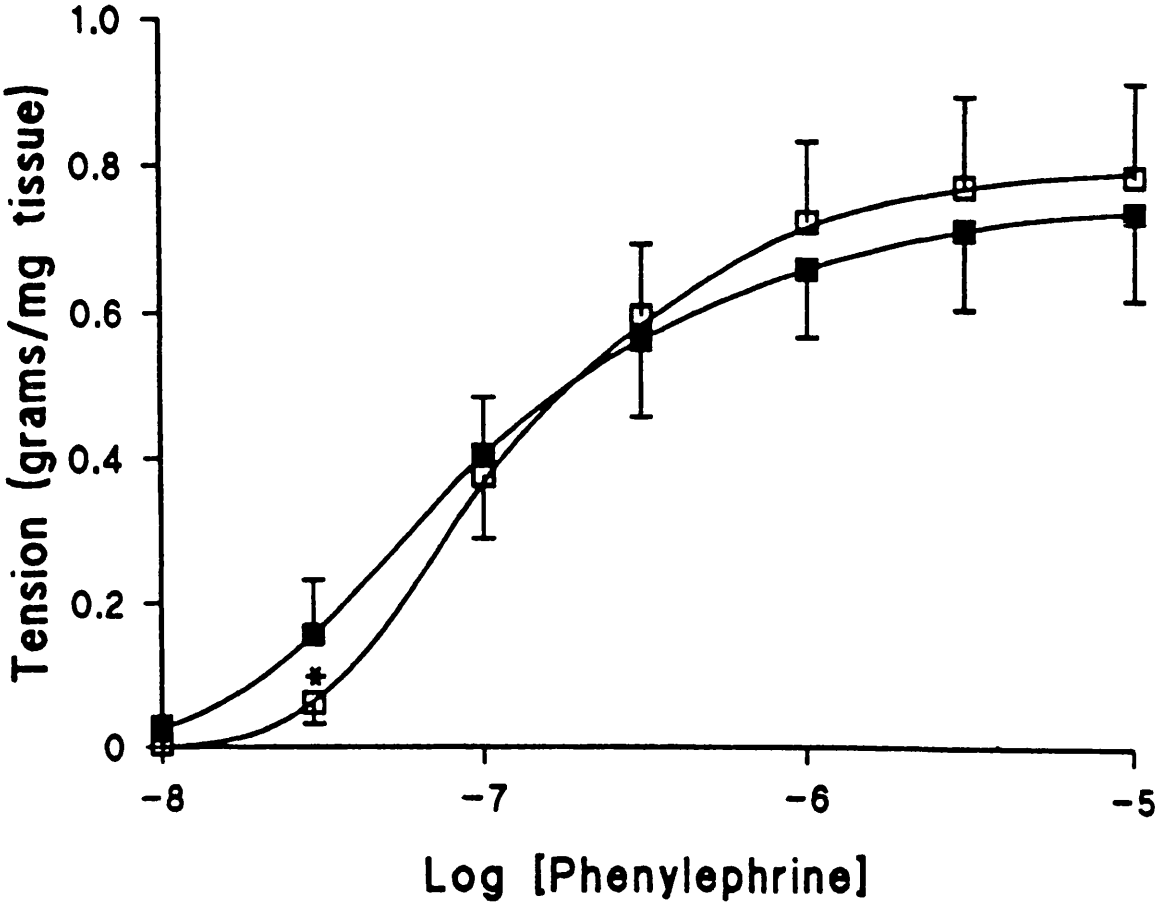


FIGURE 6.2. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

■ - control. n=12.
□ - vehicle. n=12.

* - $p < 0.05$ compared to control.

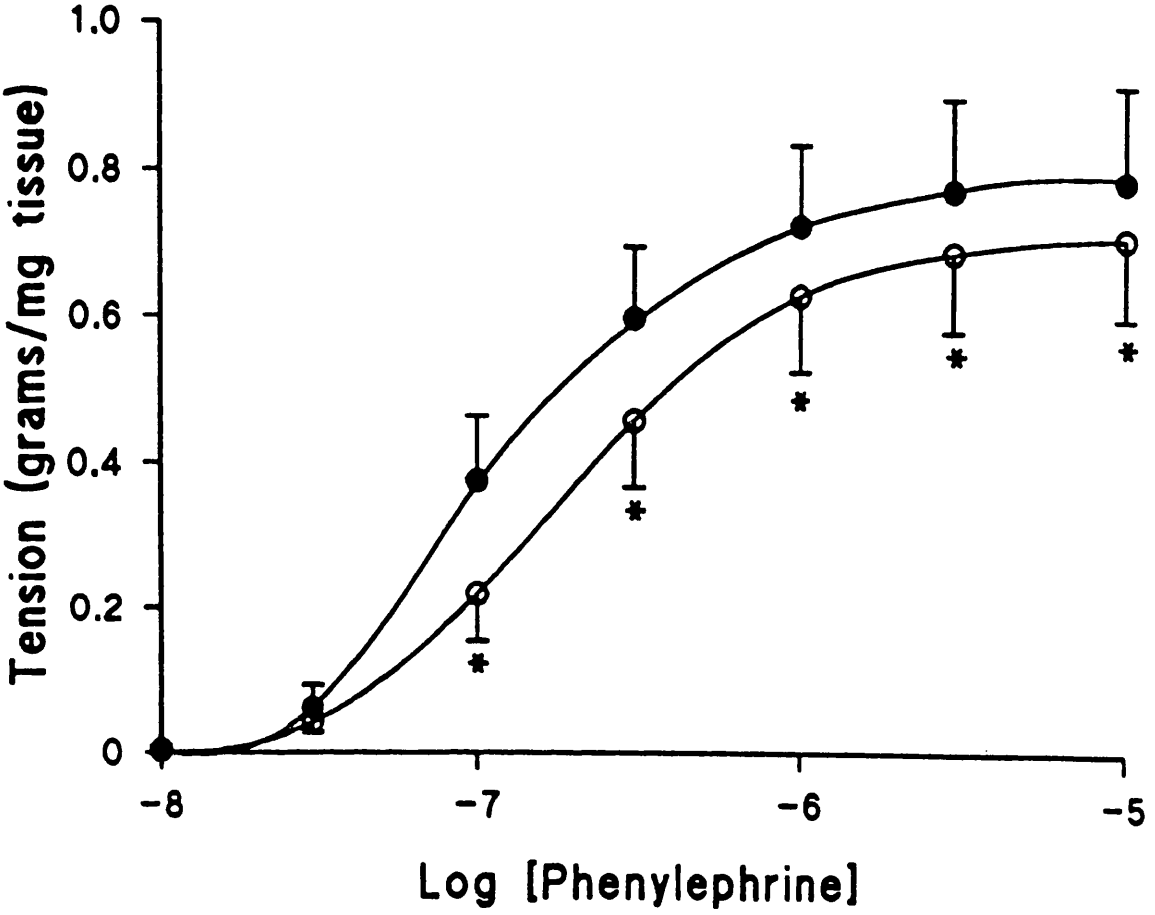


FIGURE 6.3. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

● - vehicle. n=12.
○ - XO/HX. n=12.

* - $p < 0.05$ compared to vehicle.

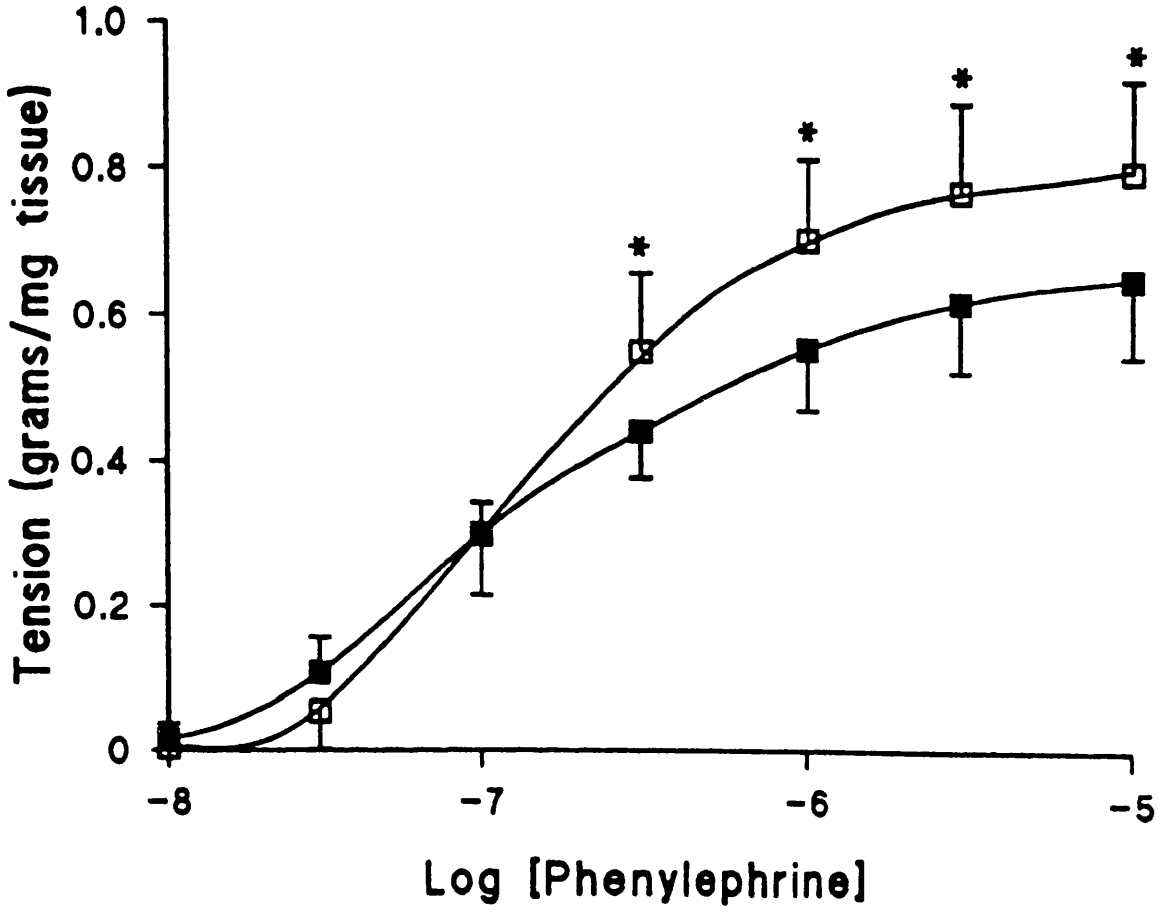


FIGURE 6.4. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in endothelium denuded rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

■ - control. n=6.
□ - vehicle. n=6.

* - $p < 0.05$ compared to control.

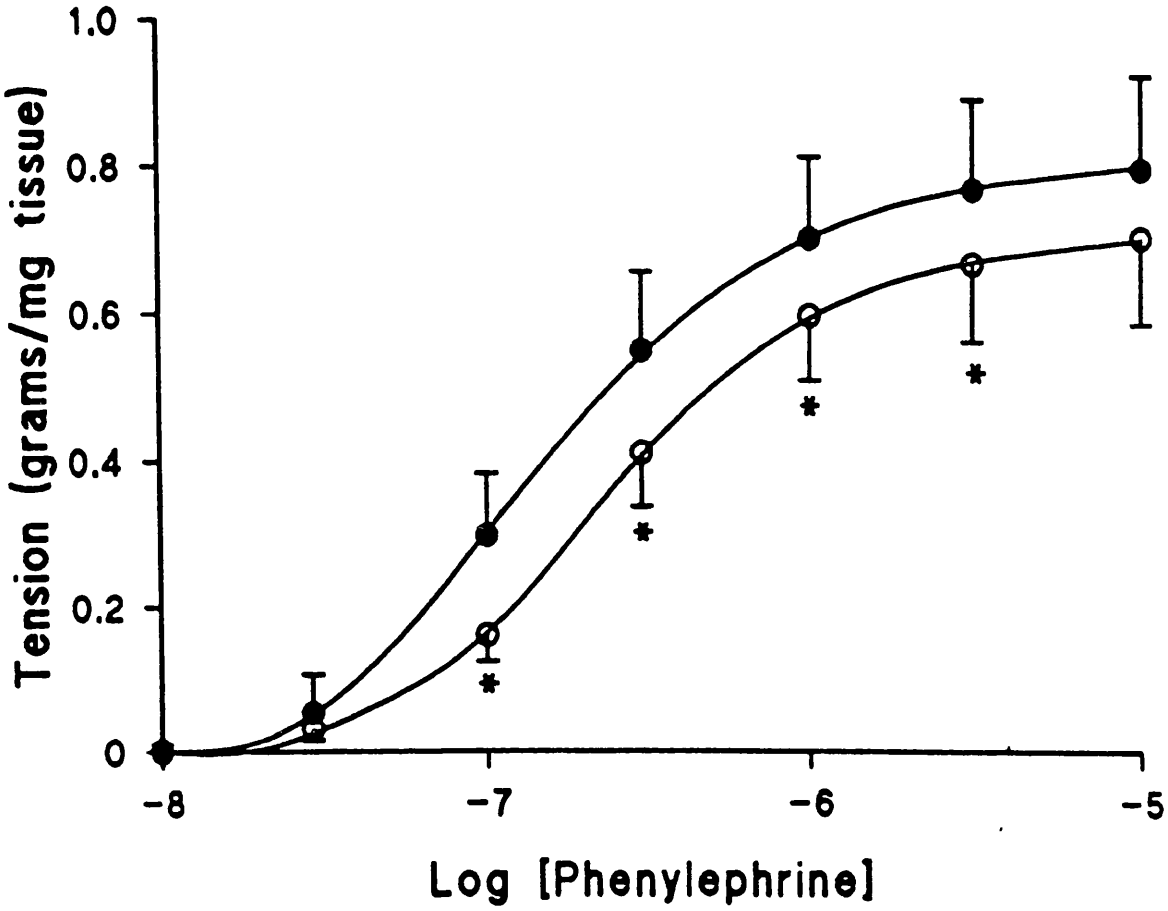


FIGURE 6.5. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in endothelium denuded rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

● - vehicle. n=8.
○ - XO/HX. n=8.

* - $p < 0.05$ compared to control.

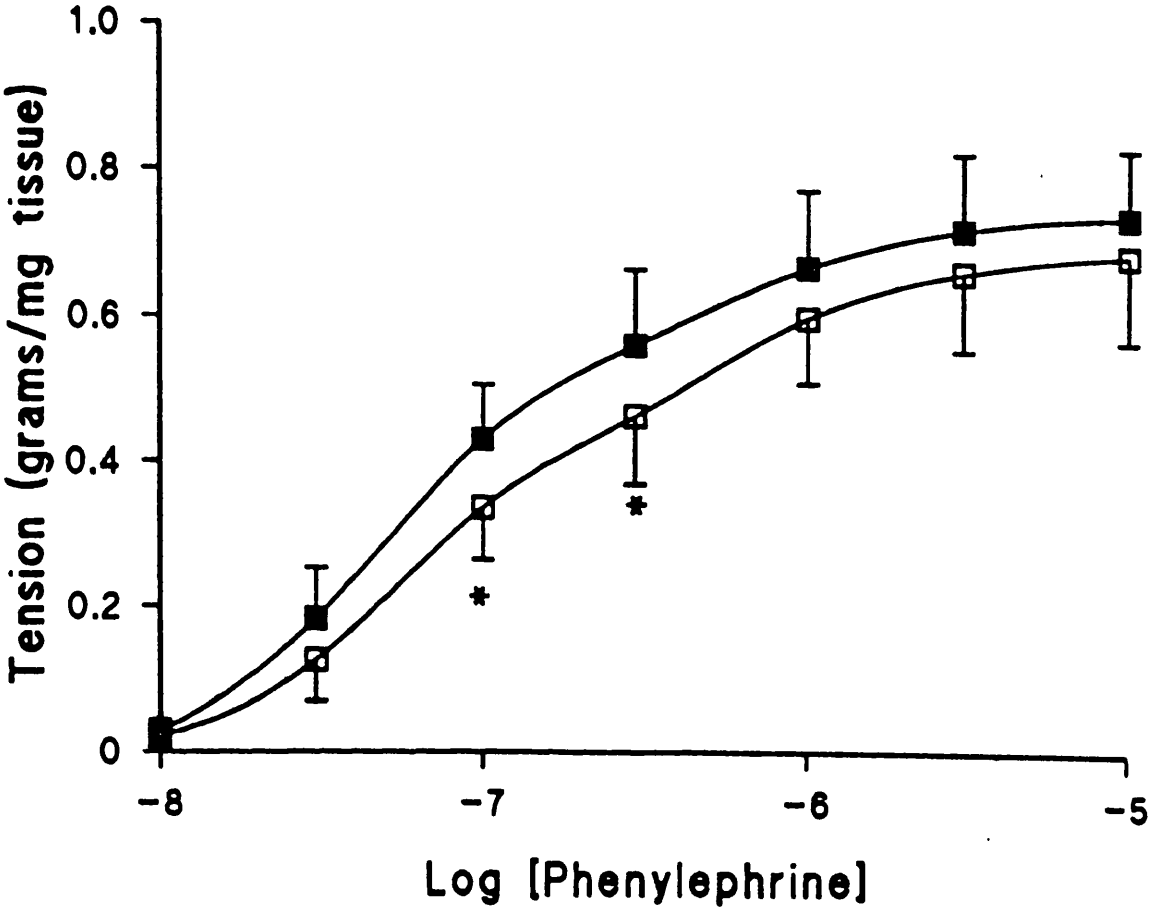


FIGURE 6.6. Control cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

■ - with endothelium. n=12.
□ - without endothelium. n=6.

* - $p < 0.05$ compared to with endothelium.

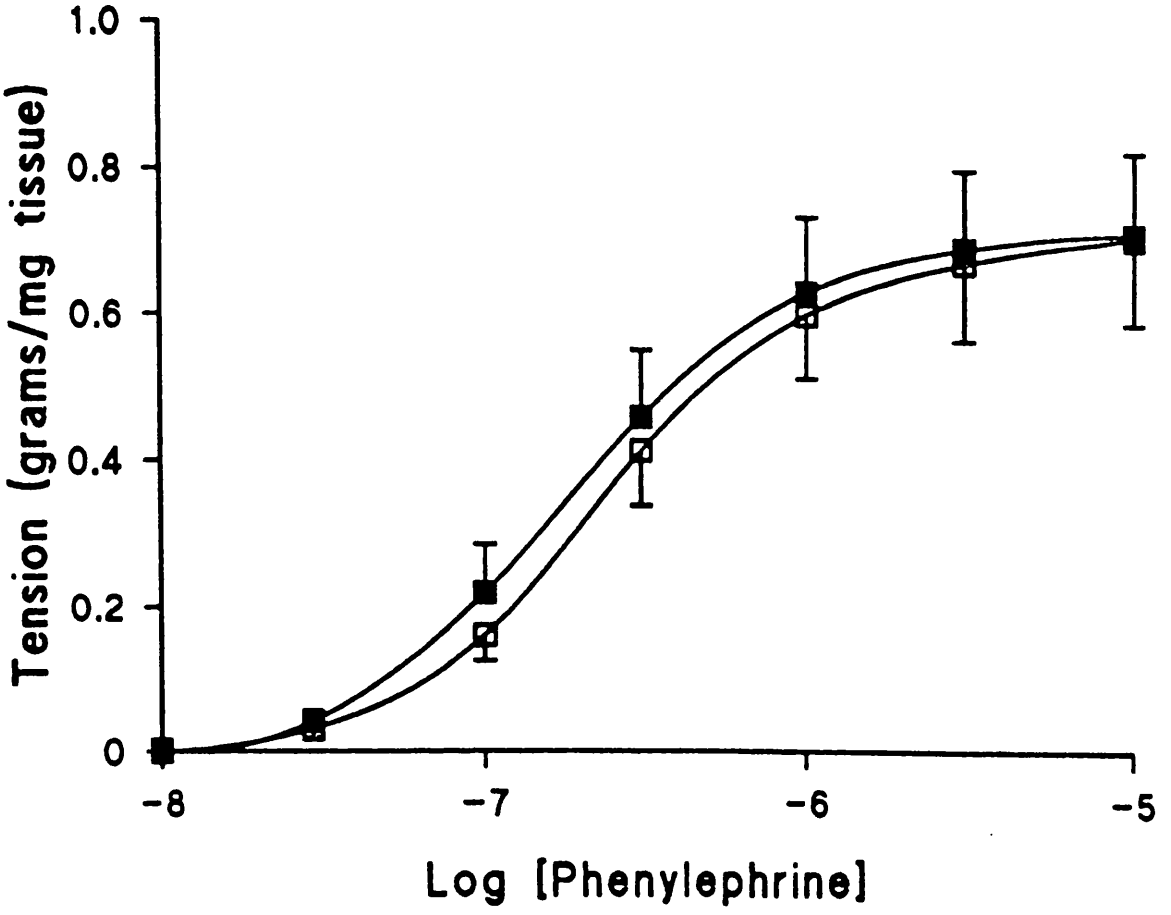


FIGURE 6.7. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits following exposure to the XO / HX system . Responses expressed in grams tension / mg tissue.

■ - with endothelium. n=12.
□ - without endothelium. n=8.

shows the control responses to phenylephrine before exposure to the xanthine oxidase / hypoxanthine system. It can be seen that there is a marginal difference in response at 10^{-7}M and $3x10^{-7}$M phenylephrine, 95% C.I. (0.023,0.166 and 0.025,0.169 respectively).

After exposure to the xanthine oxidase / hypoxanthine system, there is a decrease in response to phenylephrine in both groups (see above). However there is no statistically significant differences between the response to phenylephrine in the presence or in the absence of the endothelium (Figure 6.7).

6.4.5 Effect of Time / Vehicle on Responses to Carbachol.

Preliminary experiments showed that the addition of vehicle alone (0.5ml Krebs) to the organ bath for a 30 minute period had a small but significant effect on the cumulative concentration response curve to carbachol (Figure 6.8). As this effect was statistically significant it is this vehicle control data that is used in all further analysis as a standard control.

A cumulative concentration response curve to carbachol was still carried out before exposure of the ring to any agent to ensure that each individual ring was able to respond normally before commencement of the experimental procedure.

6.4.6 Effect of the Xanthine Oxidase / Hypoxanthine System on Responses to Carbachol.

Exposure to the xanthine oxidase / hypoxanthine system lead to around 50% inhibition of the ability of the aortic ring to relax. Figure 6.9 shows the mean data for all experiments (n=49).

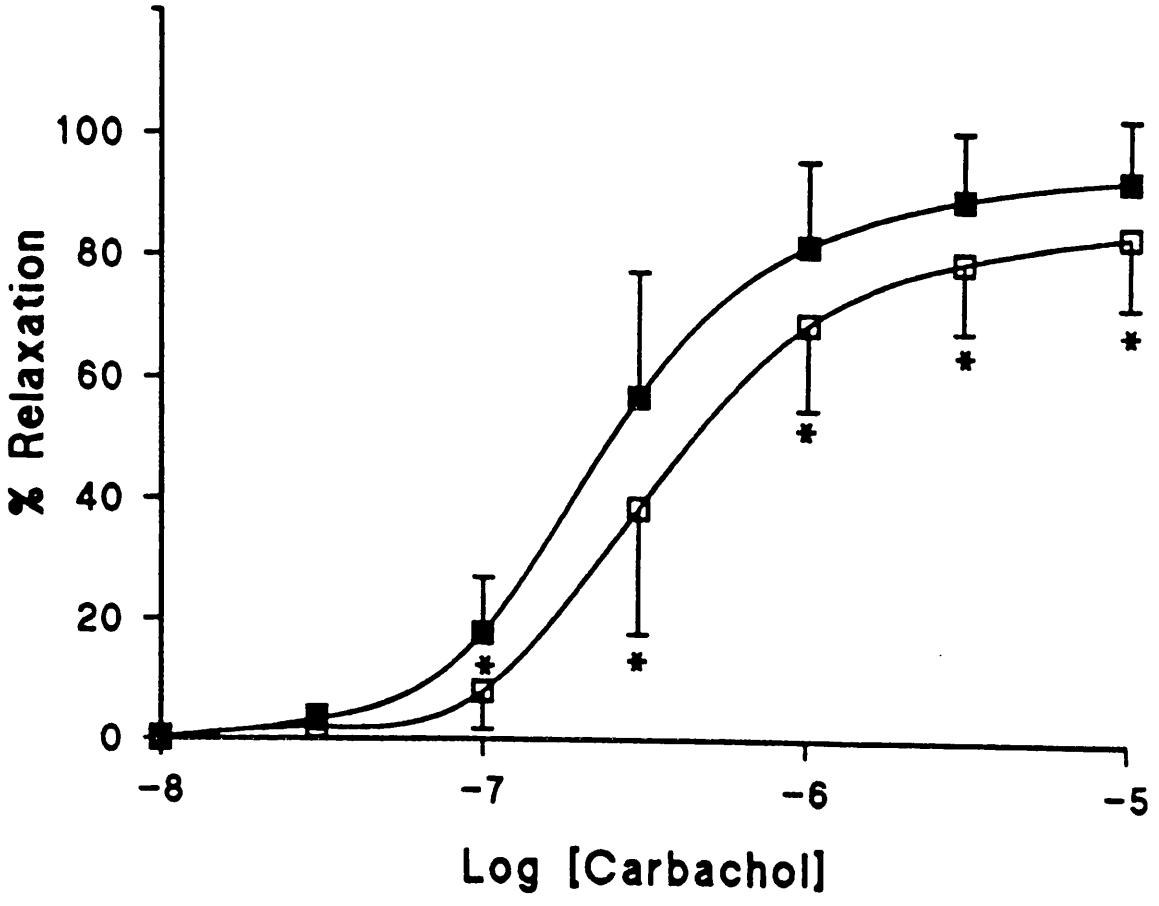


FIGURE 6.8. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

■ - control. n=9.
□ - vehicle. n=9.

* - $p < 0.05$ compared to control.

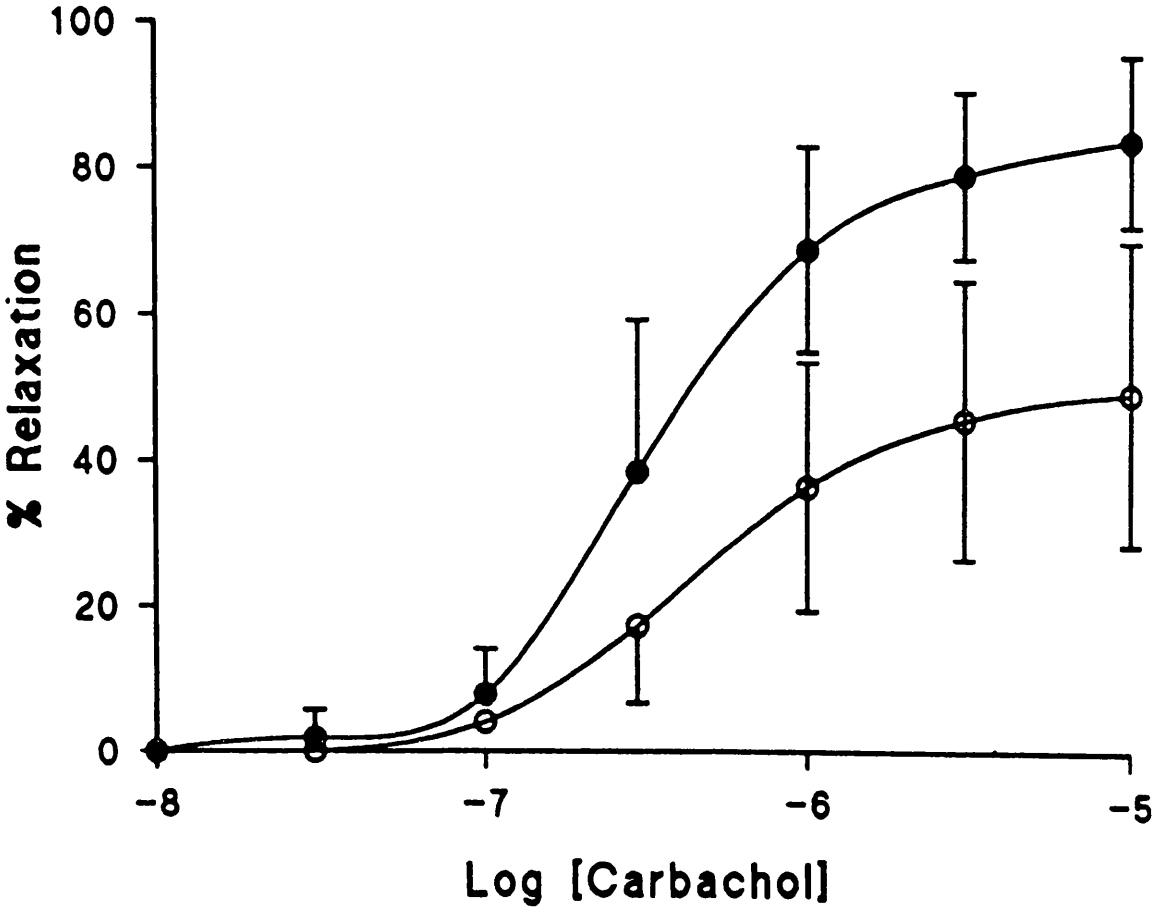


FIGURE 6.9. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

● - vehicle. n=9.
○ - XO / HX. n=49.

6.4.7 Effect of Time / Vehicle on Responses to Sodium Nitroprusside.

Addition of vehicle alone to the bath for a 30 minute period had a small but significant effect on the cumulative concentration response curve to sodium nitroprusside (Figure 6.10). So as with the carbachol data, this vehicle data was used as the standard control.

A cumulative concentration response curve to sodium nitroprusside was still carried out prior to exposure of the ring to any agent to ensue that each individual ring was able to respond normally.

6.4.8 Effect of the Xanthine Oxidase / Hypoxanthine System on Responses to Sodium Nitroprusside.

Exposure to the xanthine oxidase / hypoxanthine system lead to a significant inhibition of the response to sodium nitroprusside at lower concentrations (10^{-8}M - 10^{-6}M), the responses to $3x10^{-5}$M and 10^{-5}M were unaffected (Figure 6.11).

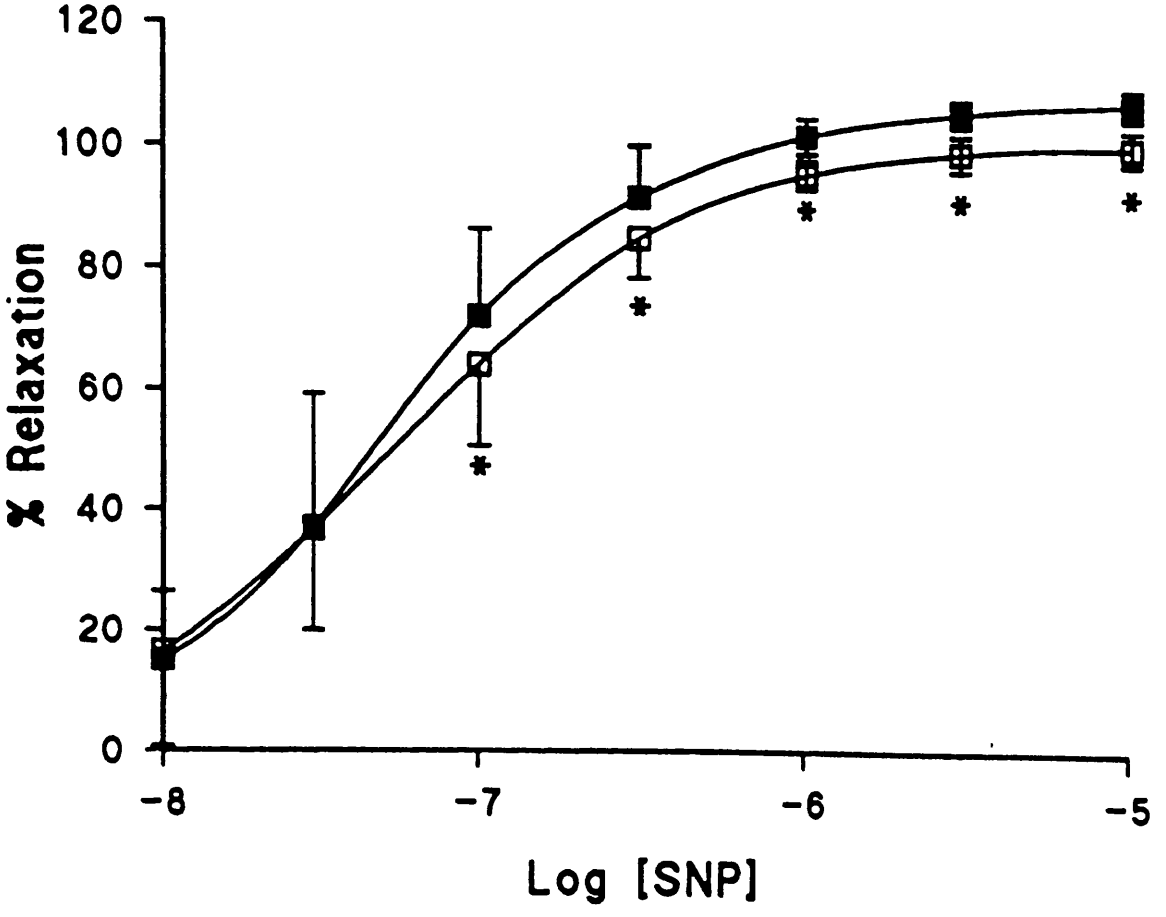


FIGURE 6.10. Cumulative concentration-response curves to SNP (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

■ - control. n=9.
□ - vehicle. n=9.

* - $p < 0.05$ compared to control.

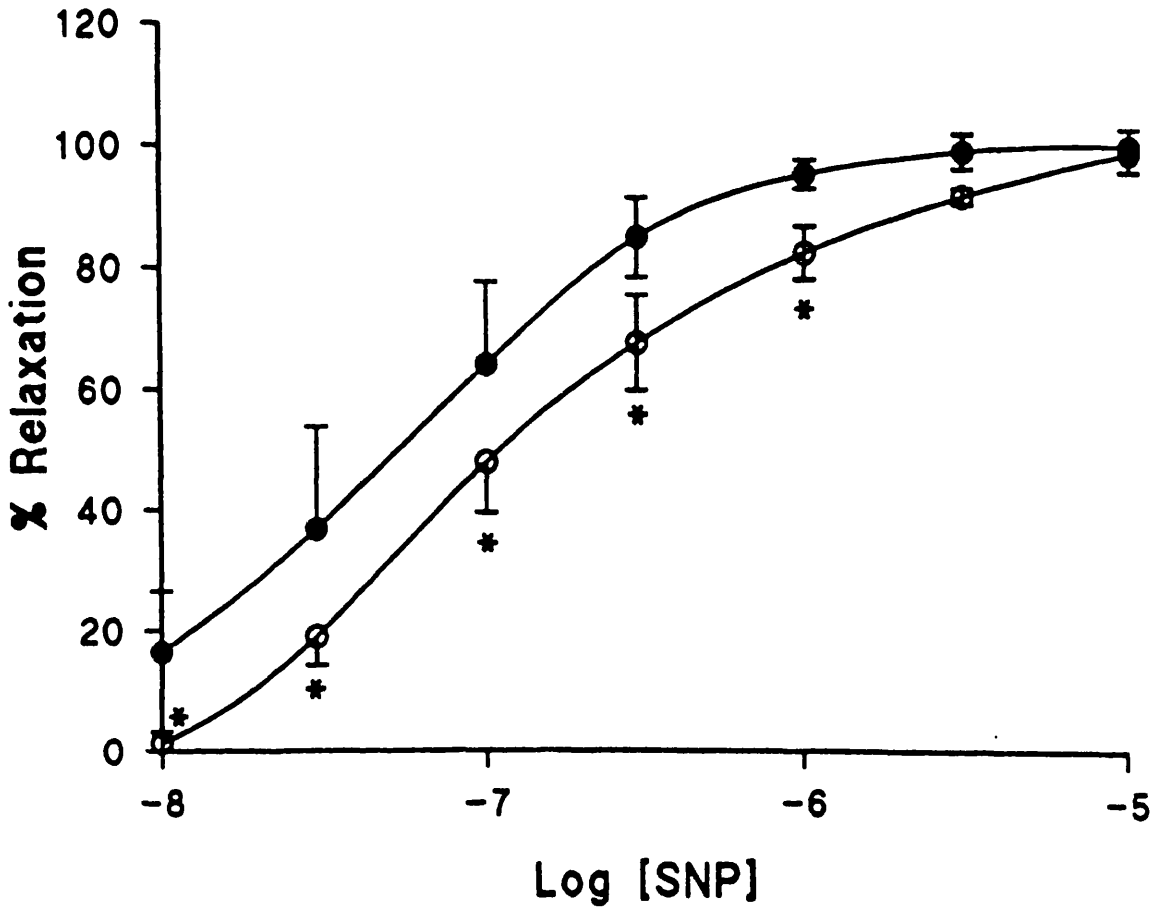


FIGURE 6.11. Cumulative concentration-response curves to SNP (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

● - vehicle. n=6.
○ - XO / HX. n=6.

* - $p < 0.05$ compared to vehicle.

6.5 DISCUSSION.

Various systems for generating free radicals / reactive oxygen species have previously been studied. However one confounding factor with many of these studies is that the various vasoactive substances are studied in the presence of the particular generating system. Under these circumstances it may be hard to determine if any observed changes in responsiveness were due to changes in cellular mechanisms or possibly due to chemical modification of the stimulating compound. Due to the experimental design this is not a problem in the work described in this study. Responses to agonists were examined after the removal of the xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system. Thus any observed change in responsiveness is due to a direct effect on the aortic ring.

In our studies, during exposure of the ring to the xanthine oxidase / hypoxanthine free radical generating system there was a small sustained rise in baseline tension of the aortic ring. Similar responses were observed by Rhoades and colleagues (1990) to a xanthine / xanthine oxidase system in rat pulmonary artery and by Auch-Schwelk and colleagues (1989) again with a xanthine / xanthine oxidase system in thoracic aorta from spontaneously hypertensive rats and also by Lawson and colleagues (1990) in rat aorta. In contrast, other groups have observed relaxation in response to exposure of rings of artery to a reactive oxygen species / free radical generating system (Rubanyi & Vanhoutte, 1986a; Rubanyi, 1988). However this response seems to be observed only in precontracted tissues. One study by Deby & Deby-Dupont (1981) suggested that at high concentrations of free radicals vasoconstriction was observed, whereas at lower concentrations a vasodilatory effect was seen.

We also showed that endothelial denudation had no significant affect on the

magnitude of this change in baseline tension, thus it cannot be due to stimulation of or impairment of release of endothelium derived substances.

Studying the effects of contractile agonists during this sustained constriction may lead to difficulties in interpretation due to the fact that contractile mechanisms are already activated. Similarly, the study of relaxation during this phase would be confusing as it is known, for example, that the superoxide anion directly inactivates nitric oxide (Rubanyi & Vanhoutte, 1986a; Katusic & Vanhoutte, 1991).

Another possible confusion may arise from chemical modification of the stimulating agonists. Wolin and colleagues (1986) reported that the superoxide anion can selectively oxidise catecholamines.

In our series of experiments, the protocol was designed to allow the effects of the xanthine oxidase / hypoxanthine system to be examined after the source of free radicals / reactive oxygen species has been removed.

It was observed with all three agonists, phenylephrine, carbachol and sodium nitroprusside, that exposure to vehicle affected the responses obtained. This effect is presumably an effect of 'time'. Any tissue will only have a limited life span after removal from the intact animal and, although the duration of the experiments is not excessive (approximately four hours), as time progresses the tissue would eventually start to lose viability. Thus the changes we observed probably reflect the very early stages of tissue degeneration. However, as explained earlier, these changes were taken into account when carrying out statistical analysis and interpretation of the results.

It was observed that the contractile response to phenylephrine was impaired after

exposure to the xanthine oxidase / hypoxanthine system. This effect was found to be quantitatively similar in the presence and absence of the endothelial layer. In retrospect, the lack of any apparent barrier function of the endothelium is not particularly surprising due to the nature of the experimental set-up. *In vivo* the endothelium is present as a physical barrier between the vascular smooth muscle layer and the blood, however in this preparation a short (2mm) ring of artery is suspended in a 10ml organ bath. Thus the Krebs and therefore the xanthine oxidase / hypoxanthine system, is in contact not only with the internal surface of the vessel but also the exterior surface and the cut ends of the ring. So, although the endothelium is present as a barrier to the internal surface of the vessel, the xanthine oxidase / hypoxanthine system has access to the muscle layer via alternative routes.

The vasorelaxant properties of the vessel were also examined. The response of the tissue to carbachol stimulated EDRF production was found to be attenuated by approximately 50% after exposure to this radical generating system. However, endothelium independent relaxation stimulated by the direct smooth muscle relaxant sodium nitroprusside was not affected in a similar manner. All tissues relaxed fully in response to higher concentrations of sodium nitroprusside, even when carbachol induced relaxation was significantly impaired.

Thus it is probable that the effect on the carbachol response is localised to the endothelium affecting either the mechanism of synthesis or release of EDRF / NO.

However, it is clear from the sodium nitroprusside and phenylephrine studies that there is also a significant impairment of the smooth muscle layer function and this cannot be fully excluded as a factor in the altered response to carbachol.

There are a range of techniques that could be used to investigate the nature of the

altered mechanisms:-

Cascade systems (Ignarro et al, 1988) could be used to determine if there is a decrease in the availability of EDRF. L-arginine, the precursor of nitric oxide and NO-synthase inhibitors such as L-N^G-nitroarginine (Moore et al, 1990) could be utilised to investigate the involvement of alterations in the synthetic pathway of NO. Further investigations using cell culture systems would also be possible. Intracellular signalling pathways such as inositol phosphate, cGMP, cAMP pathways could be studied in both vascular smooth muscle and endothelial cells.

In summary the results of this study confirm that the xanthine oxidase / hypoxanthine system generates one or more species which impair the contractile and relaxant properties of New Zealand White rabbit aortic rings. This impairment is consistent with a cytotoxic effect.

CHAPTER SEVEN.

IDENTIFICATION OF THE FREE RADICAL / REACTIVE OXYGEN SPECIES RESPONSIBLE FOR ALTERATION IN THE FUNCTION OF ISOLATED AORTAE FROM NEW ZEALAND WHITE RABBITS USING A RANGE OF DIAGNOSTIC AGENTS.

7.1 INTRODUCTION.

The studies described in chapter 6 demonstrate that the xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system produces one or more species which affect the vasoactive properties of New Zealand White rabbit thoracic aorta rings. These species significantly impair both the contractile and relaxant properties of the vessel. It is therefore, obviously of importance to investigate which of these species are responsible for these alterations in vascular function. Several highly reactive species are produced when xanthine oxidase reacts with hypoxanthine as shown in figure 1.3.

The superoxide anion is produced as the result of a one-electron reduction of molecular oxygen. This species is highly reactive in organic solutions, yet in aqueous solution it is poorly reactive. However, due to this low reactivity, the anion is able to diffuse to critical sites where it can then react. The superoxide anion is able to act in two ways. First as a single electron reducing agent, as such it is able, for example, to reduce oxidised cytochrome *c* or nitro-blue tetrazolium. Secondly as an oxidising agent, if the anion acts as an oxidising agent the superoxide itself becomes reduced to hydrogen peroxide - this reaction is known as dismutation. Dismutation of the superoxide anion will occur spontaneously or may be catalysed by the endogenous enzyme superoxide dismutase.

Superoxide has been implicated as one of the species involved in mediating ischaemia reperfusion injury. Using *in vivo* studies several groups have demonstrated that administration of superoxide dismutase before or during reperfusion of ishaemic tissue is able to reduce the detrimental effects (Werns et al, 1986; Ambrosio et al, 1986; Burton, 1985; Ytrehus et al, 1987). However, in contradiction of this other groups have reported no beneficial effect (Vanhaecke et al, 1988; Gallagher et al,

1986; Patel et al, 1988; Uraizee et al, 1987). Some *in vitro* studies have reported that superoxide produced by the xanthine oxidase / hypoxanthine system is the species responsible for impairing vascular function (Rhoades et al, 1990; Lawson et al, 1990), however these studies have examined vascular function in the presence of their generating system and thus the direct effects of the superoxide anion on adrenergic agonists and also on EDRF cannot be excluded.

Hydrogen peroxide is produced in virtually all systems which generate the superoxide anion as a result of the dismutation reaction. Hydrogen peroxide is not a free radical. Due to its uncharged nature it is able to rapidly cross biological membranes, which the charged superoxide anion can do only slowly.

The effects of hydrogen peroxide depend on the concentration examined - at low concentrations physiological reponses such as the stimulation of prostacyclin synthesis have been demonstrated, however at high concentration hydrogen peroxide has been shown to induce cell damage (Rubanyi, 1988).

The enzyme catalase can be used to study the involvement of hydrogen peroxide in biological systems. Catalase is an enzyme found in most aerobic cells, this enzyme catalyses the degredation of hydrogen peroxide to water and molecular oxygen. The effects of catalase have been examined in models of ischaemia reperfusion *in vivo*. However, these studies have generally only examined the effects of catalase in combination with superoxide dismutase. These combined studies have reported mixed results :- several studies have reported beneficial effects when a combination of superoxide dismutase and catalase are coadministered before or during a period reperfusion following a period of ischaemia (Gross et al, 1986; Myers et al, 1985; Jolly et al, 1984); whereas other studies have reported no protective effect (Przylenk et al, 1989; Richard et al, 1988; Nejima et al, 1989; Asinger et al, 1988). One study

using the perfused rat heart as a model for ischaemia reperfusion demonstrated a decrease in myocardial injury when catalase was administered alone during reperfusion (Repine, 1991).

In vitro studies of hydrogen peroxide have also demonstrated the potential role of hydrogen peroxide during ischaemia reperfusion. Hydrogen peroxide has been shown to decrease prostaglandin release (Whorton et al, 1985), induce chromium release (Simon et al, 1981) and produce vasoconstriction (Tate et al, 1984). In studies of the xanthine oxidase / hypoxanthine system several groups have proposed hydrogen peroxide as the primary cytotoxic species (Jackson et al, 1986; Rubanyi, 1986; Link and Riley, 1988; Kvietys et al, 1989; Rhoades et al, 1990; Shatos et al, 1991; Barnard and Matalon, 1992).

The hydroxyl radical is the third product of the reaction of xanthine oxidase on hypoxanthine which may potentially affect vasoactive function. The hydroxyl radical is generated by the interaction of the superoxide anion and hydrogen peroxide particularly in the presence of divalent metal cations such as iron or copper. The hydroxyl radical is highly reactive and as such will react almost immediately it is formed, close to its' site of generation.

Potential reactions of the hydroxyl radical can be inhibited by the use of specific hydroxyl radicals such as mannitol, DMSO or sulphydryl compounds. In instances, where either catalase or superoxide dismutase have been effective in decreasing the detrimental effect of the xanthine oxidase / hypoxanthine system or ischaemia reperfusion, this could be attributed to the indirect inhibition of hydroxyl radical production, thus further studies examining the effect of hydroxyl radical scavengers should be carried out.

Several *in vivo* studies have demonstrated beneficial effects of hydroxyl radical scavengers. The sulphydryl compounds MPG has been shown to reduce the detrimental effects of ischaemia reperfusion (Mitsos et al, 1986; Myers et al, 1986). Beneficial effects have also been observed using the iron chelator desferrioxamine (Bolli et al, 1987; Farber et al, 1988) which decreases hydroxyl radical production by chelating the iron which acts as a catalyst for the production of the hydoxyl radical.

In vitro studies examining free radical generating systems have however generally shown negative effects when using free radical scavengers, mannitol is most commonly used as the hydroxyl radical scavenger (Rubanyi and Vanhoutte, 1986; Link and Riley, 1988; Kvietys et al, 1989; Lawson et al, 1990). Desferrioxamine and dimethylthiourea have also been studies with negative results (Simon et al, 1988; Rhoades et al, 1990; Berman and Martin, 1993). One study by Lefer & Lefer (1991) used a combined *in vivo* and *in vitro* system. Feline hearts were subject to ischaemia followed by reperfusion *in situ*, by temporary occlusion of the coronary artery, following reperfusion the coronary artery was removed for organ bath studies. Lefer & Lefer demonstrated that MPG was able to prevent the loss of A-23187 mediated endothelium dependent relaxation, yet it was ineffective versus the loss of carbachol induced endothelium dependent relaxation.

Thus there is contradictory evidence in the literature regarding the identity of the primary cytotoxic species involved in the xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system and in the related clinical situation of ischaemia reperfusion injury.

7.2 AIMS.

The aims of the experiment described in this chapter were to identify which of the free radicals / reacive oxygen species produced by the xanthine oxidase / hypoxanthine system were responsible for the impairment of carbachol induced endothelium dependent relaxation.

7.3 METHODS.

Using the same basic study design as in Chapter 6, the effects of superoxide dismutase, catalase, MPG, captopril and mannitol were studied. Each potentially protective agent was preincubated with the tissue for 5 minutes before the addition of the xanthine oxidase / hypoxanthine system. In one series of experiments, the preincubation was extended to 1 hour to investigate the possible intracellular effects of MPG. These agents were present throughout the duration of exposure to the xanthine oxidase / hypoxanthine system and were then washed out of the bath along with the free radical generating system. Thus, both the control and experimental concentration response curves were performed in the absence of these agents. Each of the agents was also incubated alone with rings of thoracic aorta in order to assess if any had direct effects on the responsiveness of the tissue.

Hence, using these agents alone or in combination with each other, it was possible to identify the most detrimental species produced by the xanthine oxidase / hypoxanthine system.

7.4 RESULTS.

7.4.1 Effect of Mannitol in the Presence and Absence of the Xanthine Oxidase / Hypoxanthine System.

Figure 7.1 shows that the addition of mannitol (20mM) to the bath for a thirty minute period had no significant effect on the response of the aorta to carbachol.

Mannitol (20mM), when incubated with the tissue for five minutes prior to and for the duration of the exposure of the tissue to the xanthine oxidase / hypoxanthine system, had no significant effect on the rise in base line tension occuring during the exposure (Figure 7.2). There was also no protective effect against the impairment of carbachol induced relaxation of the tissue (Figure 7.3).

7.4.2 Effect of MPG in the Presence and Absence of the Xanthine Oxidase / Hypoxanthine System.

Figure 7.4 shows that the addition of MPG (300μM) to the bath for a thirty minute period had no significant effect on the response of the aorta to carbachol.

MPG (300μM), when incubated with the tissue for five minutes prior to and for the duration of the exposure of the tissue to the xanthine oxidase / hypoxanthine system, had no significant effect on the rise in baseline tension occuring during the exposure (Figure 7.2). There was also no protective effect against the impairment of carbachol induced relaxation of the tissue (Figure 7.5).

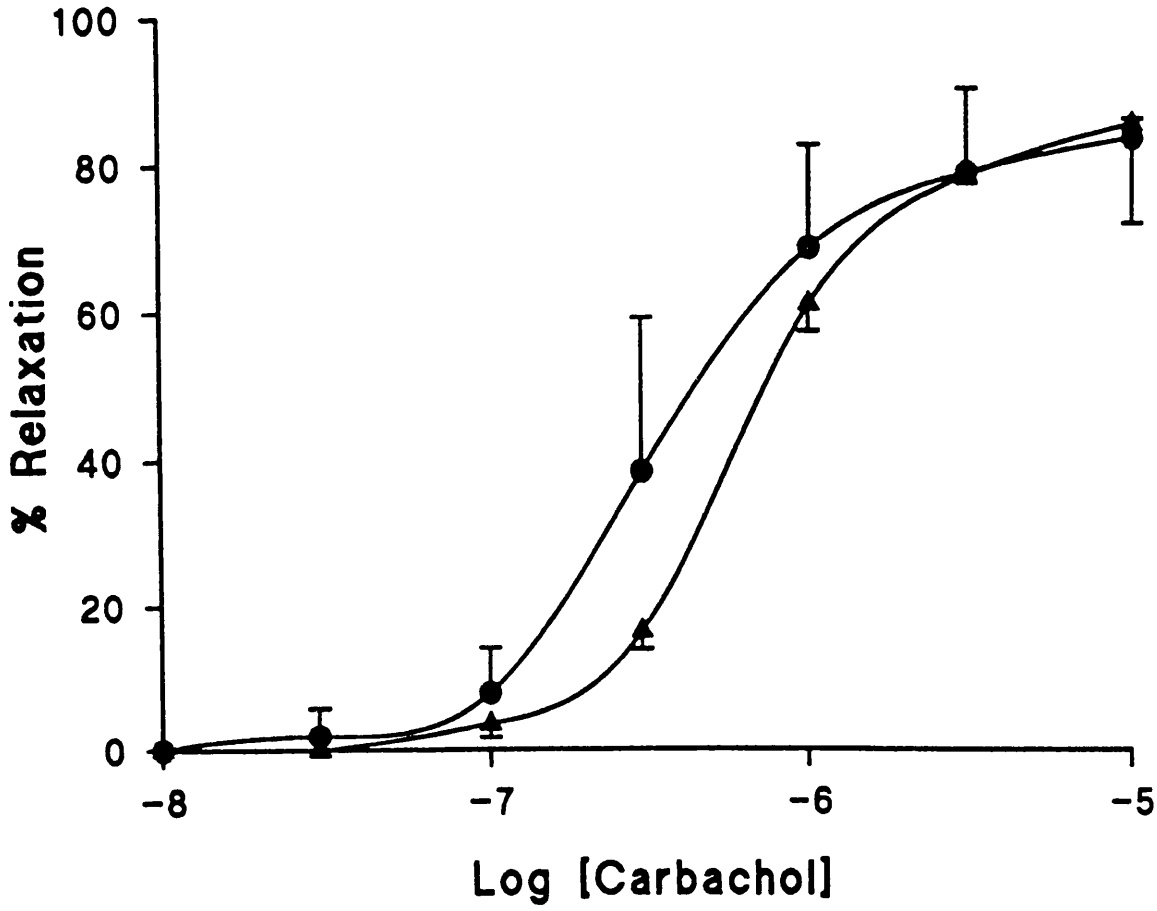


FIGURE 7.1. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=3.
▲ - mannitol. n=3.

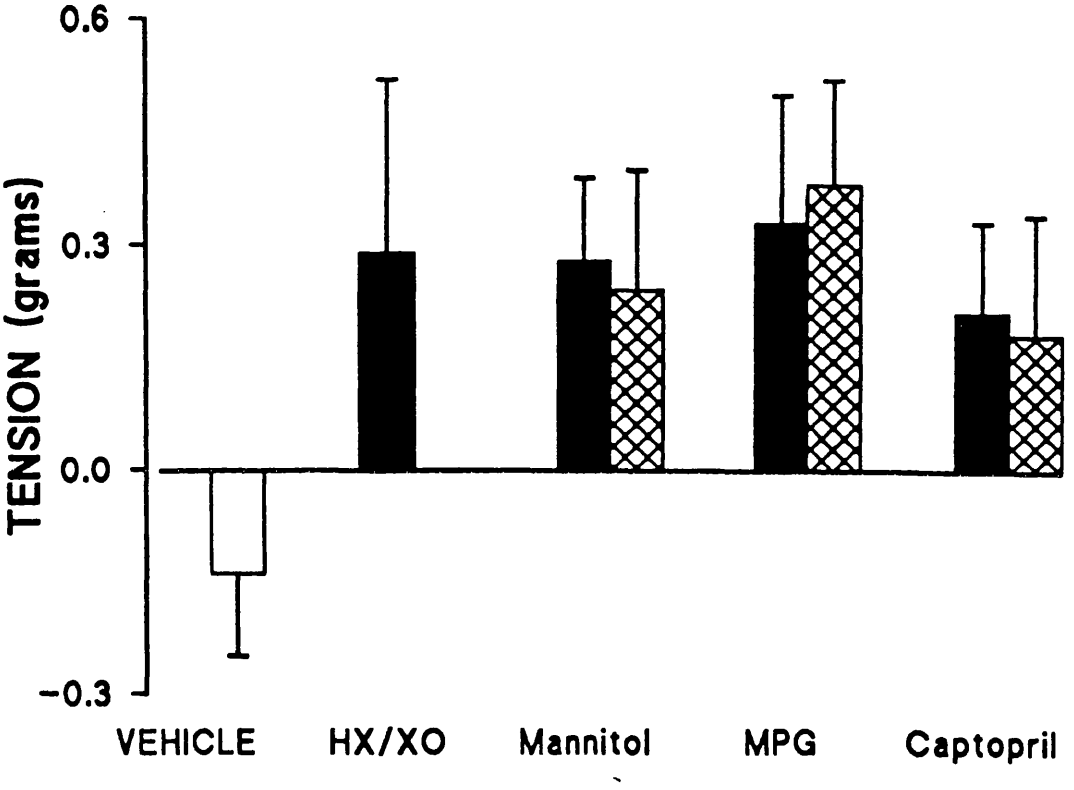


FIGURE 7.2. Change in baseline tension observed in rings of thoracic aorta during exposure to the XO / HX. Open bar represents control, solid bars represent XO / HX, hatched bars represent XO / HX plus hydroxyl radical scavengers. Data is mean ± s.d. n=8-12.

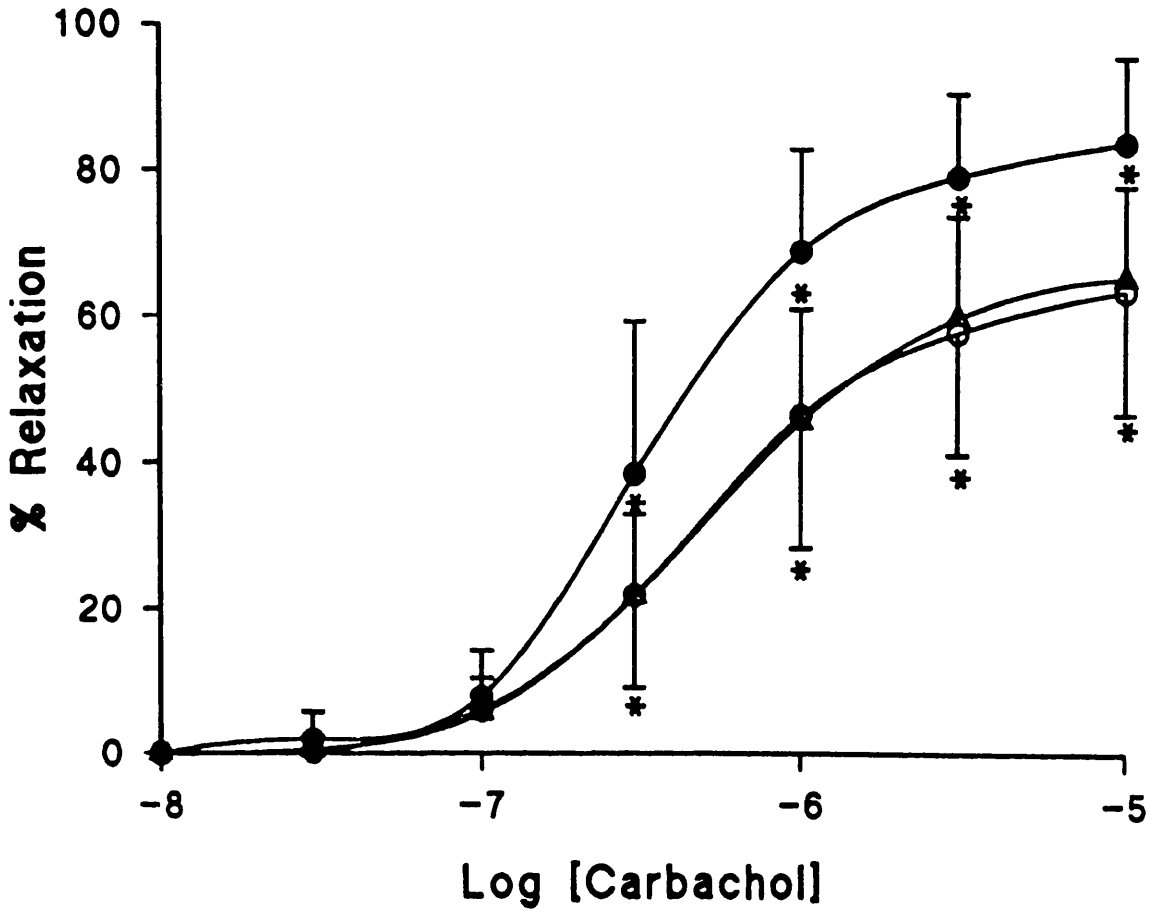


FIGURE 7.3. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
○ - XO /HX. n=12.
Δ - mannitol plus XO / HX. n=12.

* - $p < 0.05$ compared to vehicle.

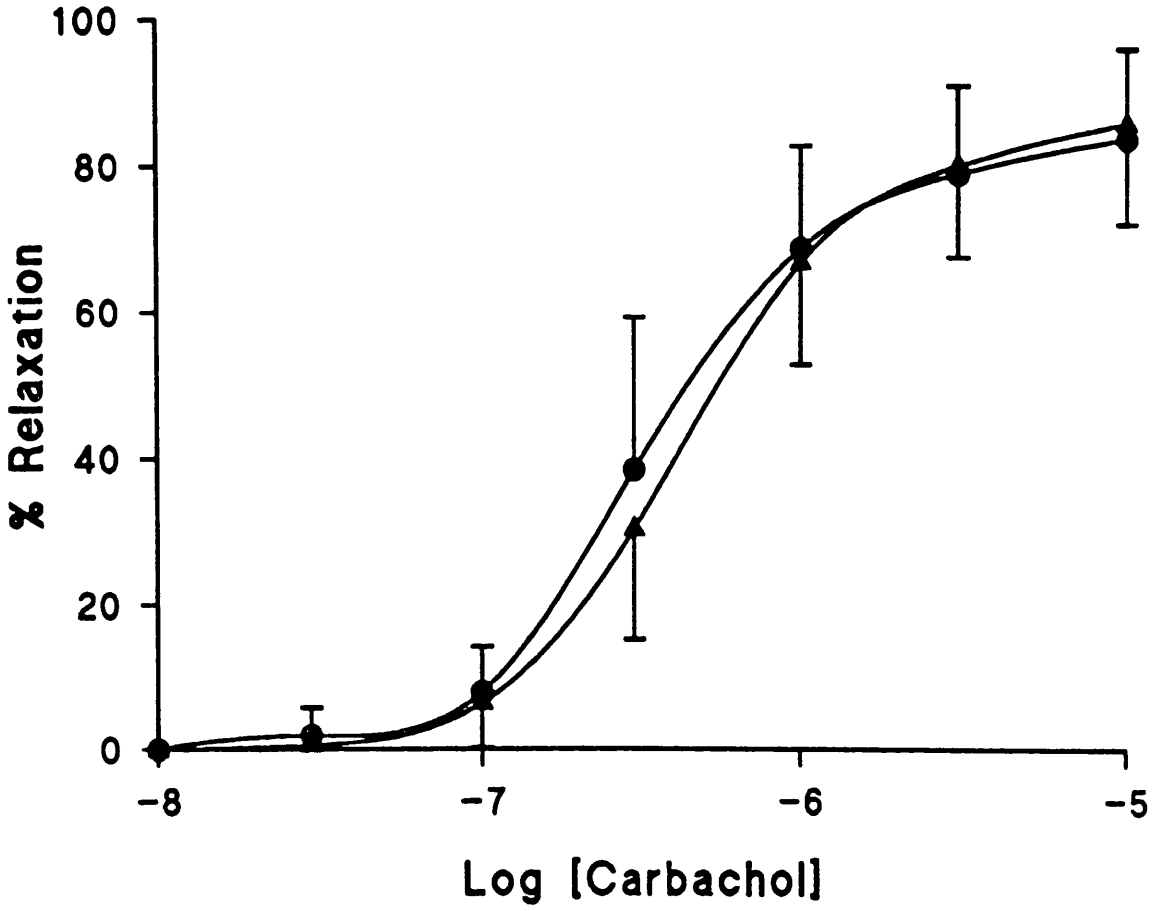


FIGURE 7.4. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
▲ - MPG. n=8.

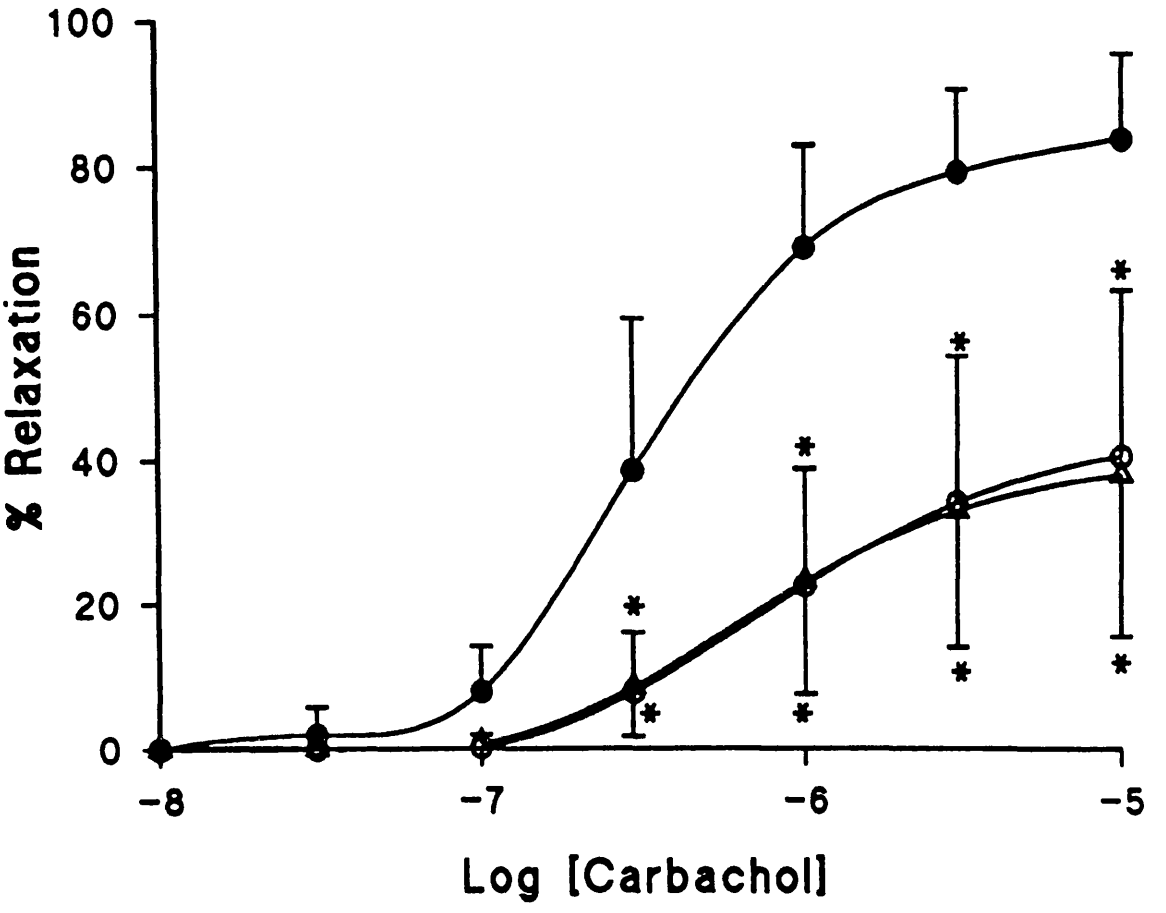


FIGURE 7.5. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
○ - XO /HX. n=9.
Δ - MPG plus XO / HX. n=9.

* - $p < 0.05$ compared to vehicle.

7.4.3 Effect of Captopril in the Presence and Absence of the Xanthine Oxidase / Hypoxanthine System.

Figure 7.6 shows that the addition of captopril (300μM) to the bath for a thirty minute period had no significant effect on the response of the aorta to carbachol.

Captopril (300μM), when incubated with the tissue for five minutes prior to and for the duration of the exposure of the tissue to the xanthine oxidase / hypoxanthine system, had no significant effect on the rise in baseline tension occuring during the exposure (Figure 7.2). There was also no protective effect against the impairment of carbachol induced relaxation of the tissue (Figure 7.7).

7.4.4 Effect of a One Hour Incubation with MPG.

Although MPG is relatively cell permeable (Devi, 1983), a 5 minute incubation is probably only sufficient for MPG to be effective in the extracellular space. Further experiments were carried out using a one hour pre-incubation of MPG (300μM) to allow more effective distribution of MPG. No significant protective effect was observed (Data not shown).

7.4.5 Effect of Superoxide Dismutase on the Xanthine Oxidase / Hypoxanthine System.

The effects of superoxide dismutase (180U/ml) were examined. Superoxide dismutase, when incubated with the tissue for thirty minutes had no significant effect on the vehicle control (Figure 7.8). When the superoxide dismutase was incubated for five minutes prior to and for the duration of the exposure to the xanthine oxidase / hypoxanthine system it was found to significantly increase the magnitude of the rise

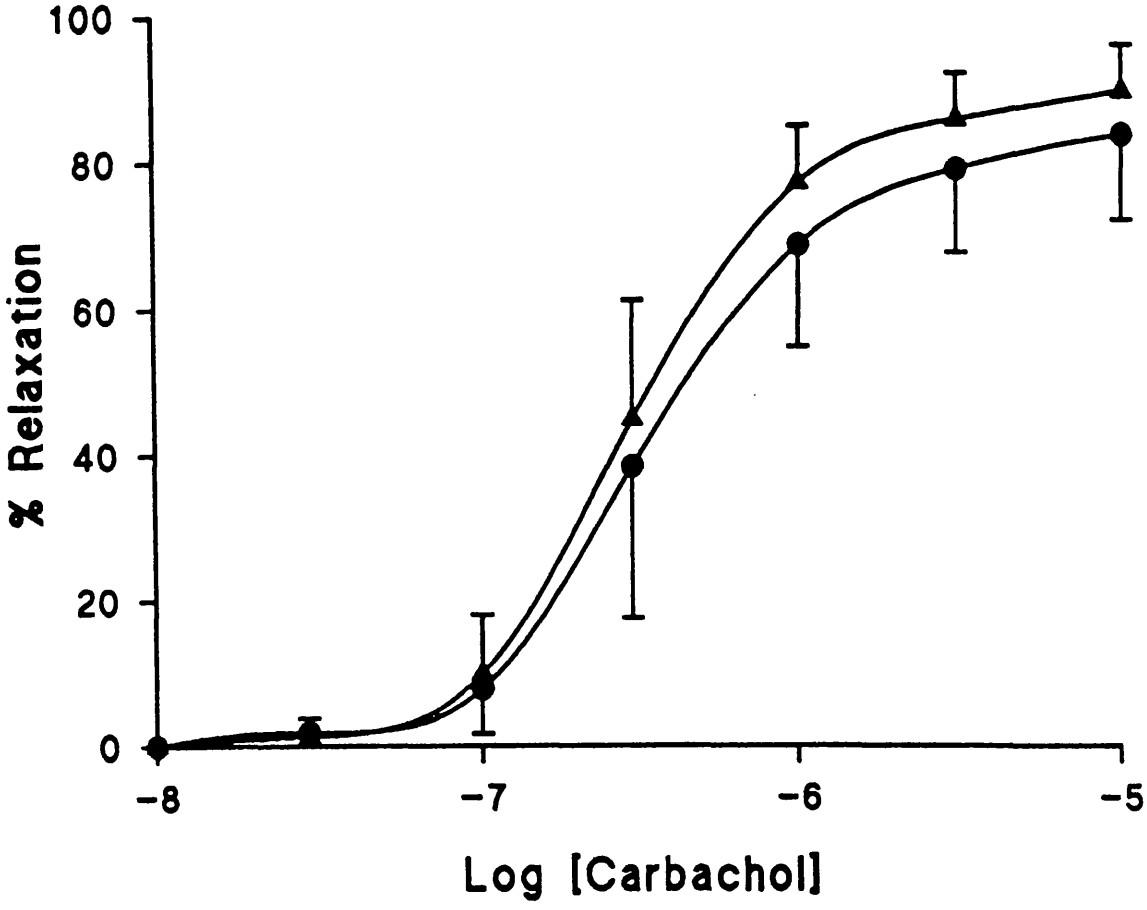


FIGURE 7.6. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
▲ - captopril. n=10.

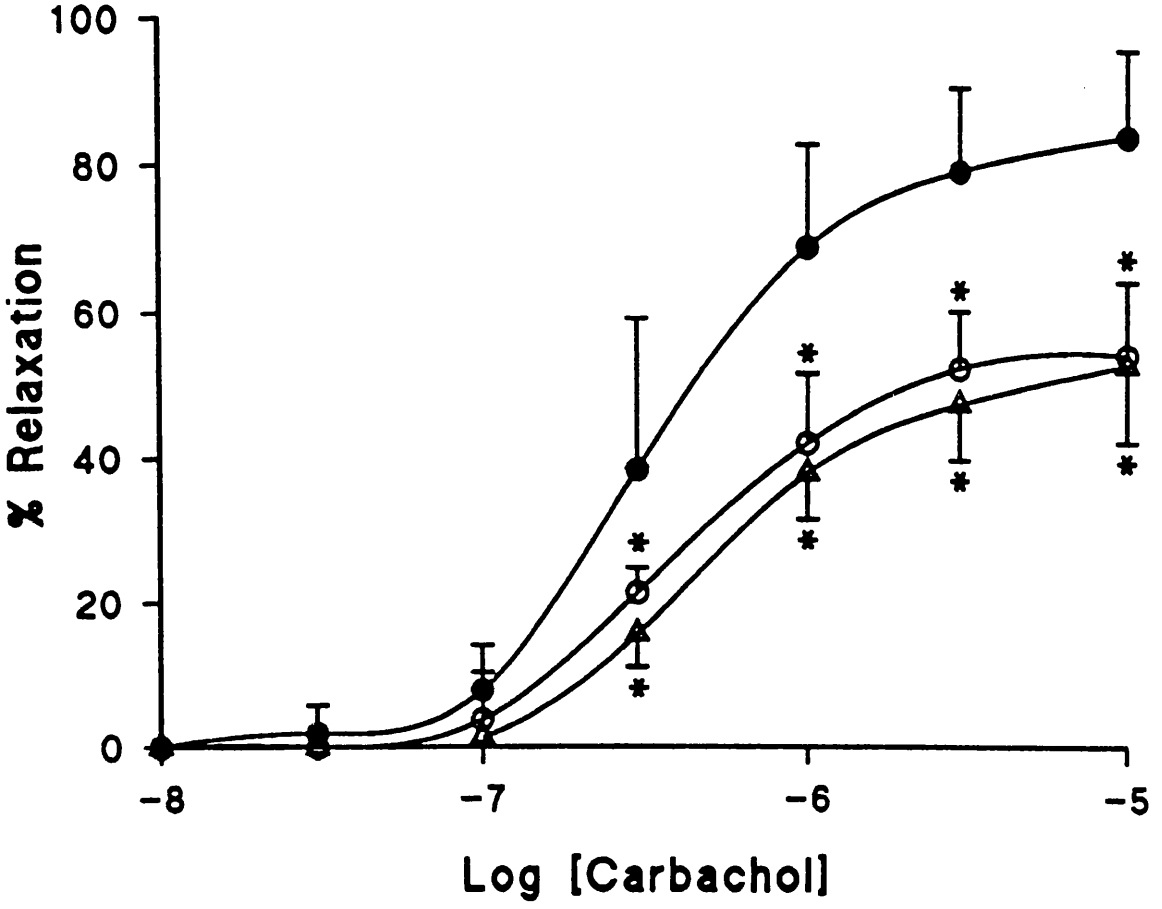


FIGURE 7.7. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
○ - XO /HX. n=8.
Δ - captopril plus XO / HX. n=9.

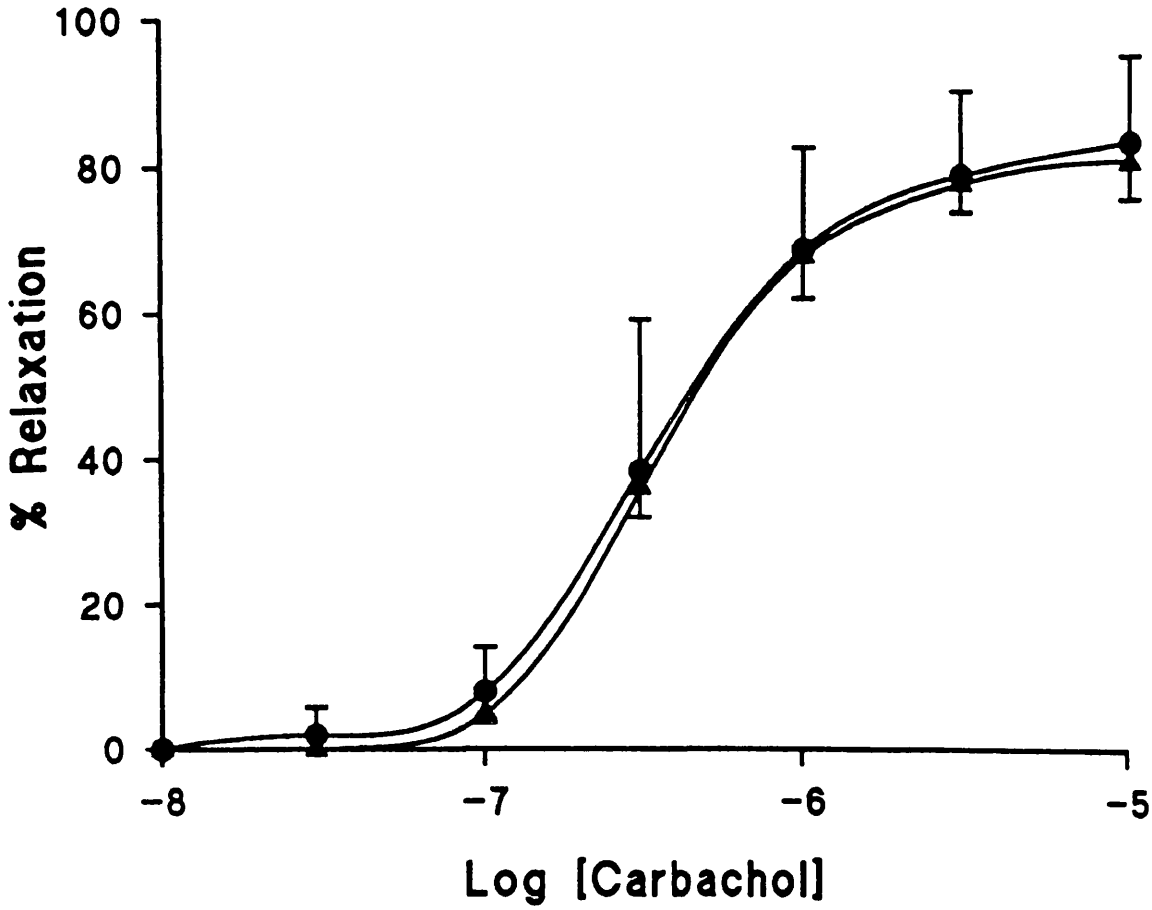


FIGURE 7.8. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
▲ - S.O.D. n=3.

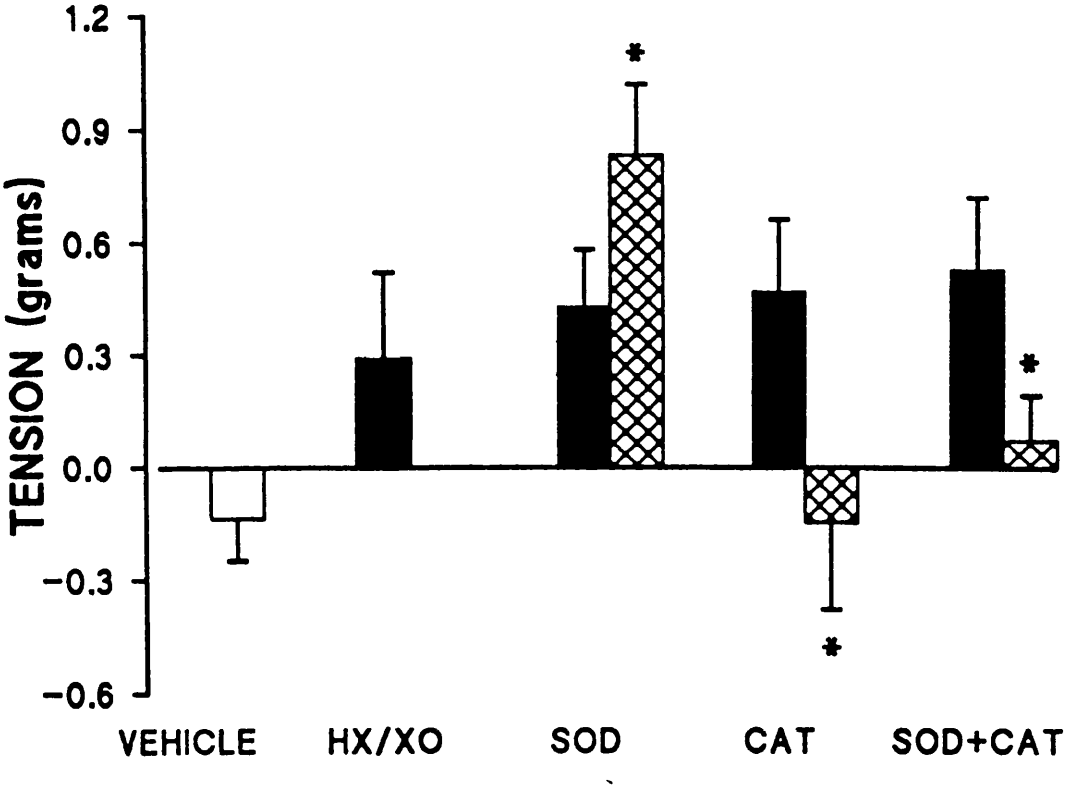


FIGURE 7.9. Change in baseline tension observed in rings of thoracic aorta during exposure to the XO / HX. Open bar represents control, solid bars represent XO /HX, hatched bars represent XO / HX plus S.O.D. or Catalase. Data is mean ± s.d. n=6-9.

* - $p < 0.05$ compared to XO / HX.

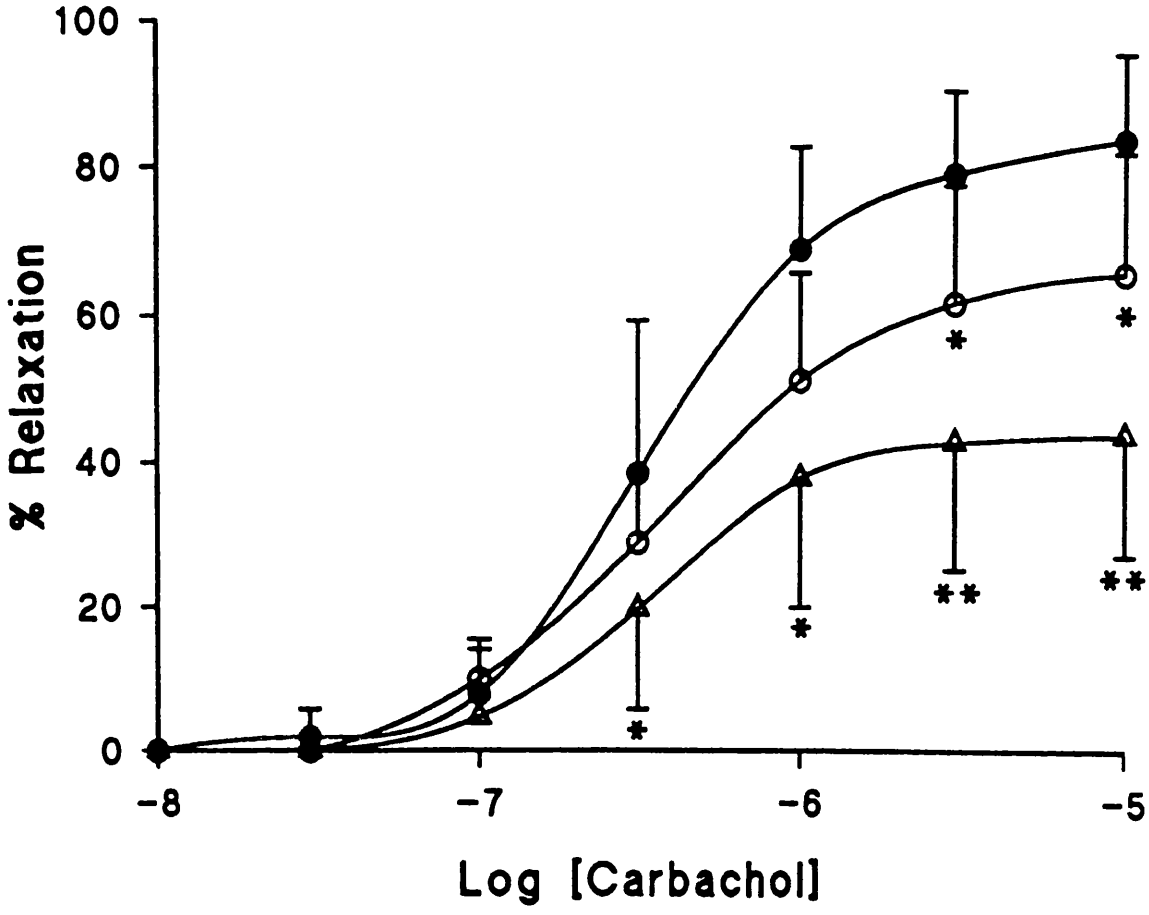


FIGURE 7.10. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
○ - XO /HX. n=6.
Δ - S.O.D. plus XO / HX. n=6.

* - $p < 0.05$ compared to vehicle.
** - $p < 0.05$ compared to vehicle and XO / HX.

in baseline tension (Figure 7.9). It was also shown to significantly increase the impairment of carbachol induced relaxation from 66% (sd 17; n=6) relaxation for the xanthine oxidase / hypoxanthine system alone to 44% (sd 17; n=6) relaxation for this system in the presence of superoxide dismutase (Figure 7.10).

7.4.6 Effect of Catalase on the Xanthine Oxidase / Hypoxanthine System.

The effects of catalase (1000U/ml) were examined. Catalase, when incubated with the tissue for thirty minutes had no significant effect on the vehicle control (Figure 7.11). When catalase was incubated with the tissue for five minutes prior to and for the duration of the exposure to the xanthine oxidase / hypoxanthine system it was found to completely inhibit the rise in baseline tension during exposure to the xanthine oxidase / hypoxanthine system (Figure 7.9). It was also shown to significantly reduce the impairment of the relaxation observed from 29% (sd 6; n=6) relaxation for the xanthine oxidase / hypoxanthine system alone to 66% (sd 14; n=6) relaxation for this system in the presence of catalase. There was however, still a significant impairment of function when compared to the vehicle control with 84% (sd 12; n=6) relaxation (Figure 7.12).

7.4.7 Combined Effect of Superoxide Dismutase and Catalase on the Xanthine Oxidase / Hypoxanthine System.

A combination of superoxide dismutase (180U/ml) and catalase (1000U/ml) was also examined. Together these enzymes were able to completely prevent the xanthine oxidase / hypoxanthine system from causing a contraction of the ring during exposure (Figure 7.9) or from impairing tissue relaxation in response to carbachol. 28% (sd 10; n=6) relaxation was seen in tissues exposed to the xanthine oxidase / hypoxanthine system alone, 81% (sd 11; n=6) relaxation was seen in tissues exposed

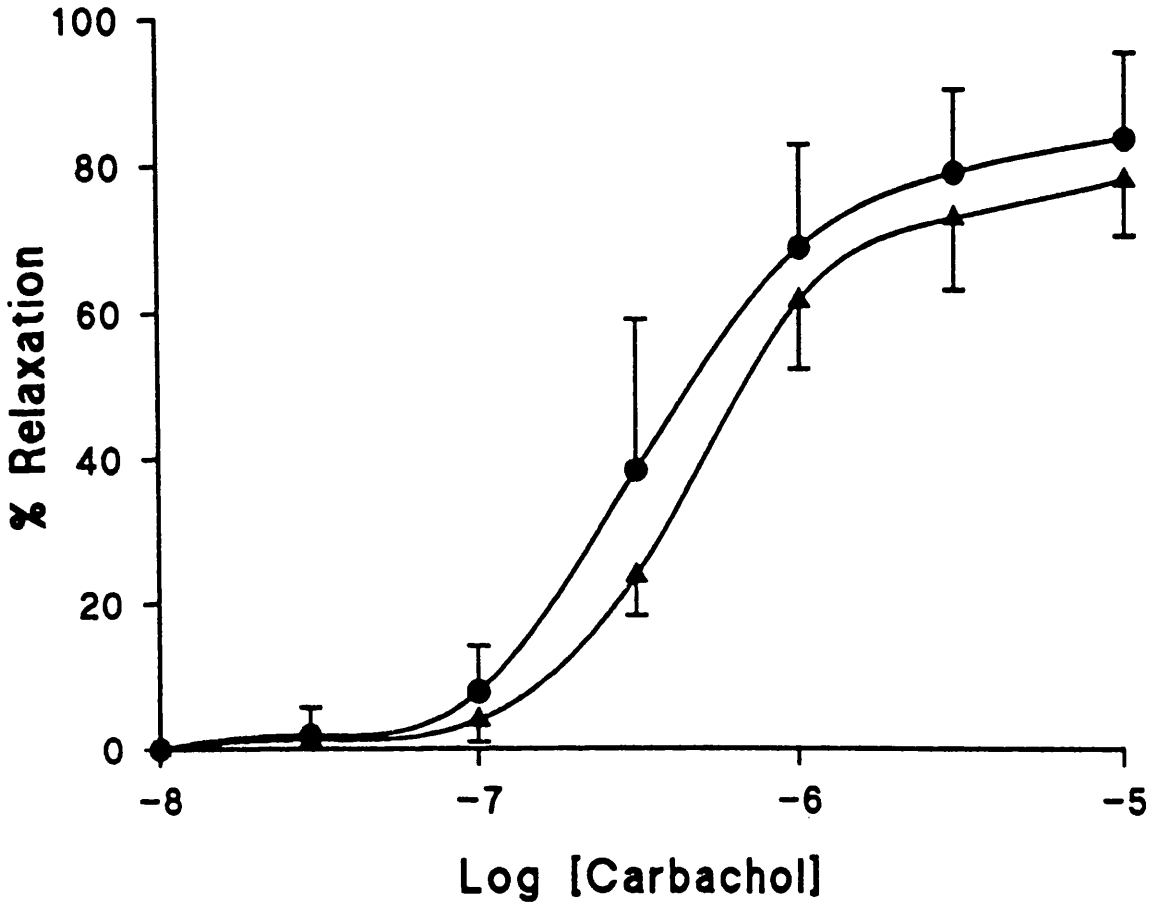


FIGURE 7.11. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
▲ - Catalase. n=3.

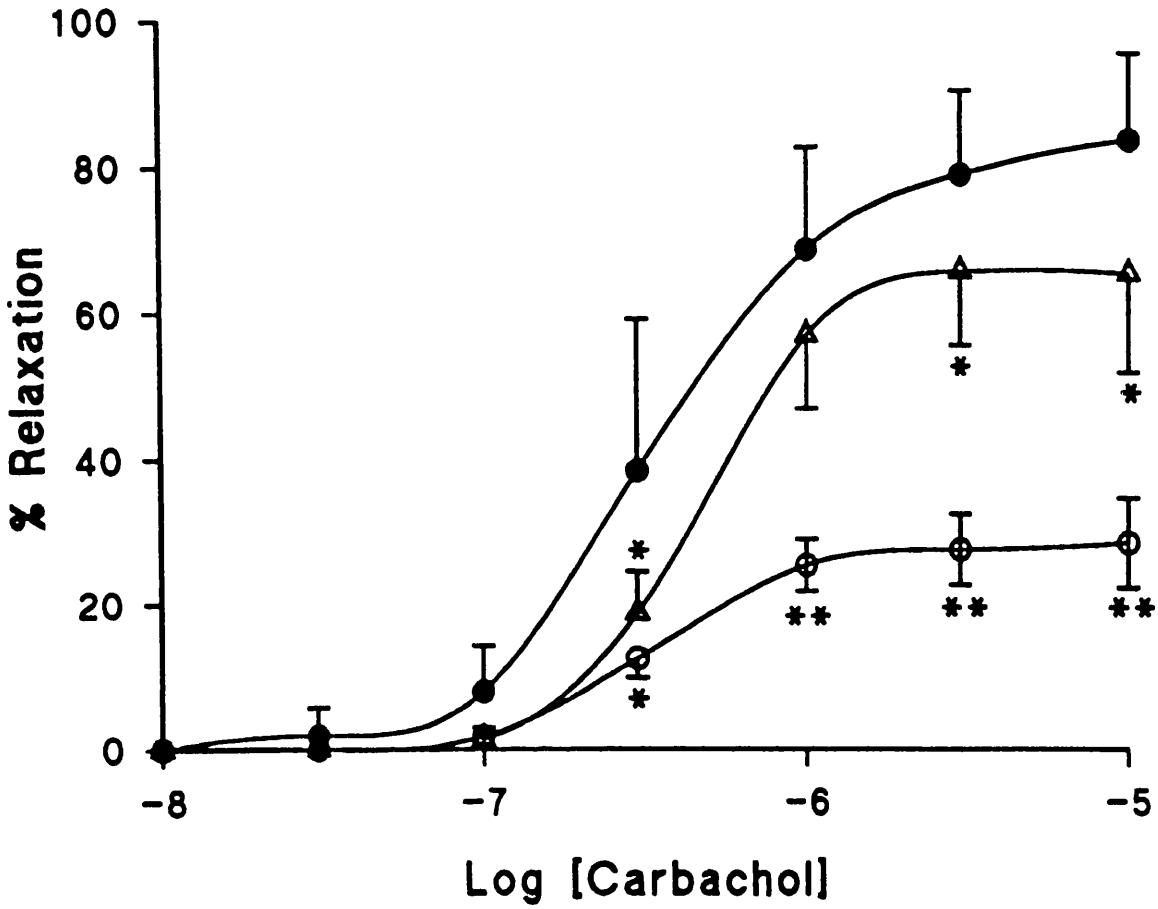


FIGURE 7.12. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
○ - XO /HX. n=6.
Δ - Catalase plus XO / HX. n=6.

* - $p < 0.05$ compared to vehicle.
** - $p < 0.05$ compared to vehicle and catalase plus XO / HX.

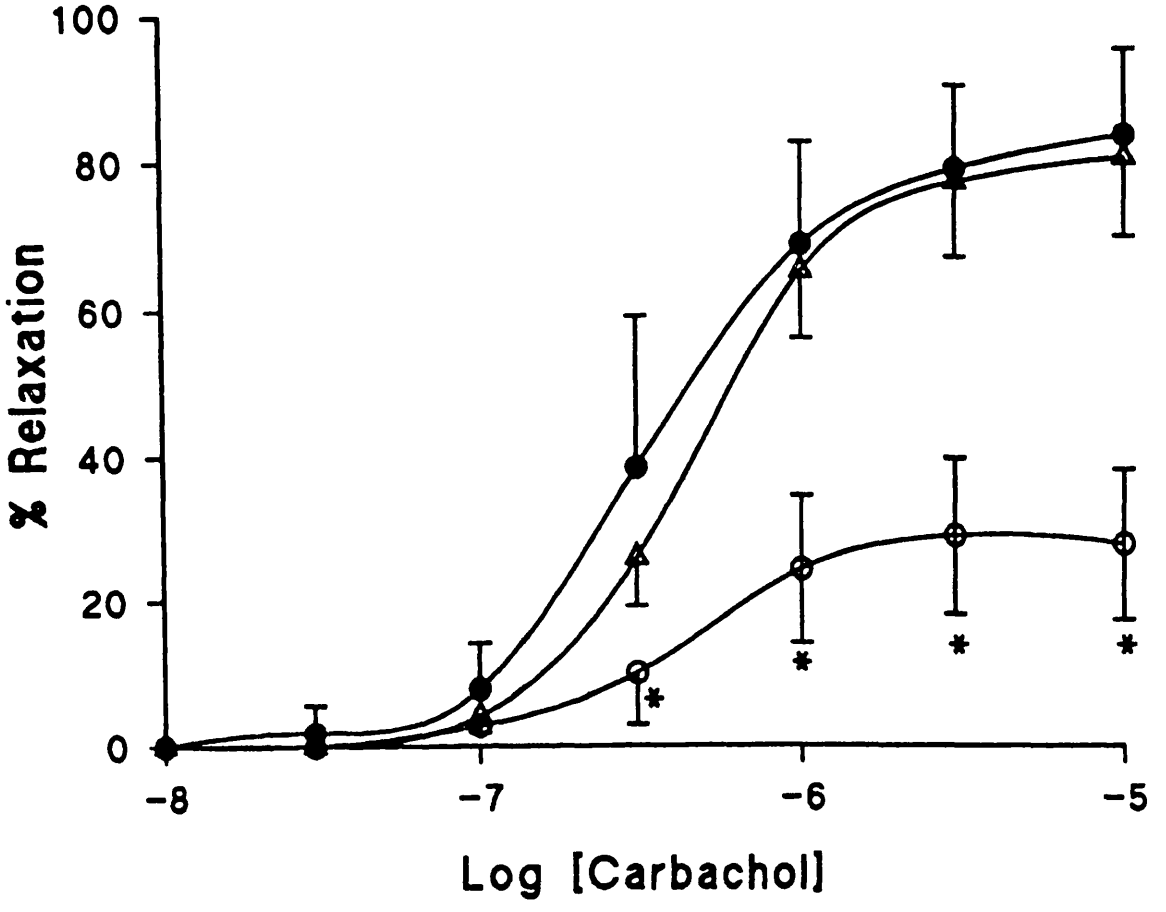


FIGURE 13. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of the phenylephrine induced tone. Data is mean ± s.d.

● - vehicle. n=9.
○ - XO /HX. n=6.
Δ - Catalase plus S.O.D. plus XO / HX. n=6.

* - $p < 0.05$ compared to vehicle and catalase plus S.O.D. plus XO / HX.

to the xanthine oxidase / hypoxanthine system in the presence of superoxide dismutase and catalase, compared with 84% (sd 12; n=6) relaxation in the vehicle control (Figure 7.13).

7.5 DISCUSSION.

In this series of experiments the identity of the cytotoxic species produced by the xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system was investigated. Scavenging the hydroxyl radical with either mannitol or the sulphydryl compounds captopril or MPG did not prevent impairment of the relaxation to carbachol, hence the hydroxyl radical is unlikely to be the injurious agent, unless it is generated intracellularly. We attempted to investigate this possibilty be using a one hour incubation with MPG, but this still offered no protective influence against the xanthine oxidase / hypoxanthine system. However it is still not possible to fully exclude the possibility of intracellular hydroxyl radical generation.

Superoxide dismutase, which catalyses the dismutation of the superoxide anion to hydrogen peroxide, accentuated the impairment of the carbachol-stimulated relaxation. Thus if a further loss of function is observed when levels of the superoxide anion are decreased, then the superoxide anion cannot be the primary injurious agent. Previous studies have shown that superoxide dismutase offers protection against the xanthine oxidase / hypoxanthine system, (Rhoades et al, 1990; Lawson et al, 1990) however these other studies examined the responses to carbachol whilst the tissue was still in the presence of the xanthine oxidase / hypoxanthine system. As both the superoxide anion and NO are free radical species, when they are produced concurrently in a system they will interact, hence the superoxide anion may be acting directly to inactivate any EDRF / NO that is produced, rather than having

any actual effect on the tissue.

If hydrogen peroxide was responsible for causing the impairment of function, then this could explain the further loss of function in the presence of superoxide dismutase, as there may have been a more rapid accumulation of hydrogen peroxide as the the superoxide anion was broken down.

Other data in our studies also support the hypothesis that hydrogen peroxide is the cytotoxic agent. Catalase, which breaks down hydrogen peroxide to water and oxygen, significantly reduced the impairment of responses. However this protection was incomplete. Yet, a combination of superoxide dismutase and catalase provided complete protection against the xanthine oxidase / hypoxanthine system. The sequence of reactions shown in Figure 1.3 would be consistent with the superoxide anion being responsible for some of the damage to the tissue. Catalase would remove the hydrogen peroxide as it was formed, yet it would not affect the amount of the superoxide anion present; Hence the superoxide anion could still exert an effect on the tissue. However, a combination of superoxide dismutase and catalase would drive the reaction through from hypoxanthine to water and oxygen.

The effects of the various protective agents could also be seen on the rise in baseline tension during the exposure of the rings to the xanthine oxidase / hypoxanthine system. As with the effect on the response to carbachol, neither mannitol, MPG nor captopril prevented the sustained contraction seen during the xanthine oxidase / hypoxanthine exposure. Again, in parallel with the relaxation studies, superoxide dismutase was seen to increase the effects of the xanthine oxidase / hypoxanthine system, whereas catalase alone or a combination of superoxide dismutase and catalase were seen to diminish the effects.

These observations suggest that hydrogen peroxide is the primary reactive oxygen species responsible for the impairment of the carbachol induced endothelium dependent relaxation in aortic rings in this system, but that the superoxide anion may also have a detrimental effect. The relative effect of these two species may be exaggerated by their respective rates of diffusion which are dependent on charge. However, it seems that hydrogen peroxide alone is responsible for the sustained elevation of baseline tension observed during incubation with the xanthine oxidase / hypoxanthine system, as catalase alone was able to block this effect completely.

Other work supporting the view that hydrogen peroxide is the primary cytotoxic species generated by xanthine oxidase has been carried out in several systems. A study in rat pulmonary artery rings examined the contractile response to a xanthine oxidase / xanthine system (Rhoades et al, 1990). Using the enzymes catalase and SOD, and also direct studies with hydrogen peroxide, this study concluded that hydrogen peroxide was the species responsible for the observed contraction. Another study examined the effects of a xanthine oxidase / xanthine system on the contractile response to $PGF_{2\alpha}$, inhibition of contraction was observed following exposure to the free radical / reactive oxygen species generating system (Rubanyi, 1986). This inhibition could be blocked by catalase and mimicked by hydrogen peroxide. Studies of xanthine oxidase based systems have also been conducted in endothelial cell systems. Hydrogen peroxide has been implicated as the injurious agent with three different techniques for assessing endothelial cell injury :- permeability of trypan-blue labelled albumin (Berman and Martin, 1993), platelet adherence and prostacyclin release (Shatos et al, 1991), chromium^{51} release (Kvietys et al, 1989). Chromium^{51} release was also used as an indicator of cellular injury in fibroblasts exposed to xanthine oxidase / xanthine, again hydrogen peroxide was implicated (Simon et al, 1981). Link and coworkers (1988) examined the effects of xanthine

oxidase / xanthine on survival of epithelial cells, again with the use of catalase, hydrogen peroxide was proposed as the species responsible for lethal injury.

Two groups have used the isolated perfused lung to study the effects of xanthine oxidase. Barnard and colleagues (1992) studied changes in permeability in response to a xanthine oxidase / hypoxanthine system. Using catalase, SOD and DMSO it was concluded that hydrogen peroxide was responsible for changes in vascular permeability. Kjæve and colleagues (1991) assessed changes in pulmonary pressure and increases in oedema following exposure to a xanthine oxidase / hypoxanthine system. Chemiluminesence techniques demonstrated that hydrogen peroxide was the major reactive oxygen species produced, and catalase was able to prevent the effects of the xanthine oxidase / hypoxanthine system.

Two *in vitro* studies of ischaemia reperfusion have also implicated hydrogen peroxide. In 1991 Repine and colleagues published a study in the isolated, perfused rat heart, in which catalase or GSH were able to prevent the detrimental effects of 20 minutes global ischaemia followed by 40 minutes reperfusion. SOD was ineffective. In 1992 Lum and colleagues reported the beneficial effects of catalase in preventing increases in ^{125}I-albumin permeability in endothelial cells following a period of hypoxia and reoxygenation.

Thus in several different *in vitro* models studying xanthine oxidase based systems, hydrogen peroxide has been implicated as the primary injurious agent.

CHAPTER EIGHT.

DIRECT EFFECTS OF HYDROGEN PEROXIDE ON THE FUNCTION OF ISOLATED AORTAE FROM NEW ZEALAND WHITE RABBITS.

8.1 INTRODUCTION.

The xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system is known to produce the superoxide anion, hydrogen peroxide and the hydroxyl radical. The experimental data presented in the previous two chapters has shown that this xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system is able to impair the vasoactive properties of rings of thoracic aorta from New Zealand White rabbits. Phenylephrine induced contraction is impaired and both carbachol induced endothelium dependent and SNP induced non-endothelium dependent relaxation are significantly reduced following exposure to this system. Further work using a range of enzymes and free radical scavengers has demonstrated that hydrogen peroxide is the primary cytotoxic species produced by this xanthine oxidase / hypoxanthine system.

The direct effects of hydrogen peroxide have previously been examined in a number of situations. Several studies which have used xanthine oxidase to generate free radicals and reactive oxygen species have demonstrated that hydrogen peroxide is able to mimic the observed effects of the xanthine oxidase in the different models, for example in isolated tissue studies (Rubanyi and Vanhoutte, 1986; Rhoades et al, 1990), endothelial cells (Kvietys et al, 1989; Berman and Martin, 1993), epithelial cells (Link and Riley, 1988), fibroblasts (Simon et al, 1981), and isolated perfused lung (Kjæve et al, 1991; Barnard and Matalon, 1992). However, none of these studies have examined the permanent effects of the xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system on the relaxant properties of arterial rings. Following an ischaemic attack, the ability of the vessel to relax is crucial for the restoration and maintenance of blood flow through the vessel and supply of oxygen to the surrounding tissue. Endothelium dependent relaxation is possibly most susceptible to the detrimental effects of a burst of free radicals /

reactive oxygen species due to the physical nature of the endothelium.

8.2 AIMS.

Hence the aims of this study were to examine the direct effect of a range of concentrations of hydrogen peroxide on carbachol induced endothelium dependent relaxation in rings of thoracic aorta from New Zealand White rabbits. Also to compare the effects of hydrogen peroxide both quantitatively and qualitatively to the effects of the xanthine oxidase / hypoxanthine system described previously.

8.3 METHODS.

Following the same protocol as for the xanthine oxidase / hypoxanthine experiments, tissues were exposed to a range of concentrations of hydrogen peroxide as described in detail in Chapter Two.

8.4 RESULTS

8.4.1 Effect of Hydrogen Peroxide on Baseline Tension.

Figure 8.1 shows the effect on the aortic rings of a thirty minute incubation with a range of concentrations of hydrogen peroxide (100 - 1000μM) on the baseline tension. It can be seen that 100μM hydrogen peroxide has no significant effect on baseline tension when compared to control. There was no change in baseline tension with 300μM hydrogen peroxide and that 500, 700 and 1000μM hydrogen peroxide cause an increase in baseline tension. The responses to 500 and 700μM hydrogen peroxide are similar in magnitude to the response observed during incubation with the xanthine oxidase / hypoxanthine system.

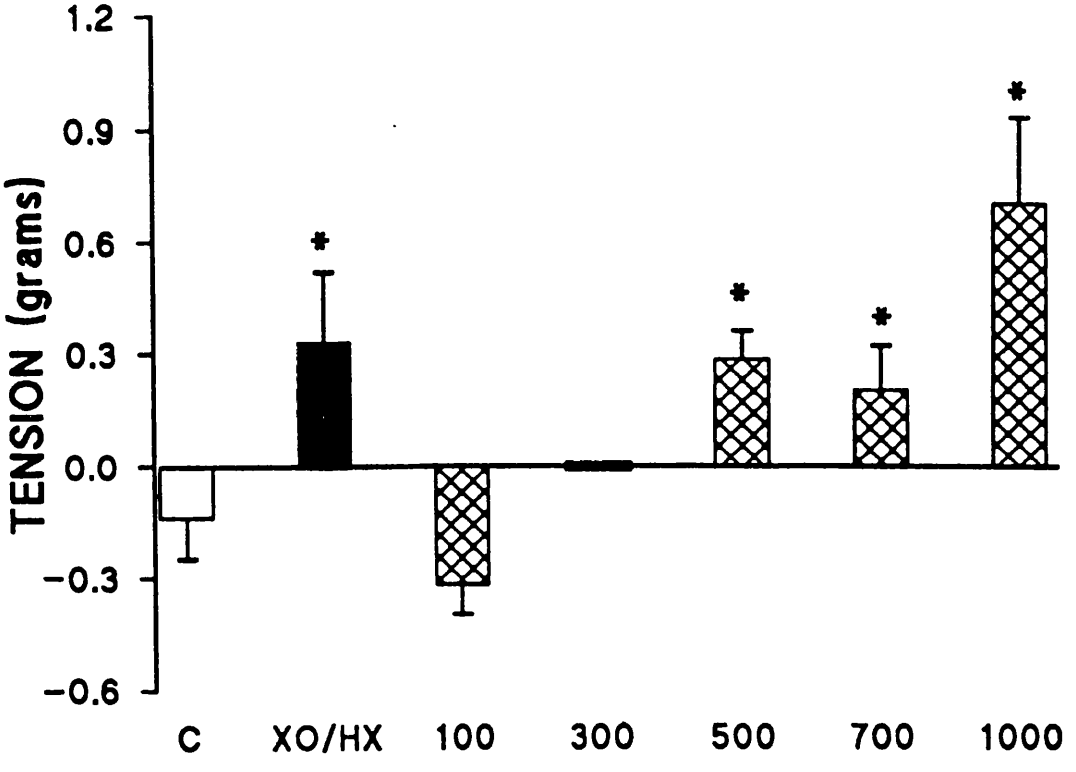


FIGURE 8.1. Change in baseline tension observed in rings of thoracic aorta during exposure to hydrogen peroxide. Open bar represents control (C), solid bar represents XO/HX, hatched bars represent varying concentrations of hydrogen peroxide (μM) as labelled. Data is mean ± s.d. n=5-9.

* - $p < 0.05$ compared to control.

8.4.2 Effect of Hydrogen Peroxide on the Response to Carbachol.

As discussed in Chapter Six, there was a significant decrease in relaxation after a thirty minute incubation with vehicle (0.5ml Krebs) (Figure 8.2). Hence as before the vehicle control data was used in all statistical analysis.

Figure 8.3 shows the effect of a range of concentrations of hydrogen peroxide from 100μM to 1000μM on carbachol induced relaxation. It can be seen that there is a clear dose dependent decrease in the ability of the ring to relax in response to carbachol after exposure to hydrogen peroxide. At 100μM hydrogen peroxide there is little impairment of response - 76 ± 4% relaxation compared with 83 ± 12% relaxation in the control rings. The effect on the relaxation increases with increasing hydrogen peroxide concentration up to 1000μM hydrogen peroxide which completely abolishes the ability of the tissue to respond to carbachol.

This effect was seen to be qualitatively similar to the effect of the xanthine oxidase / hypoxanthine system and at 500μM hydrogen peroxide was also seen to be quantitatively similar to the xanthine oxidase / hypoxanthine system (Figure 8.4).

From this it was found that 500μM hydrogen peroxide closely mimicked the effects of the xanthine oxidase / hypoxanthine system. Hence the effects of 500μM hydrogen peroxide were also examined on the phenylephrine induced contraction and on SNP induced relaxation in rings of thoracic aorta from New Zealand White rabbits.

8.4.3 Effect of Hydrogen Peroxide on the Response to Phenylephrine.

As in previous experiments, a thirty minute incubation with vehicle (0.5ml Krebs)

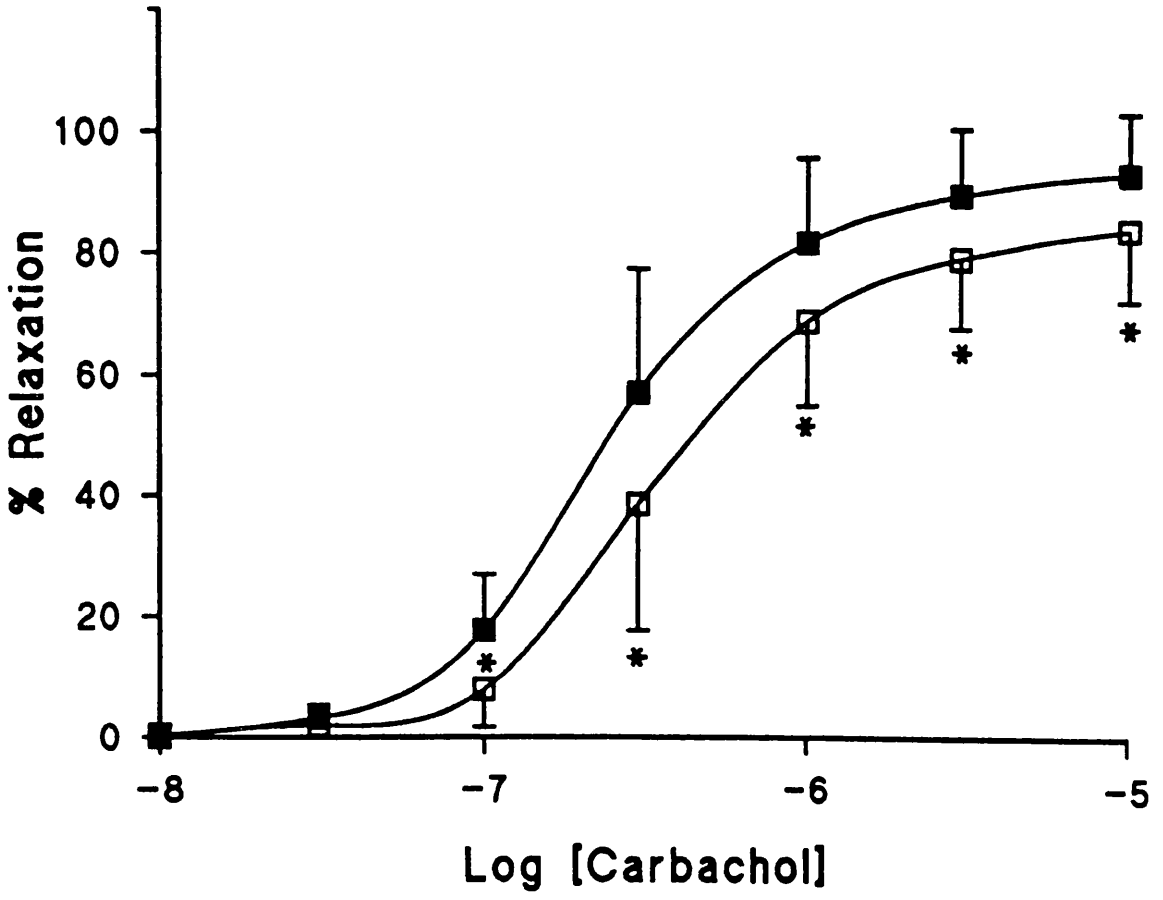


FIGURE 8.2. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

■ - control. n=9.
□ - vehicle. n=9.

* - $p < 0.05$ compared to control.

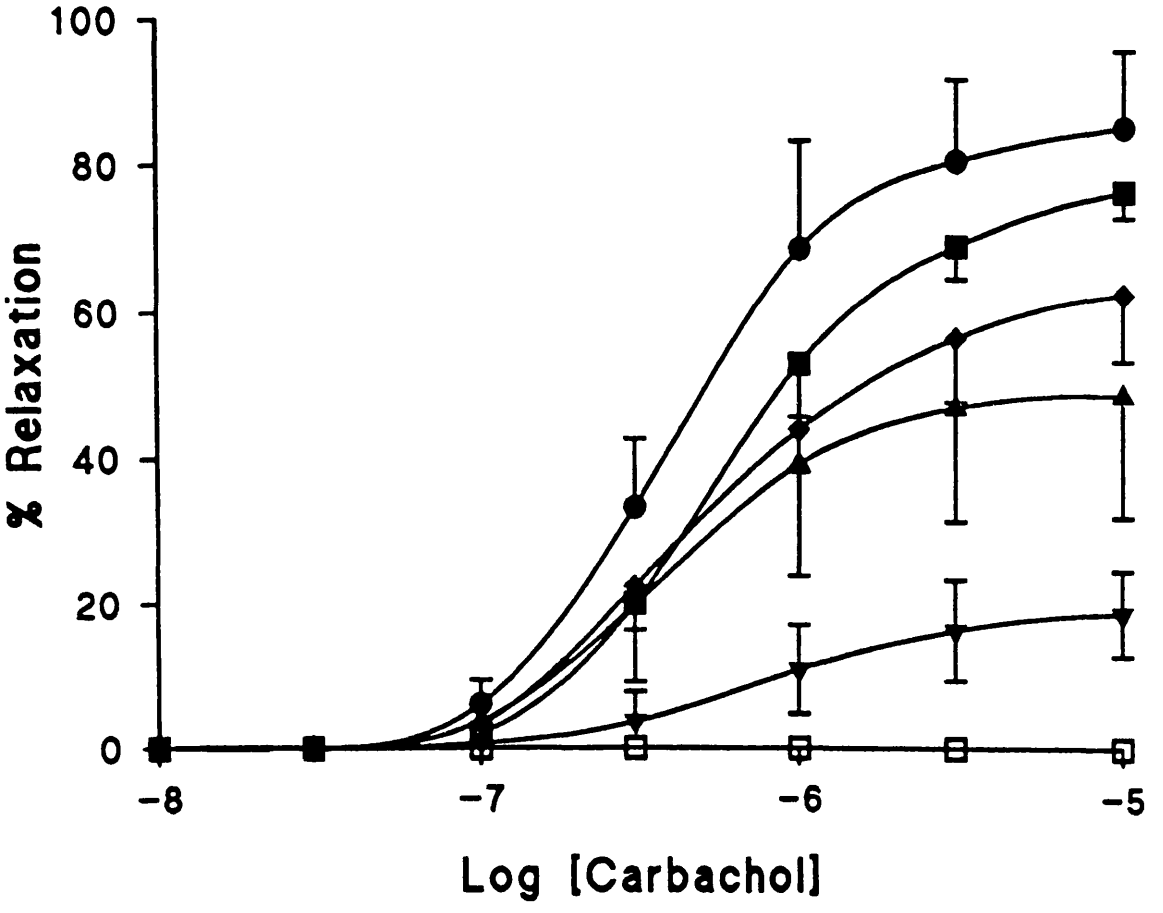


FIGURE 8.3. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

- ● - control. n=9.
- ■ - 100μM H_2O_2. n=5.
- ◆ - 300μM H_2O_2. n=5.
- ▲ - 500μM H_2O_2. n=18.
- ▼ - 700μM H_2O_2. n=5.
- □ - 1000μM H_2O_2. n=5.

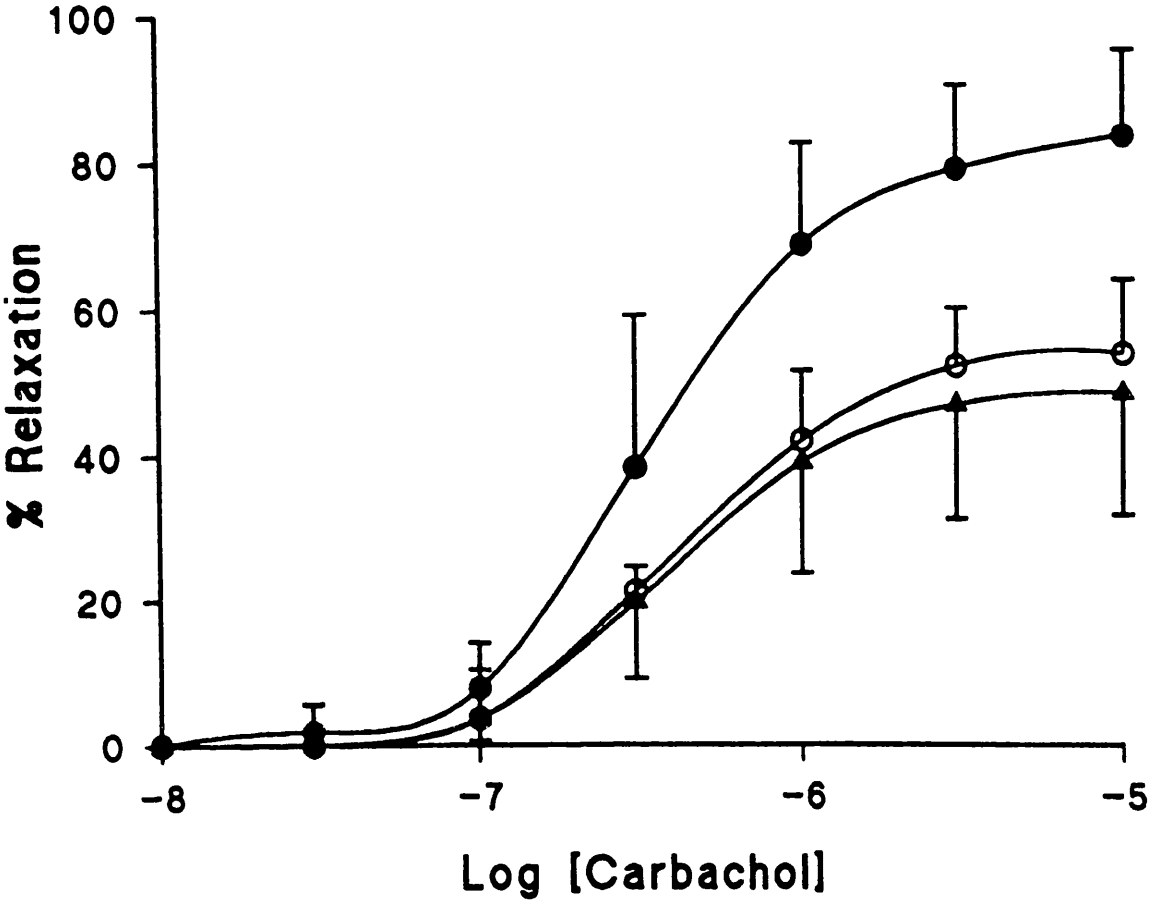


FIGURE 8.4. Cumulative concentration-response curves to carbachol (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

● - control. n=9.
▲ - 500μM H_2O_2. n=18.
○ - XO/HX n=49.

led to a significant decrease in contraction (Figure 8.5). Hence as before the vehicle control data was used in all statistical analysis.

Exposure of the rings to 500μM hydrogen peroxide lead to a significant impairment of the ability of the ring to constrict to phenylephrine. This effect was statistically significant at all but the two lowest concentrations of phenylephrine and also the highest dose where the difference was found to be marginally non significant - 95% C.I. (-0.165,0.002) (Figure 8.6).

These effects were found to be quantitatively and qualitatively similar to the effects of the xanthine oxidase / hypoxanthine system. Figure 8.7 shows the control dose response curves for the hydrogen peroxide and xanthine oxidase / hypoxanthine experiments before exposure to any free radicals / reactive oxygen species. There is a slight difference in the curves at $3x10^{-6}$M phenylephrine - 95% C.I. (-0.155,-0.014). This presumably reflects experimental variation.

Comparing the effects of the xanthine oxidase / hypoxanthine system and the effects of hydrogen peroxide (Figure 8.8), it can be seen that the effects of the two systems are similar. The effects of the xanthine oxidase / hypoxanthine system are marginally greater at $3x10^{-6}$M and 10^{-5}M phenylephrine - 95% C.I. (-0.167,-0.026) and (-0.194,-0.053) respectively.

8.4.3 Effect of Hydrogen Peroxide on the Response to Sodium Nitroprusside.

In this set of experiments exposure to vehicle (0.5ml Krebs) for a thirty minute period was not seen to have a statistically significant effect (Figure 8.9). However as can be seen from Figure 8.9 the response to sodium nitroprusside tended to be lower after the thirty minute exposure to vehicle. Although this was not found to be

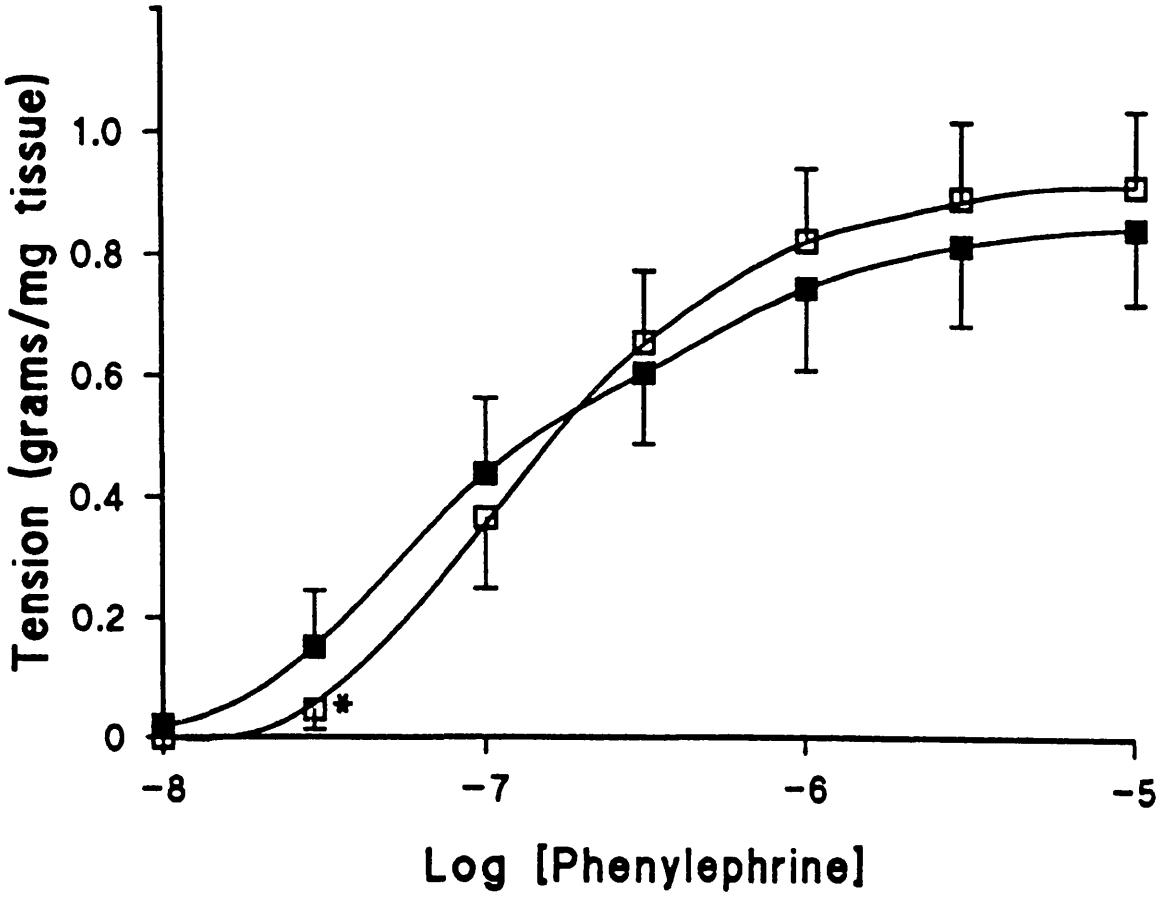


FIGURE 8.5. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

■ - control. n=6.
□ - vehicle. n=6.

* - $p < 0.05$ compared to control.

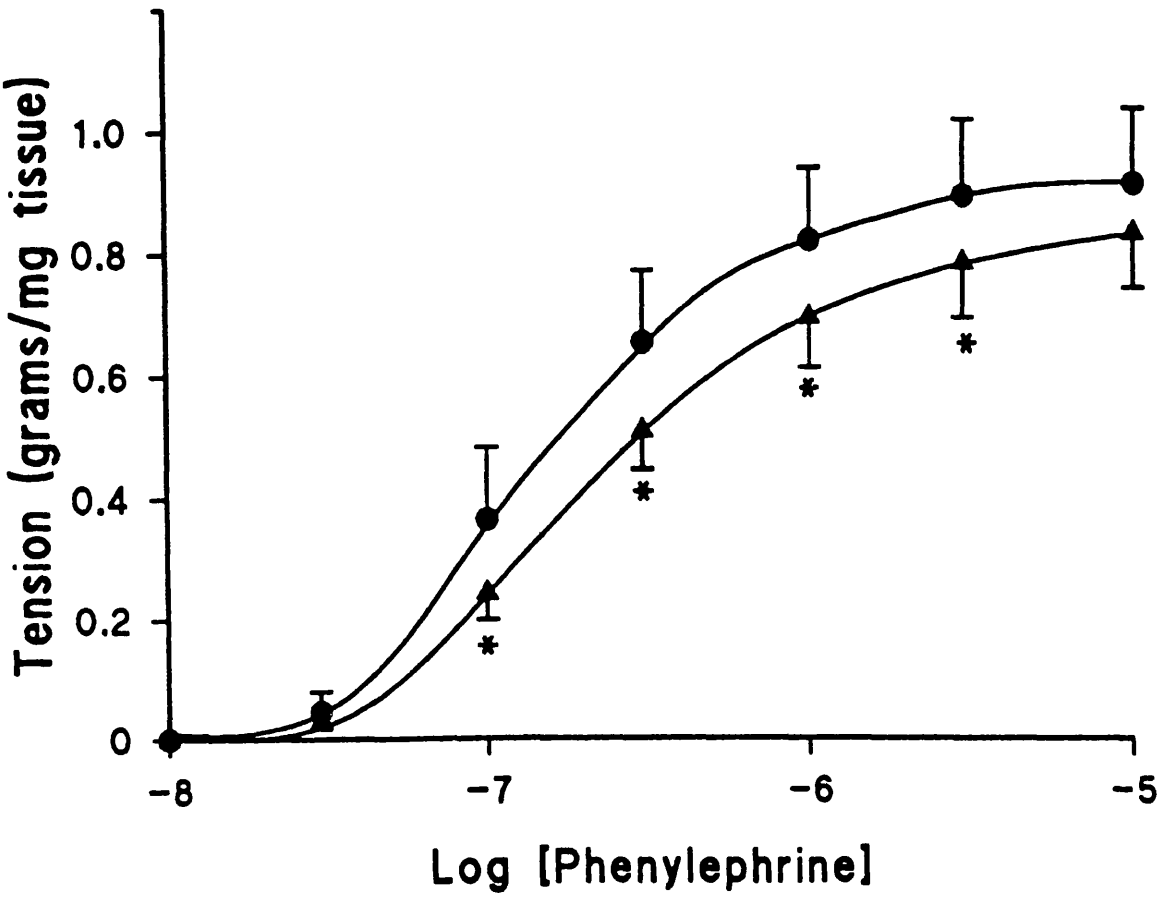


FIGURE 8.6. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

● - vehicle. n=6.
▲ - 500μM H_2O_2. n=6.

* - $p < 0.05$ compared to vehicle.

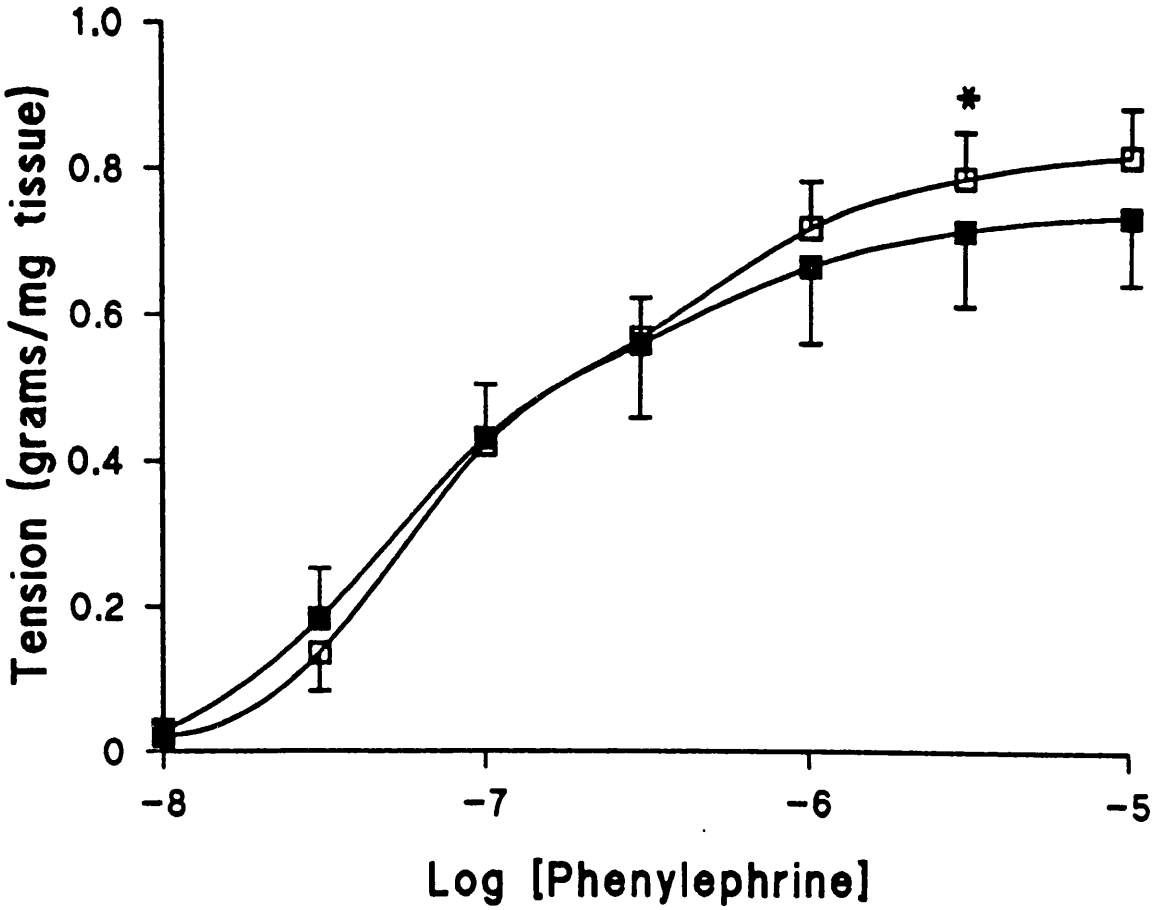


FIGURE 8.7. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

■ - control for XO / HX study. n=12.
□ - control for H_2O_2 study. n=6.

* - $p < 0.05$ compared to control for XO / HX study.

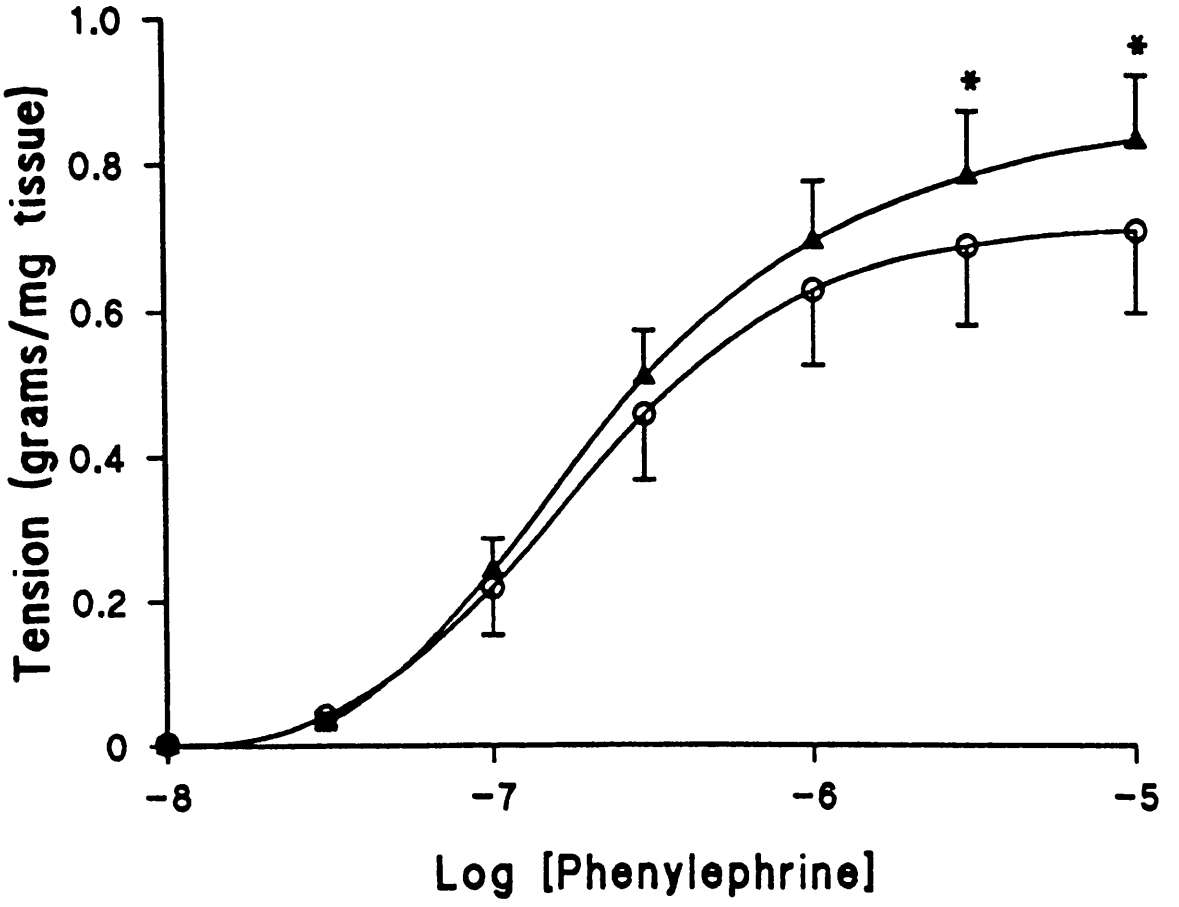


FIGURE 8.8. Cumulative concentration-response curves to phenylephrine (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed in grams tension / mg tissue.

○ - XO / HX. n=12.
▲ - H_2O_2. n=6.

* - $p < 0.05$ compared to XO / HX.

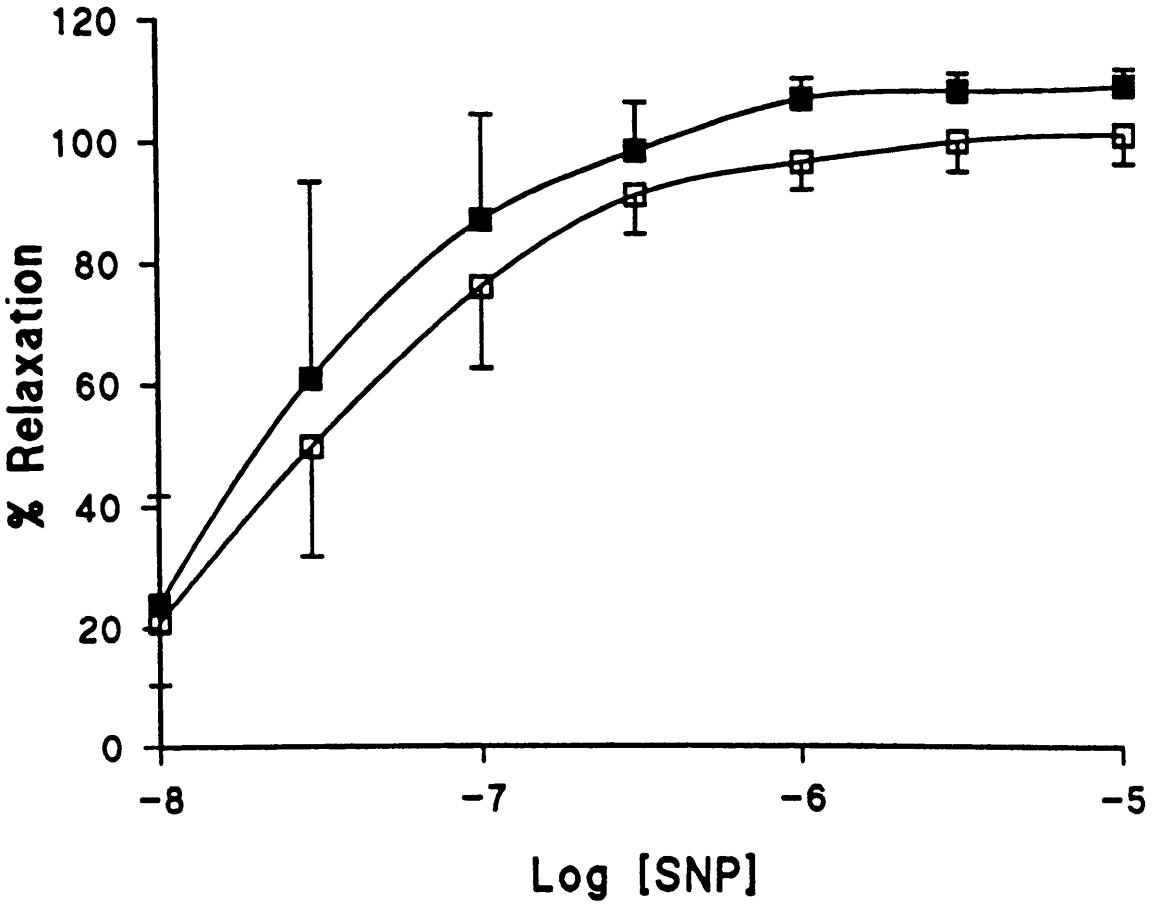


FIGURE 8.9. Cumulative concentration-response curves to SNP (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

■ - control. n=9.
□ - vehicle. n=9.

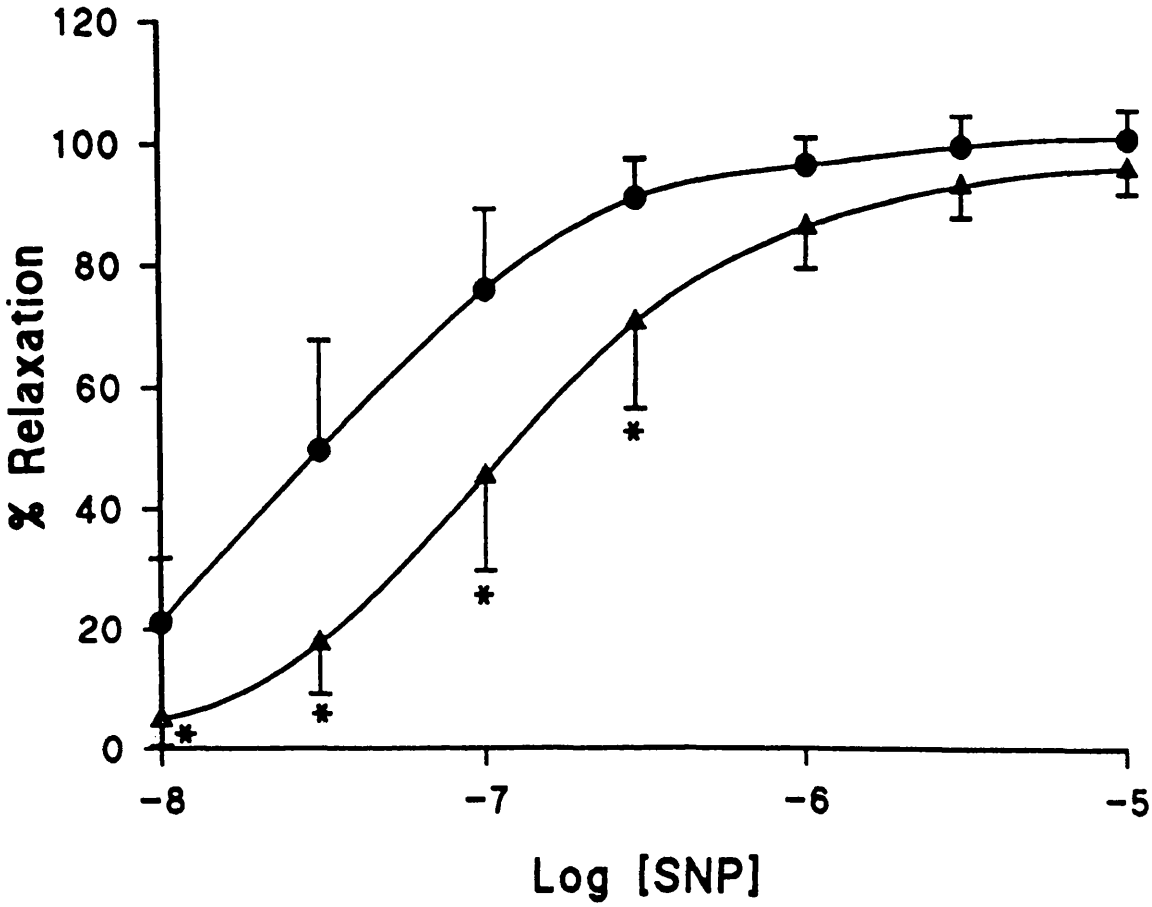


FIGURE 8.10. Cumulative concentration-response curves to SNP (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

● - control. n=9.
▲ - H_2O_2. n=9.

* - $p < 0.05$ compared to vehicle.

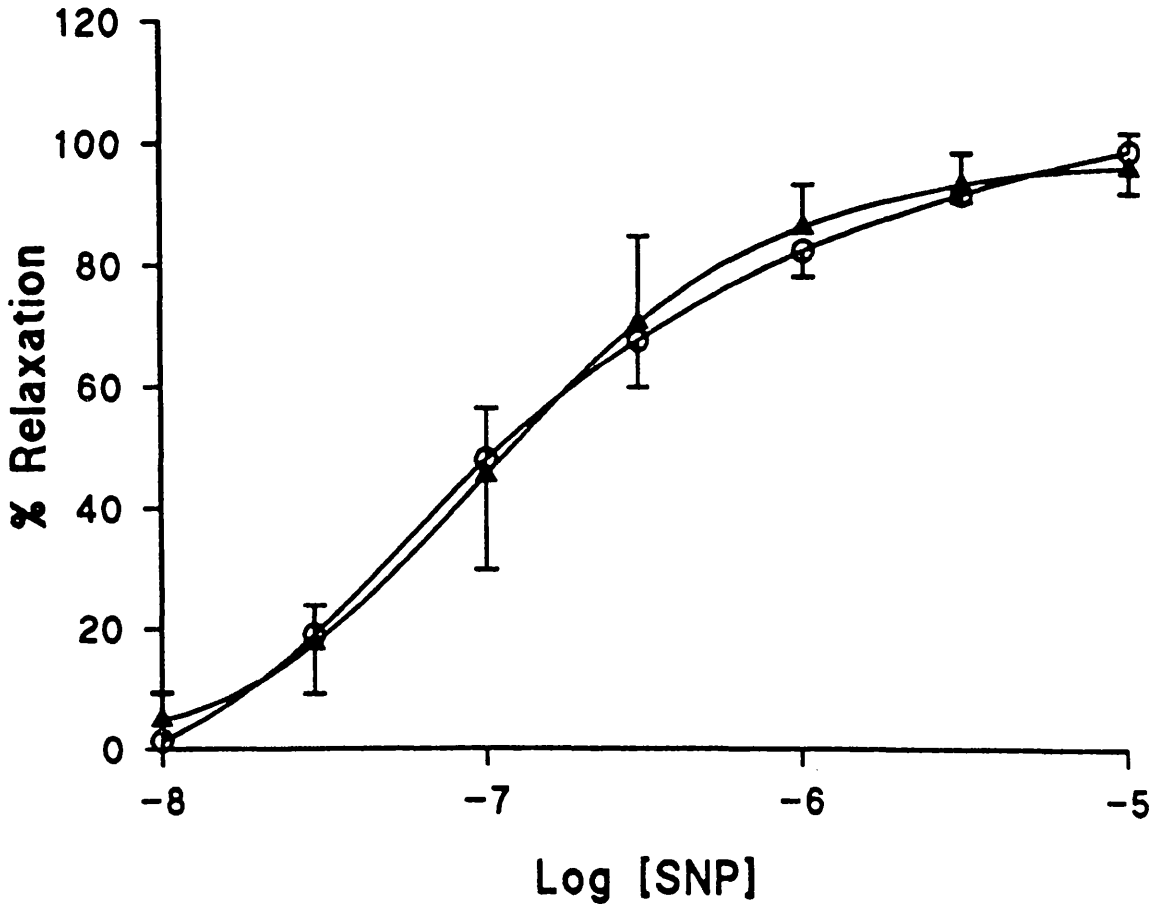


FIGURE 8.11. Cumulative concentration-response curves to SNP (10^{-8}M-10^{-5}M) in rings of thoracic aorta from New Zealand White rabbits. Responses expressed as percentage relaxation of phenylephrine induced tone.

○ - XO / HX. n=6.
▲ - H_2O_2. n=9.

significant, this vehicle control data was used for comparison in the statistical analysis as before.

Exposure of the rings to 500μM hydrogen peroxide lead to a significant decrease in the response to sodium nitroprusside from 10^{-8}M through to $3x10^{-7}$M. There was no statistically significant difference in response to sodium nitroprusside from 10^{-6}M to 10^{-5}M (Figure 8.10).

Again these effects were seen to be quantitatively and qualitatively similar to the effects of exposure to the xanthine oxidase / hypoxanthine system (Figure 8.11).

8.5 DISCUSSION.

The studies discussed in Chapter Seven concluded that hydrogen peroxide is the primary reactive oxygen species responsible for the impairment of the carbachol induced, endothelium dependent relaxation in aortic rings in this xanthine oxidase / hypoxanthine based free radical / reactive oxygen species generating system. Also that hydrogen peroxide is responsible for the elevation of baseline tension during incubation with the xanthine oxidase / hypoxanthine system.

This conclusion is further supported by the studies described in this chapter. These studies examined the direct effects of hydrogen peroxide on the responses of rings of thoracic aorta from New Zealand White rabbits. As in the xanthine oxidase / hypoxanthine studies (Chapter Six), the responses to phenylephrine, carbachol and sodium nitroprusside were examined.

A range of concentrations of hydrogen peroxide were added directly to the baths and their effects on the carbachol induced endothelium dependent relaxation were

examined. From this data it can be seen that 500μM hydrogen peroxide causes similar impairment of the response as the xanthine oxidase / hypoxanthine system. The effect was not only quantitatively similar but was also qualitatively similar, in that the shape of the responses to carbachol were affected in a similar manner by the hydrogen peroxide as they were by the xanthine oxidase / hypoxanthine system.

The effects of 500μM hydrogen peroxide on the responses to phenylephrine and sodium nitroprusside were also studied. As with the responses to carbachol it can be seen that hydrogen peroxide can qualitatively and quantitatively mimic the effects of the xanthine oxidase / hypoxanthine system.

Hydrogen peroxide was also able to mimic the increase in baseline tension observed during incubation of the aortic ring with the xanthine oxidase / hypoxanthine system. As with the other effects 500μM hydrogen peroxide was the most comparable dose.

The effect of hydrogen peroxide on baseline tension was found to be dose dependent - increasing concentrations produce greater elevation of baseline tension. However, the concentration response curve (Figure 8.1) exhibits a slight deviation from the expected line at 700μM. This is probably simply be due to the low numbers involved in this section of the study. However, a biphasic response to hydrogen peroxide has also been observed in studies of arterial endothelial barrier dysfunction (Berman and Martin, 1993).

There are many technical problems associated with quantifying the hydrogen peroxide produced by the xanthine oxidase / hypoxanthine system, due to the on going synthesis and degradation of hydrogen peroxide. However, based on the enzyme and substrate concentrations, and other reports in the literature (Kvietys et al, 1989; Link and Riley, 1988), this system should produce approximately 500μM

hydrogen peroxide, consistent with the above findings.

The mechanism by which hydrogen peroxide exerts its effects on the endothelium is unclear. It has been suggested that hydrogen peroxide acts by rapidly diffusing into the tissue where it comes into contact with Fe^{2+} (Whorton et al, 1985). Hydroxyl radicals are then produced by the iron catalysed Haber-Weiss reaction (Halliwell, 1989). To investigate this possibility, MPG was pre-incubated with the tissue for 1 hour before exposure to the xanthine oxidase / hypoxanthine system, to allow more effective distribution of the sulphydryl compound into the intracellular cytoplasm where it would be able to scavenge any hydroxyl radical produced within the cells, but still no effective protection was seen (see Chapter 7). Another approach to examine intracellular hydroxyl radical production, would be the use of an iron chelating compound such as desferrioxamine, to inhibit the Haber-Weiss reaction. If the intracellular Fe^{2+} was chelated then the Haber-Weiss reaction could not produce hydroxyl radical. However desferrioxamine is less cell permeable than MPG and in this system the endothelium does not remain viable for sufficiently long periods to allow uptake of desferrioxamine and the subsequent study of the endothelium dependent relaxation. Due to these limitations it is not possible to completely exclude intracellular generation of hydroxyl radicals. Further investigation into the mechanism by which hydrogen peroxide impairs endothelial function could be carried out in cultured cell systems where scavenging of the hydroxyl radical could be studied more effectively.

Hydrogen peroxide has been shown to increase intracellular calcium levels. Several mechanisms for this increase have been proposed:- Sheenan and colleagues (1993) proposed that hydrogen peroxide activates a serine esterase and / or phospholipase C to induce an increase in intracellular calcium concentration; Suzuki and coworkers (1991) suggested that hydrogen peroxide inhibits the Ca^{2+}-ATPase in smooth muscle

which would consequently induce an increase in intracellular calcium levels; another study demonstrated that hydrogen peroxide induced calcium influx through voltage operated calcium channels (Josephson et al, 1991). Elevation of intracellular calcium could explain the observed increase in baseline tension observed during exposure to either the xanthine oxidase / hypoxanthine system or hydrogen peroxide. The study by Josephson and colleagues was in rat ventricular myocytes. They also observed that hydrogen peroxide impaired glycolytic substrate metabolism which would lead to further ATP depletion - this in turn would decrease the cellular capability to respond to agonist induced signals as energy is required for cellular activity.

In summary, the xanthine oxidase / hypoxanthine free radical generating system causes impairment of phenylephrine induced contraction, carbachol induced, EDRF dependent relaxation and SNP induced, non-endothelium dependent relaxation of rabbit aortic rings. The results of this study indicate that hydrogen peroxide is the primary species responsible for this impairment, but that the superoxide anion may also exert some detrimental effect on the tissue.

These studies were carried out on isolated tissues in an organ bath system, however, the generating system used was based on the physiological situation that arises during ischaemia and subsequent reperfusion *in vivo*. The results of these studies could, therefore be relevant to clinical states, where intervention with a combination of SOD and catalase could be beneficial and help to minimise the abnormal vascular function associated with ischaemia-reperfusion injury.

CHAPTER NINE.

GENERAL DISCUSSION.

9.1 SUMMARY.

The work presented in this thesis has examined changes in vascular function arising in two areas both of which can be related to clinical situations. Firstly the vasoactive properties of arterial tissue were examined during the development, progression and potentially the regression of atherosclerotic disease. Two animal model of atherosclerosis were used :- the cholesterol fed New Zealand White rabbit and the genetically hyperlipidaemic Watanabe Heritable Hyperlipidaemic (WHHL) rabbit. The second situation investigated was that exposure to free radicals / reactive oxygen species. Using a xanthine oxidase / hypoxanthine based system an *in vitro* model of free radical / reactive oxygen species exposure was investigated in aortic tissue from New Zealand White rabbits. Both the contractile and relaxant properties of the vasculature were examined in these models.

The first model presented is the cholesterol fed New Zealand White rabbit. Unfortunately, a high proportion of the rabbits in the study displayed hypercholesterolaemia resistance and as a result serum cholesterol levels were not significantly elevated in several animals. However, bearing in mind the limitation of the study, it was still possible to draw some conclusions from the experimental data obtained. When compared to age matched control rabbits it was found that the period of increased dietary intake of cholesterol had no effect on phenylephrine induced contraction in the thoracic aorta. However, it was observed that carbachol induced relaxation was significantly impaired in relation to the obtained elevation of serum cholesterol. All tissues retained the ability to relax 100% when exposed to SNP, regardless of the impairment of the carbachol response. The second part of this study examined the effects of normalising dietary cholesterol intake following the period of elevation. The relaxant properties of the thoracic aorta were then examined. It was observed that the endothelium dependent response to carbachol had significantly

improved returning towards normal levels. Thus, in this model of atherosclerosis it can be concluded that, in some cases, dietary supplementation of cholesterol will induce hypercholesterolaemia. In these instances, where cholesterol becomes elevated, contractile responses are unaffected at the time point studied, however endothelium dependent relaxation is significantly impaired. Non-endothelium dependent relaxation is unaffected. Following normalisation of dietary cholesterol intake the endothelium can initiate repair mechanisms which, in time, allow the restoration of endothelium dependent relaxation.

The cholesterol fed rabbit, although widely studied as a model of atherosclerosis, is limited as a model for human atherosclerosis. This is due to the fact, that although high dietary intake of cholesterol and fatty acids are often present as an aggravating influence, the human condition usually arises as the result of genetic or acquired defects in cholesterol or lipid metabolism. Thus the discovery in the 1970's of the WHHL rabbit provided a good, accurate model for a specific form of the human disease - familial hypercholesterolaemia. Several aspects of vascular function have been assessed in WHHL rabbits since their discovery, however many of the reports in the literature present studies that are limited due to the design and more commonly due to lack of adequate numbers in sufficiently narrow age groups.

Hence the next portion of this work was carried out in WHHL rabbits and was designed to provide a detailed picture of the sequential events occurring in vascular tissue during the progressive development of atherosclerosis in the WHHL rabbit model of human familial hypercholesterolaemia. The study was designed to examine contractile responses to the α_1-adrenoceptor agonist phenylephrine, and endothelium dependent relaxation in response to the muscarinic agonist carbachol, in rings of three arterial tissues (thoracic aorta, aortic arch and carotid artery), at four individual age points (3, 6, 9 & 12 months), in three categories of rabbit (control New Zealand

White, homozygous WHHL and heterozygous WHHL).

One of the most novel aspects of the work presented in this thesis is the study in heterozygous WHHL rabbits. To date the heterozygous WHHL rabbit has rarely been studied despite the obvious clinical relevance to the heterozygous form of human familial hypercholesterolaemia. This study examined three categories of rabbit :- the New Zealand White as a normocholesterolaemic control and both the heterozygote and homozygote WHHL rabbits as a model for familial hypercholesterolaemia.

Contractile function of the vasculature was examined using phenylephrine. In the thoracic aorta contractile function was found to decline progressively as the age of the animals increased in all three categories of rabbit. The rate of decline of function was found to be most dramatic in the homozygous WHHL rabbits, least so in the New Zealand White rabbits. Age matched comparisons of contractile function revealed an initial hyper-reactivity in the homozygous WHHL rabbit thoracic aorta compared to the New Zealand White control thoracic aorta. However with increasing age the decline in function observed in the homozygous and heterozygous WHHL rabbits results in a clear reversal of this trend such that the contraction in the homozygous and to a lesser extent in the heterozygous WHHL rabbit thoracic aorta is significantly reduced when compared to the New Zealand White rabbit thoracic aorta. Thus an initial hyper-reactivity to phenylephrine observed in the homozygous WHHL rabbits is reversed to a clear decline in function associated with the development of disease.

Studies of endothelium dependent relaxation were also conducted in rings of thoracic aorta from these three categories of rabbit. Relaxation was found to be unaffected by progressing age in New Zealand White rabbits but to decline progressively with age

in the heterozygous WHHL rabbits and at an accelerated rate in the homozygous WHHL rabbits. Even as early as three months of age (the earliest time point in this study) endothelium dependent relaxation was found to be impaired in both homozygous and heterozygous WHHL rabbit thoracic aorta when compared to the New Zealand White control group.

Similar patterns of impairment of both contractile and relaxant function were observed in ring segments of aortic arch from WHHL rabbits. However, no alteration in responsiveness was observed in ring segments of carotid artery in the genetically hyperlipidaemic rabbits.

Concurrent histological studies were conducted to assess the development and progression of atheroma in the thoracic aorta of the WHHL rabbits. Several macro- and microscopic techniques were used to quantitatively and qualitatively assess the extent of atheroma in the thoracic aorta in each of the WHHL rabbits included in the above study. This assessment revealed the progressive development of atheroma in the thoracic aorta of the homozygous WHHL rabbits. The extent of atheroma increased with age. In the heterozygous WHHL rabbits early stages of atheroma were visible and were also found to progress with age. The atheroma present was found to closely resemble classic human atheroma.

Statistical analysis of linear correlation and regression was used to examine the potential relationship between the elevation in serum cholesterol, modification of vasoactive function and structural development of atheroma. Elevation of serum cholesterol was found to be significantly related to both the decline in vasoactive function and the physical development of atheroma. The relationship between functional and structural parameters was also found to be significant. With regard to contractile function intimal thickening appeared to exert the greatest influence on the

ability of the vessel to contract. This probably reflects the fact that smooth muscle cells migrate from the media to the intima changing orientation and phenotype. Thus, under areas of intimal thickening, the media becomes thinner and proportionally weaker. In addition as the intima thickens the existing media has greater physical mass to constrict, thus increasing the load and potentially decreasing the force generated.

Decline of endothelium dependent relaxation was found to be significantly related to both the proportion of intimal involvement and the relative thickness of the developed plaque. This indicates that the decline of endothelium dependent relaxation is not simply due to an increase of the diffusion distance for EDRF / NO, as if this were the case, intimal thickness would exert a greater influence on the response than the proportion of intimal involvement.

Thus the studies presented in Chapter Four have provided a clear, progressive picture of the structural and functional modifications occurring in the vasculature of the WHHL rabbit during the development of atheroma. Furthermore, for the first time, data is presented on the clinically relevant heterozygous WHHL rabbit.

Having established this basic data it was subsequently possible to investigate a potentially beneficial therapeutic strategy. The effect of a period of treatment with the HMG CoA reductase inhibitor simvastatin was examined. Four groups of WHHL rabbits were treated :- young (3-6 month) heterozygous, older (9-12 month) heterozygous, young (3-6 month) homozygous and older (9-12 month) homozygous WHHL rabbits. The treatment regime was based on preliminary studies in the department. Simvastatin was found to significantly reduce serum cholesterol levels in the young heterozygous WHHL rabbits and a non-significant reduction was observed in the older heterozygous WHHL rabbits. In the homozygous WHHL

rabbit groups no significant fall was observed.

Following treatment with simvastatin vasoactive function was examined in rings of thoracic aorta as for the control studies. It was observed that simvastatin was clearly able to retard the progressive loss of both contractile and relaxant function in the young heterozygous WHHL rabbit group, similar but smaller trends were observed in the young homozygous groups and older heterozygous groups. However, no beneficial effect was observed in the older homozygous group.

Quantitative assessment of the structural development of atherosclerosis was also conducted in these simvastatin treated groups. No significant effect of treatment was observed. Thus the observed retardation of the loss of vasoactive function was not parallelled by inhibition of the structural atheroma development. So, in conclusion, this study provides evidence that the use of the HMG CoA reductase inhibitors such as simvastatin may be clinically beneficial in familial hypercholesterolaemia, especially in the heterozygous form of the disease.

One further observation made in these studies is that the effects of the disease on the function of the vasculature are apparent before the development of atheroma is visible. In the three month homozygous WHHL there was an apparent hyper-reactivity to phenylephrine, this is supported by evidence from direct studies with LDL which demonstrate increased intracellular calcium and agonist stimulated PI turnover following cell / tissue incubation with lipoproteins. Similar studies with LDL have shown that endothelium dependent relaxation can be impaired in tissue following a period of LDL exposure. In the WHHL rabbit the vasculature is exposed to elevated serum cholesterol / LDL from birth and thus these direct effects of LDL could account for the observed alterations in vascular reactivity in these rabbits.

Direct comparison of the two models of atheroma is impaired by the fact that only one time point of development was examined in the cholesterol fed model. The two models are consistent with regard to the observed decline in relaxant function associated with the development of atherosclerosis. With regard to contractile function - in the WHHL rabbits an initial hyper-reactivity to phenylephrine was progressively overcome by the development of disease, such that by 12 months of age the phenylephrine induced contraction was dramatically reduced. The single time point studied in the cholesterol fed model exhibited no change in phenylephrine induced contraction. This could correlate with a stage intermediate between the hyper-reactivity and the decline of the response or it may simply reflect a difference between the two models of atherosclerosis, further studies would be necessary to investigate this.

Free radicals / reactive oxygen species have been implicated in the pathogenesis of atherosclerosis from several perspectives. It has been suggested that free radicals / reactive oxygen species may be responsible for the initial vascular injury which subsequently leads to the development of atheroma. It has also been proposed that free radicals / reactive oxygen species induce LDL oxidation and thus increase its atherogenicity. It is almost certain that free radicals are involved in the self propagating chain reaction which cascades within LDL once oxidation has been initiated. Further roles for free radicals / reactive oxygen species have been proposed in the death of lipid laden macrophages leading to the release of lipid into the extracellular space thus forming the necrotic cholesteryl ester filled core of the advanced atherosclerotic plaque. Free radicals / reactive oxygen species are implicated in many diverse disease / pathogenic processes. However one which is related to the cardiovascular system and also to atherosclerosis is that of ischaemia reperfusion injury. The series of events occurring in a tissue during a period of

ischaemia followed by subsequent reperfusion can interact to produce a burst of free radicals / reactive oxygen species including the superoxide anion, hydrogen peroxide and the hydroxyl radical. Thus the final series of experiments presented in this thesis were designed to create and study an *in vitro* model of free radical / reactive oxygen species exposure.

Rings of thoracic aorta from young, male, New Zealand White rabbits were exposed to a xanthine oxidase / hypoxanthine free radical / reactive oxygen species generating system. This system mimics the environment in ischaemic tissue upon reperfusion and as such generates the free radicals / reactive oxygen species described above. The vasoactive properties of the thoracic aorta were examined before and after exposure to this system. It was observed that phenylephrine induced contraction, carbachol induced endothelium dependent relaxation and SNP induced non-endothelium dependent relaxation were impaired. Carbachol induced relaxation was most severely affected with an approximately 50% reduction in maximum response. The maximum response to phenylephrine was also reduced but to a lesser extent. The response to SNP was reduced but at the lower end of the concentration response curve, with the maximum response remaining unaffected.

Subsequent experiment using a series of inhibitors / enzymes :- mannitol, MPG, captopril, catalase and SOD allowed the elucidation of the primary cytotoxic species causing the impairment of carbachol induced relaxation. The primary cytotoxic agent was identified as hydrogen peroxide. If availability of time had not been limited it would have been useful to conduct a similar series of experiments with the "protective agents" to establish if similar conclusions would be drawn regarding the phenylephrine and SNP responses.

However direct studies with hydrogen peroxide suggest that this would indeed be the

case. Hydrogen peroxide was able to directly mimic the observed effects of the xanthine oxidase / hypoxanthine system with all three agonists. The concentration which most closely mimic the effect of the xanthine oxidase / hypoxanthine system is 500μM hydrogen peroxide - this is approximately equivalent to the theoretical quantity of hydrogen peroxide produced by the free radicals / reactive oxygen species generating system.

The mechanism by which hydrogen peroxide impairs vasoactive function was not directly examined in the experiments presented in this thesis. Reports in the literature suggest that hydrogen peroxide can impair glycolytic substrate metabolism thus decreasing the ability of the cell to respond to agonist stimulation due to energy depletion. A range of other mechanisms may also be involved - further studies possibly in tissue culture systems, would allow insight into the mode of impairment.

Thus these studies suggest that in the clinical situation intervention with enzymes such as catalase may be potentially beneficial in clinical situations, such as ischaemia reperfusion, if administered during or soon after reperfusion.

9.2 CONCLUSIONS.

Thus, in conclusion, the work presented in this thesis has provided novel information on several aspects of cardiovascular disease. Firstly, it has been demonstrated that dietary elevation of serum cholesterol can, in some cases, lead to the development of hyperlipidaemia and atherosclerosis. This atherosclerosis can potentially be reversed by reduction of dietary cholesterol intake.

Secondly, a wide ranging study of the process of atherosclerotic disease in both homozygous and heterozygous WHHL rabbits has been presented. Thus, providing

documentation of both structural and functional changes occurring in severe and mild forms of the same disease - an animal model for familial hypercholesterolaemia.

Furthermore the potential therapeutic benefit of the relatively new drug simvastatin has been demonstrated in the WHHL rabbit.

Finally, an investigation into the identity of the cytotoxic agent responsible for alterations in vascular function following exposure to a xanthine oxidase / hypoxanthine *in vitro* model was conducted. The evidence that catalase could prevent and hydrogen peroxide could mimic the effects of the xanthine oxidase / hypoxanthine system has implicated hydrogen peroxide as the primary deleterious species.

9.3 FUTURE DIRECTIONS.

Further studies with the cholesterol fed New Zealand White rabbit model are required to increase the number of rabbits in which serum cholesterol is effectively elevated. This would subsequently allow improved analysis and interpretation of the study.

The potential for future work related to the WHHL rabbit studies is wide ranging. Studies could be designed to further investigate the alterations in vascular function, possibly investigating other agonists; using cascade systems to examine the change in the EDRF / NO response; ligand binding studies could be used to examine receptor density; tissue culture systems could be used to study intracellular signalling and response pathways in both vascular smooth muscle and endothelial cells. Further studies with the HMG CoA reductase inhibitors could be conducted, examining

higher dosage and longer treatment schedules. In addition the effects of the other HMG CoA reductase inhibitors, such as pravastatin or lovastatin, could be investigated.

The precise mechanism by which hydrogen peroxide affects the vasoactive properties of the thoracic aorta could also be investigated using cell culture systems. Vascular smooth muscle cells, endothelial cells and potentially cocultures could be used to investigate the interaction between hydrogen peroxide and cellular signalling and response pathways. Regulation of intracellular second messengers such as IP_3, cGMP and cAMP could be investigated.

The potential role of intracellular hydroxyl radical generation could also be more easily investigated using cell culture systems. This is due to the fact that, in cell culture systems, it would be possible to use longer incubation periods with hydroxyl radical scavengers and / or inhibitors, which, due to the initiation of tissue degeneration, the organ bath type experiments do not permit.

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