GUINEA PIGS AS A MODEL OF CHOLESTEROL-INDUCED CARDIAC CONCENTRIC REMODELING

by

JOY RENÉE OWEN

(Under the Direction of Stephen J. Lewis)

ABSTRACT

Hypercholesterolemia contributes to the progression of atherosclerosis, coronary heart disease, and hypertension in humans. Normotensive hypercholesterolemic patients develop cardiac (left-ventricular) concentric remodeling by unknown mechanisms. We used the cholesterol-fed guinea pig to examine the effects of hypercholesterolemia on cardiovascular homeostatic mechanisms.

Guinea pigs that consumed a 1% dietary cholesterol regimen for 13 weeks developed cardiac concentric remodeling of the left ventricle, which was characterized by an increase in relative left ventricular wall thickness without an increase in left ventricular mass. Fractional shortening, a determinant of cardiac contractility, was unchanged. The baroreceptor reflex (BR) modulates mean arterial pressure by regulating sympathetic and parasympathetic discharge to the heart and vasculature. BR activity was impaired in hypercholesterolemic guinea pigs although these animals were normotensive. The gain and sensitivity of the BR were markedly diminished.

A loss of BR activity in guinea pigs with cholesterol-induced cardiac concentric remodeling may be attributed to many mechanisms involved in autonomic regulation. Our studies determined that

hyperholesterolemia may disrupt central processing of baroafferent function, and causes both down-regulation of cardiac β-adrenoceptors and elevations in intrinsic heart rate. Systemic injections of the 5-HT_3 receptor agonist phenylbiguanide, elicit Bezold-Jarisch reflex (BJR)-mediated falls in heart rate and mean arterial blood pressure. The BJR responses elicited by phenylbiguanide in conscious control and cholesterol-fed guinea pigs were due primarily to increases in cardiovagal drive. The BJR responses elicited by phenylbiguanide were reduced in hypercholesterolemic animals perhaps by down-regulation of 5-HT_3 receptors rather than diminished central/efferent processing of the BJR reflex. We also obtained evidence that hypercholesterolemia markedly affects the rate of desensitization/resensitization of 5-HT_3 receptors and that endogenous nitrosyl factors in vagal afferents of the rat regulate the rate of desensitization/resensitization of 5-HT_3 receptors on vagal afferents mediating the BJR.

Finally, we found that endothelium-dependent relaxation was reduced in cholesterol-fed guinea pigs via the reduced potency of endothelium-derived L-S-nitrosocysteine and that these guinea pigs have diminished vesicular stores of L-S-nitrosocysteine.

Keywords: Hypercholesterolemia, concentric cardiac remodeling, echocardiography, cardiac morphology, baroreceptor heart rate reflex, Bezold-Jarisch reflex, endothelium-derived relaxing factors, nitric oxide, S-nitrosothiols.

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DEDICATION

To my dearest parents, Ronald and Barbara Owen.

For their unconditional love and support, magnanimous understanding,

encouragement and inspiration.

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Trust in the Lord, with all thine heart and lean not on your own understanding. In all thy ways acknowledge Him and He shall direct thy path.

Proverbs 3:5-6

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CHAPTER 1

INTRODUCTION

CHOLESTEROL

Cholesterol plays a vital role in biological processes such as the formation of membrane micro-domains known as lipid rafts. These lipid rafts regulate cell signaling pathways by promoting the formation of scaffolds, which stabilize trimeric G-proteins, their receptors and their effectors (Miura et al., 2001). Disruption of cholesterol homeostasis by dietary means or as occurs in hereditary hyperlipidemias affects receptor-mediated signaling pathways that regulate normal cardiovascular function (Miura et al., 2001). These receptor systems are not only involved in the moment-to-moment regulation of cardiovascular function (e.g., heart rate, cardiac contractility and vascular tone) but also in the morphological status of the heart and blood vessels.

HYPERCHOLESTEROLEMIA

Hypercholesterolemic, normotensive humans are subject to concentric remodeling of the left ventricle (Celentano et al., 2001). Concentric remodeling is an increase in relative wall thickness with normal left ventricular mass (Krumholz et al., 1995). This change in ventricular structure is likely to be important in hypercholesterolemic patients since left ventricular remodeling is correlated with increased mortality and morbidity in hypertensive patients (Devereux et al., 1994; Verdecchia et al., 1999), reduced coronary flow reserve (Shafer et al., 200), and abnormal diastolic (Qu et al., 2001) and systolic function (Sadler et al., 1997). Hypercholesterolemia also increases the stiffness of systemic blood vessels in humans thereby increasing central pulse pressure (Roman et al., 2000; Wilkinson et al., 2002). These factors contribute to a decreased coronary perfusion during diastole. Moreover, there is considerable clinical and experimental evidence that cardiovascular homeostatic mechanisms including baroreflex sensitivity are abnormal in hypercholesterolemic patients (Piccirillo et al., 2001; Wronski et al., 2002).

DEVELOPMENT OF A NORMOTENSIVE MODEL OF HYPERCHOLESTEROLEMIA

This dissertation will provide evidence that we have developed a normotensive model of hypercholesterolemia, the cholesterol-fed guinea pig that develops a concentric left ventricular remodeling. There is now compelling evidence that the guinea pig is the best small animal model to investigate the effects of hypercholesterolemia on cardiovascular function (see Fernandez, 2001). Most importantly, guinea pigs and humans have similar lipid profiles. The guinea pig, unlike rabbits, rats and mice, transport most of their cholesterol in the form of low density lipoproteins (LDL). Moreover, guinea pigs have high LDL to high density lipoprotein (HDL) ratios and similar rates of hepatic cholesterol catabolism and synthesis as compared to humans (Fernandez, 2001). The exact time-course of the progression of cholesterol-induced cardiac concentric remodeling has not been established. Schwemmer et al. (2000) found that placing guinea pigs on a 1% cholesterol diet for 8 weeks produced an increase in baseline coronary artery flow but markedly impaired coronary vasorelaxation elicited by the endothelium-dependent vasodilator, bradykinin. Most importantly, major cholesterol-induced changes in cardiac structure were not reported to occur in these cholesterol-fed guinea pigs. These findings suggest that concentric remodeling takes longer than 8 weeks of cholesterol-feeding to develop.

OBJECTIVES

Understanding the mechanisms and consequences of hypercholesterolemia-induced concentric remodeling of the heart will provide information as to the underlying cellular mechanisms that contribute to the changes in cardiac structure and function. We expect that the novel data yielded from our studies will form a basis of strategies designed to develop novel therapeutic agents to ameliorate hypercholesterolemia-induced cardiovascular disorders.

The **objective** of this dissertation project is to test our **major hypothesis** that cholesterol-induced cardiac concentric remodeling modifies cardiovascular homeostatic mechanisms. The following **major specific aims** will be addressed to accomplish the objective.

1. To use echocardiography and morphological techniques to determine the effects of high cholesterol diet on cardiac geometry in guinea pigs.

2. To use cardiovascular techniques to determine the effects of high cholesterol diet on baroreceptor reflex function in conscious guinea pigs.

3. To use cardiovascular techniques to determine the effects of high cholesterol diet on afferent and autonomic effector mechanisms in conscious and anesthetized guinea pigs.

4. To use cardiovascular techniques to determine the effects of high cholesterol diet on Bezold-Jarisch reflex function in conscious guinea pigs.

5. To use cardiovascular techniques to determine the effects of high cholesterol diet on endothelium-*dependent* vasodilator mechanisms in conscious guinea pigs.

The specific **Chapters** in this dissertation are listed on the next page. This **Chapter 1** provides the rationale for our studies and a brief description of our objectives. **Chapter 2** provides a literature review on the effects of high dietary cholesterol on cardiovascular function and explains the importance of our studies in hypercholesterolemic guinea pigs. **Chapters 3-7** detail our experimental findings on the effects of high cholesterol diet on cardiovascular function in conscious and anesthetized guinea pigs. **Chapter 8** details our findings as to the mechanisms regulating 5-HT_3 receptors desensitization in rats (Owen et al., Endogenous nitrosyl factors inhibits the desensitization of 5-HT_3 receptors on vagal cardiopulmonary afferents. *Brain Res. 1059: 167-172).* **Chapter 9** provides an overall summary of our findings.

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CHAPTER 2

LITERATURE REVIEW

CHOLESTEROL

Cholesterol (cholest-5-en-3β-ol) was originally discovered in 1769 by Poulletier de la Salle as a hard, white solid occurring in bile and gallstones. Cholesterine, a term first used by Michel Chevreul in 1815, was derived from the Greek words "chole" and "steros", which mean "bile" and "solid", respectively (Dam, 1958). In 1900, the word "cholesterine" was changed to the present term of "cholesterol" to reflect the presence of hydroxyl groups on the molecule (Sabine, 1977). Cholesterol is the primary sterol found in eukaryotes. As a major constituent of cellular membranes, it also occurs in the membranes of the brain, nervous system, peripheral nerves and the spinal cord. Of primary physiological significance is that cholesterol is a sole precursor of ovarian and testicular hormones, bile acids, vitamin D and steroid hormones (Sabine, 1977). Cholesterol ($C_{27}H_{46}O$, see **Figure 2.1**) is a non-polar steroid alcohol that is a waxy solid at room temperature, essentially insoluble in aqueous solutions and soluble in a myriad of organic solvents. Dietary cholesterol is exclusively of animal origin, and encompasses meat, poultry, seafood, diary products, lard, shortening and eggs (Feeley et al., 1972).

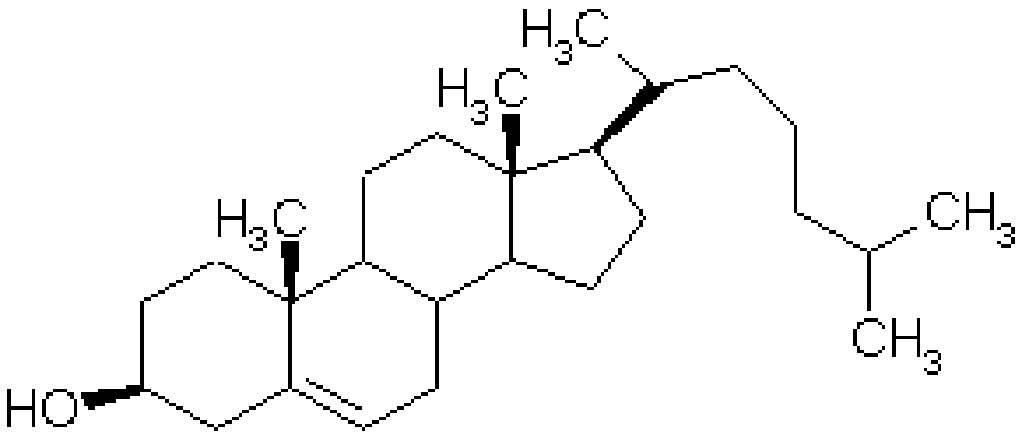

Figure 2.1: Structure of Cholesterol

CHOLESTEROL BIOSYNTHESIS

Cholesterol synthesis occurs primarily in the liver although all tissues including the intestine, skin, adrenal cortex and gonads, can produce cholesterol. A diagrammatic presentation of the cholesterol biosynthetic pathway is shown in **Figure 2.2** (see next page). *De novo* cholesterol synthesis proceeds in the cytoplasm and from the two carbon acetate group of acetyl-CoA (Decker and Barth, 1973). Acetyl-CoA derived from an oxidation reaction in mitochondria, is transported to the cytoplasm, where two moles of acetyl-CoA are actively condensed by acetoacetyl-CoA thiolase to form acetoacetyl-CoA (Middleton, 1973). Acetoacetyl-CoA combines with a third mole of acetyl-CoA and is converted to β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase (Clinkenbeard et al., 1975).

As the rate limiting reaction of cholesterol synthesis, HMG-CoA reductase, catalyzes the conversion of HMG-CoA to mevalonate (Goldstein and Brown, 1990). HMG-CoA reductase is an integral protein of endoplasmic reticulum membranes (Goldstein and Brown, 1990). NADPH is an essential cofactor for HMG-CoA reductase in this two step reaction and hence two moles of NADPH are consumed (Goldstein and Brown, 1990). HMG-CoA reductase is highly regulated and pharmacological therapies have been designed to target this vital enzyme. In a series of two successive phosphorylation steps using adenosine triphosphate (ATP), mevalonate generates a 5-pyrophosphomevalonate derivative. Pyrophosphomevalonate decarboxylase catalyzes an ATP dependent decarboxylation with dehydration to yield isopentenyl pyrophosphate (IPP), which is an activated isoprenoid molecule (Sabine, 1977). The inter-conversion of IPP and dimethylallyl pyrophosphate (DMPP) is catalyzed by isopentenyl pyrophosphate isomerase, which shifts the double bond in IPP to form DMPP.

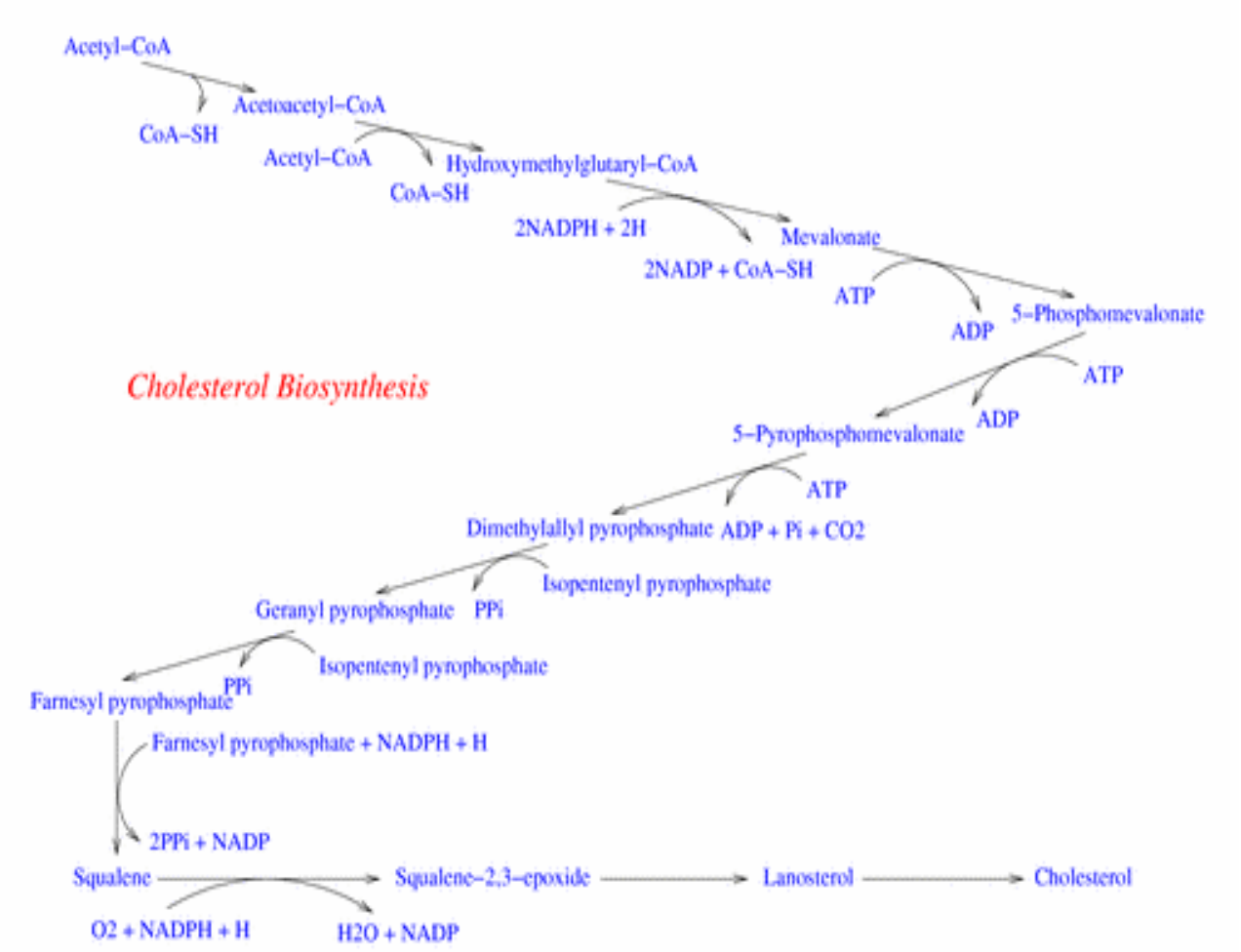

Figure 2.2. Cholesterol Biosynthetic Pathway

Lloyd, C. (Bioengineering Institute, University of Auckland).

http://www.cellml.org/examples/repository/qualitative/metabolic_models_doc.html

In a series of head to tail condensation steps catalyzed by prenyl transferase, DMPP combines with IPP to generate geranyl pyrophosphate (GPP). Another molecule of IPP then combines with GPP to yield farnesyl pyrophosphate (FPP) (Yeagle, 1988). Squalene synthase, a NADPH dependent enzyme, condenses two molecules of FPP to form squalene (Rilling, 1966). As the first in a two step cyclization, squalene epoxidase uses NADPH to introduce molecular oxygen as an epoxide to yield squalene 2,3 epoxide. In a second cyclization step, squalene oxidocyclase causes a series of electron shifts to produce the sterol, lanosterol (Rillings, 1985; Trzaskos and Gaylor, 1985). Finally, through a series of 19 additional reactions catalyzed by enzymes in the membrane of the endoplasmic reticulum, lanosterol is converted to cholesterol (Yeagle, 1988). Hepatic synthesis of the primary bile acids cholic and chenodeoxycholic acid is catalyzed by the rate limiting enzyme cholesterol 7α-hydroxylase (Cyp7) and is the dominant mechanism of cholesterol excretion. However, cholesterol may be excreted in bile, milk and urine, while unabsorbed cholesterol is eliminated via the feces (Sabine, 1977; Nguyen et al., 1999).

CHOLESTEROL TRANSPORT

Due to the aqueous environment of plasma, lipids such as cholesterol are transported via lipoprotein complexes that contain lipids in association with specific transport proteins called apoproteins (Apo) (Horton et al. 2005). These lipoprotein complexes are transported in two pathways, the exogenous pathway, which transports lipids from the intestine to the liver and the endogenous pathway, which transports dietary and *de novo* synthesized lipids from the liver to extrahepatic tissues. As depicted in **Figure 2.3** on the following page, the generalized structure of a plasma lipoprotein is a hydrophobic core of cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipids and cholesterol which contain integral apoproteins.

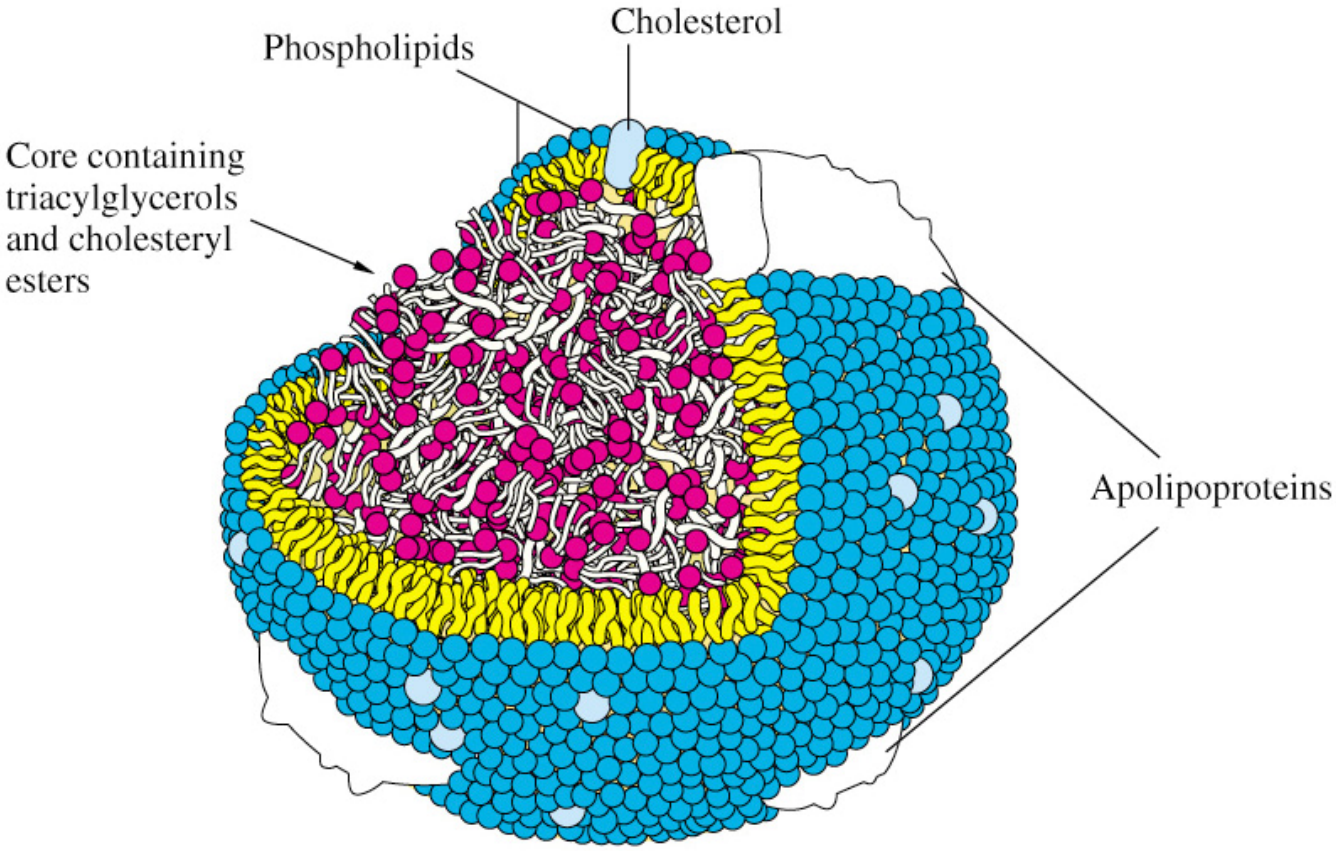

Figure 2.3: **Structure of an apoprotein**

http://cwx.prenhall.com/horton/medialib/media_portfolio/16.html

Principles of Biochemistry, 4/E

Lipoproteins are distinguished by protein content and relative density (Horton et al., 2005). The primary apoproteins involved in lipid transport are, ApoA-I, ApoA-II, ApoA-IV, ApoB-48, ApoB-100, ApoC-I, ApoC-II, ApoC-III, ApoD and ApoE. However, ApoB-48, which is exclusive to the exogenous pathway, is only associated with dietary lipid transport (Jackson et al., 1976). After eating lipids, chylomicrons (formed by intestinal mucosa) transport triglycerides and a small percentage of cholesterol to extrahepatic tissues and the liver. Nascent chylomicrons primarily consist of triglycerides, ApoB-48, ApoA-I, ApoA-II, ApoA-IV, minute amounts of cholesterol and acquire ApoC-II and Apo E from plasma high density lipoproteins (HDL). After leaving the intestines by the lymphatics, chylomicrons enter the circulation via the subclavian vein. The heparin dependent enzyme, lipoprotein lipase (LPL), on the surface of the capillary endothelium, breaks down triglycerides in the chylomicrons to form free fatty acids (FFA) and glycerol. LPL is activated by the ApoC-II complex in chylomicrons and very low density lipoproteins (VLDL). Free fatty acids remain in the blood bound to albumin or are absorbed by extrahepatic tissues, while glycerol returns via the blood to the liver and kidney. Once chylomicrons are depleted of triglycerides, the chylomicron remnants containing cholesterol, ApoB-48 and ApoE, are delivered to the liver where they bind to chylomicron remnant and low density lipoprotein receptors. Upon activation these receptors are immediately internalized by receptor-mediated endocytosis and degraded by lysosomes (Ganong, 1999; Jackson et al., 1976).

In the endogenous lipoprotein pathway, VLDL transport endogenously synthesized cholesterol and triglycerides formed from fatty acids and carbohydrates in the liver to extrahepatic tissues. VLDL consist of cholesterol, triglycerides, and apoproteins ApoB-100, ApoC-I, ApoC-II, ApoC-III and ApoE. Similar to chylomicrons, lipoprotein lipase releases FFA and glycerol from

triglycerides in VLDL, which converts these lipoprotein complexes to intermediate density lipoproteins (IDL). IDLs release phospholipids and via the plasma enzyme lecithin-cholesterol acyltransferase (LCAT), obtain cholesterol esters from HDL. At this point, IDL is converted to LDL or is taken up by the liver. Conversion of IDL to LDL requires the loss of triglyceride and protein, primarily Apo E. Hepatic uptake of IDL by the LDL receptor depends upon the presence of ApoB-100 and ApoE. Cholesterol is the primary constituent of LDL along with ApoB-100, which must be present for these lipoproteins to supply extrahepatic tissues with cholesterol by LDL receptor mediated endocytosis in clathrin coated pits (Ganong, 1999). The human LDL receptor consists of 839 amino acids with multiple functional regions that include a cysteine-rich ligand binding domain; an amino acid sequence homologous to the precursor for epidermal growth factor; a glycosylation site enriched in serine and threonine to which carbohydrates are attached; a hydrophobic amino acid region that spans the cellular membrane; and an intracellular domain that extends into the cytoplasm (Rudenko and Deisenhofer, 2003).

During receptor mediated endocytosis, caveolae are internalized to form coated vesicles and then endosomes. As pH falls via ATP dependent protein pumps located on the membrane of the endosomes, LDL receptor dissociation is triggered to allow recycling to the cellular membrane. Endosomal fusion with lysosomes occurs and cholesterol formed from cholesteryl esters by acid lipases located in the lysosomes yield cholesterol to meet the physiological requirements in tissues. Exuberant amounts of intracellular cholesterol is re-esterified by acyl-CoA-cholesterol acyltransferase (ACAT) for intracellular conservation. In addition, macrophages have a low affinity system for LDL uptake, which contributes to atherosclerotic plaque formation (Ganong, 1999). The relevant steps are shown in **Figure 2.4** on the following page.

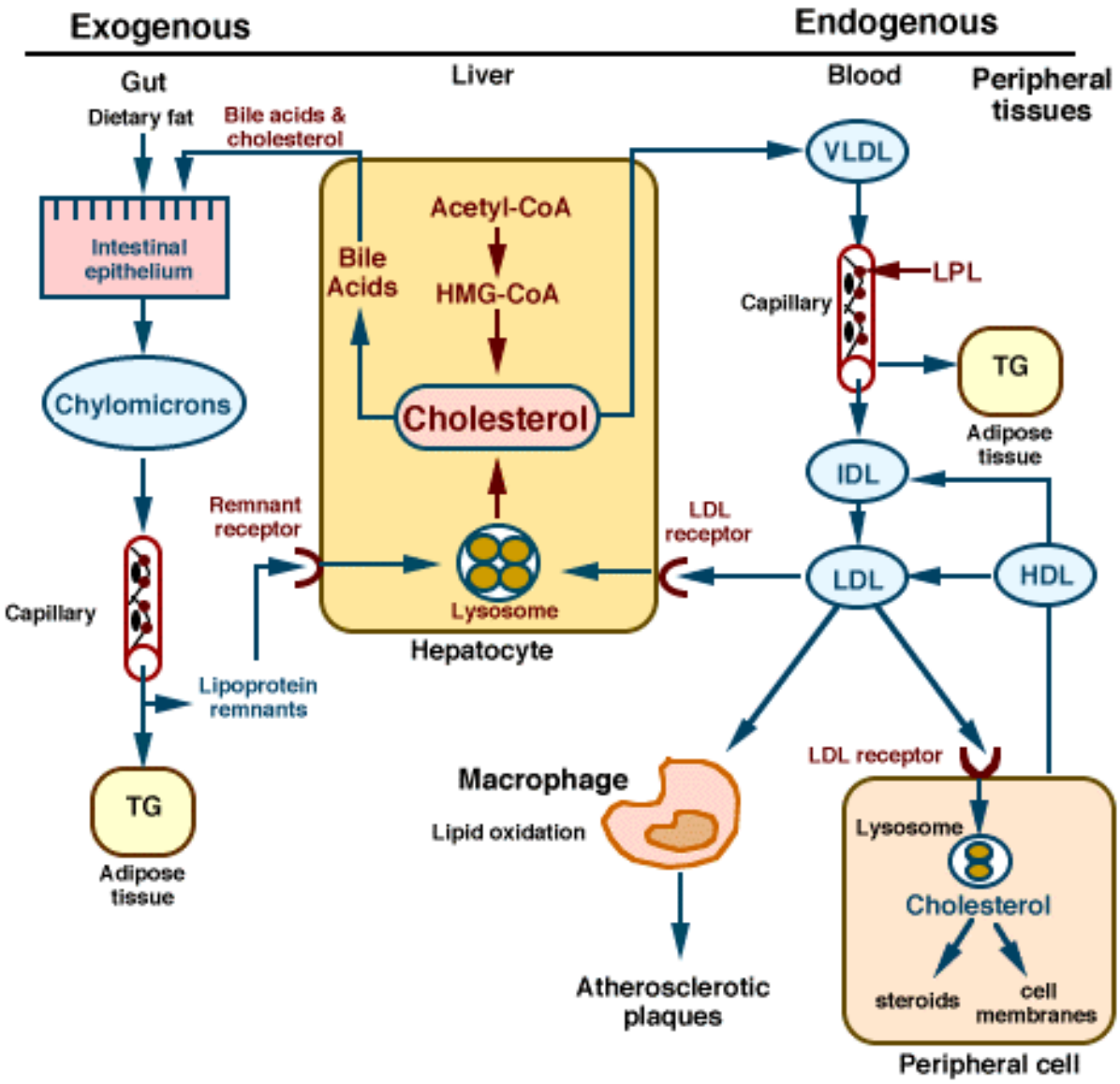

Figure 2.4. Cholesterol metabolism

http://www.ovc.uoguelph.ca/BioMed/Courses/Public/Pharmacology/pharmsite/98-409/Blood/hyperlipidemia.html

Nascent HDLs are synthesized in the liver and small intestine practically devoid of cholesterol and cholesteryl esters. The primary lipoproteins associated with HDLs are ApoA-I, ApoC-I, ApoC-II and ApoE, and in addition to cholesterol and cholesteryl ester sequestering, a significant function of HDL is to act as a circulating reservoir of ApoC-I, ApoC-II and ApoE. Nascent HDL are small protein-rich disc shaped particles, however mature HDL are converted to spherical lipoprotein particles via the accumulation of cholesteryl esters. LCAT, which requires ApoA-I for activation, is an HDL associated enzyme that catalyzes the conversion of free cholesterol to esterified cholesterol in chylomicron remnants and IDL. HDL are also responsible for the acquisition of cholesterol from cell surface membranes, which in turn lowers intracellular cholesterol levels due to the mobilization of cholesteryl esters to the plasma membrane.

Reverse cholesterol transport allows for cholesterol enriched HDL to undergo receptor mediated endocytosis in the liver via HDL specific ApoA-I receptors or by lipid-lipid interactions. Excess cellular cholesterol in the form of HDL associated cholesteryl esters may be transferred to VLDL and LDL through the HDL associated enzyme, cholesterol ester transfer protein (CETP). This unique cellular capability allows for hepatic delivery of cholesterol via LDL and HDL receptor mediated pathways. In addition to the liver, macrophages have the ability to process HDL particles (Ganong, 1999; Jackson et al., 1976).

The primary aim of the studies in this dissertation was to investigate the effects of high cholesterol diet on hemodynamic function in guinea pigs. Accordingly, the next sections detail important cardiovascular control mechanisms such as the baroreceptor reflex and what is known about the effects of high cholesterol intake on these mechanisms.

BARORECEPTOR REFLEX

The regulation of arterial blood pressure by the baroreflex allows for optimal perfusion of tissues during various physiological conditions on a beat-to-beat basis (Vasquez et al., 1997). Baroreceptor afferents are embedded in the adventitia of the carotid sinus and aortic arch. An increase in arterial pressure accelerates baroafferent impulses, which in turn causes an increase in vagal efferent activity, thereby resulting in bradycardia. During systole, an increase in arterial blood pressure causes cytoskeletal rearrangement of baroafferent terminals, therefore opening stretch activated Na^+ channels involved in depolarization. The cell bodies of baroreceptor afferents located in the aortic arch and carotid sinus are of the nodose ganglia in the left and right vagus (Xth cranial nerve) and the petrosal ganglia of the left and right glossopharyngeal nerve (IXth cranial nerve), respectively. These baroafferents centrally project through each tractus solitarius in the brainstem and terminate in the nucleus of the tractus solitarius (NTS), located bilaterally in the dorsal region of the medulla oblongata. Glutamate is the excitatory neurotransmitter released in the NTS and upon activation of kainite and N-methyl-D-aspartate (NMDA) receptors, these neurons project to the dorsal motor nucleus (DMX).

In the DMX glutamate is released and activates preganglionic parasympathetic neurons that project through the vagus directly to the heart. In the heart, preganglionic fibers release acetylcholine which activates nicotinic receptors involved in depolarization of postganglionic parasympathetic neurons. Following depolarization of postganglionic parasympathetic neurons, acetylcholine activates muscarinic receptors innervating pacemaker cells, including the sinoatrial (SA) node, atrioventricular (AV) node and the Purkinje fibers, causing bradycardia. Baroreceptor reflex activation of the sympathetic nervous system proceeds via the same pathway as vagal

activation of baroreceptor afferents until termination at the NTS. Once in the NTS, glutamate activates kainate and NMDA receptors, which depolarize neurons projecting to the caudal ventral lateral medulla (CVLM). Glutamate is released in the CVLM, which elicits a depolarization projecting to the rostral ventral lateral medulla (RVLM). The RVLM is unique due to the release of the inhibitory neurotransmitters γ-aminobutyrate (GABA) and glycine which increase the inhibitory stimulus regulating pacemaker activity of the RVLM cells. $GABA_A$ and glycine receptors mediate inhibitory neurotransmission in the brain and spinal cord via ligand gated Cl^- channels which cause hyperpolarization of these neurons. Neural projections subsequently terminate in the intermedial lateral (IML) portion of the spinal cord where glutamate drives preganglionic sympathetic neurons projecting to various ganglia such as the stellate and superior cervical ganglia. Acetylcholine is released from these ganglia and activates nicotinic receptors located on postganglionic sympathetic nerves.

After depolarization, norepinephrine is released and activates β_1-adrenoceptors on cardiac pacemaker cells and muscle cells, resulting in a decrease in the rate and force of contraction. Also, norepinephrine elicits α_1-adrenoceptor mediated responses on vascular smooth muscle, which decrease peripheral vascular resistance. Activation of baroreceptors in response to increases in mean arterial pressure results in a myriad of cardiovascular responses that lead to a rapid reduction in blood pressure. On the contrary, a decrease in mean arterial pressure reduces baroreceptor afferent impulses and consequently via the same mechanisms in opposition, raises blood pressure (Spyer, 1981; Brody et al., 1991). Baroreceptor adaption or resetting occurs after prolonged exposure to elevated mean arterial blood pressure (McCubbin et al., 1956). In this situation, neural reflex mechanisms regulating fluctuations in mean arterial blood pressure are

reset at a new elevated level and subsequently maintained in the same manner as described previously. The mechanisms responsible for this resetting are not known but are thought to occur within the central nervous system (McCubbin et al., 1956; Wolk and Summers, 2002).

EFFECTS OF CHOLESTEROL ON BARORECEPTOR REFLEX FUNCTION

Cholesterol is associated with alterations in cardiac baroreceptor reflex sensitivities in animals and humans (Piccirillo et al., 2001; Wronski et al., 2002). An emergence of literature suggests that decreased baroreflex sensitivity may be indicative of cardiac (Minami and Head, 1993; Lantelme et al., 1998) or vascular abnormalities (Lantelme et al., 1994). Chapleau et al. (1995) determined that increased levels of LDL cholesterol reduced baroreflex sensitivity, potentially from cholesterol-induced endothelial dysfunction. Thus, hypercholesterolemia may induce vascular changes in the absence of atherosclerotic lesions, therefore rendering baroreflex sensitivity as an excellent prognostic method for identifying individuals at increased risk for cardiovascular morbidity and mortality (Piccirillo et al., 2001).

Interestingly, Straznicky et al. (1997) determined that individuals consuming a high fat diet for 2 weeks in an open, randomized crossover study of 6 weeks duration had similar baroreceptor reflex sensitivities as compared to the normocholesterolemic group. In concordance with these studies, Koskinen et al., (1995) observed a slight inverse trend in baroreceptor reflex sensitivity in hypercholesterolemic subjects as compared to subjects with normal serum cholesterol levels. However, Koskinen et al. (1995) concluded that hypercholesterolemia does not significantly alter autonomic neural regulation of the cardiovascular system as assessed by the examination of baroreceptor reflex sensitivity.

BEZOLD JARISCH REFLEX

The main role of vagal cardiopulmonary afferents in the atria and pulmonary circulation is to protect the pulmonary circulation from rapid and marked increases in blood pressure (Thoren, 1979; Aviado and Guevara Aviado, 2001). Cardiopulmonary afferents are stretch afferents and are activated by elevations in atrial and pulmonary pressures (Thoren, 1979; Aviado and Guevara Aviado, 2001). Activation of these afferents elicits prompt falls in systemic arterial pressure via falls in heart rate and cardiac output, due mainly to activation of cardiovagal efferents (Thoren, 1979; Aviado and Guevara Aviado, 2001). The rapid fall in systemic arterial pressure unloads pulmonary pressures to protect the delicate pulmonary capillaries from pressure-induced damage. These responses are part of a co-coordinated series of hemodynamic and respiratory responses known as the Bezold-Jarisch reflex.

The Bezold Jarisch reflex can be induced in experimental animals such as rats and guinea pigs by inflating a non-occlusive balloon catheter in the right atrium (Thoren, 1979; Aviado and Guevara Aviado, 2001). The Bezold Jarisch reflex can also be activated by systemic injections of 5-hydroxytryptamine (5-HT), which stimulates 5-$HT_{3A,3B}$ receptors on cardiopulmonary vagal afferent terminals (Fozard, 1984; Richardson and Engel, 1986; Dabire et al., 1990). 5-$HT_{3A,3B}$ receptors are ligand-gated ion-channels which conduct Na^+ and K^+ ions (Hartig et al., 1990; Julius, 1991; Hartig, 1992; Zifa and Fillion, 1992). Since 5-HT elicits other hemodynamic responses by activation of 5-$HT_{1A,B,D}$ and 5-$HT_{2A,B}$ receptors in the cardiovascular system, it is often preferable to inject selective 5-HT_3 receptor agonists such as phenylbiguanide or 2-methyl-5HT (Whalen et al., 2000), which elicit the Bezold Jarisch reflex only since these receptors do not exist on blood vessels or cardiac tissues (Richardson and Engel, 1986; Whalen et al., 2000).

EFFECTS OF CHOLESTEROL ON BEZOLD-JARISCH REFLEX FUNCTION

Changes in cardiopulmonary afferent function are sensitive indicators of the progression of cardiopulmonary disease processes associated with pulmonary and systemic hypertension, atherosclerosis and hypercholesterolemia (Whalen et al., 2000; Lewis and Bates, 2004a,b). These disease processes can directly affect cardiopulmonary afferent function by altering metabolic or signal transduction processes (i.e., disposition/function of membrane-associated receptors and ion-channels) in the afferent terminals (Whalen et al., 2000; Aviado and Guevara Aviado, 2001; Lewis and Bates, 2004a,b). These disease processes can also indirectly alter afferent responses by changing the distensibility of atria and arteries in the cardiopulmonary circulation (Aviado and Guevara Aviado, 2001).

At present, there are no data concerning the effects of hypercholesterolemia on cardiopulmonary reflexes such as the Bezold Jarisch reflex, however, these studies are warranted due to increasing evidence that subtle alterations in cardiovascular reflex mechanisms may be of prognostic significance (Piccirillo et al., 2001).

ADRENOCEPTORS IN THE CARDIOVASCULAR SYSTEM

Adrenoceptors located on cardiac muscle and in the vasculature sub-serve the effects of catecholamines released by post-ganglionic sympathetic nerves and from the adrenal medulla (Guimaraes and Moura, 2001). Ahlquist (1948) first proposed the existence of distinct α- and β-adrenoceptors. β-adrenoceptors include β_1-, β_2-, and β_3-adrenoceptors, with each predominantly existing in the heart, respiratory system and adipose tissue, respectively. However, each specific β-adrenoceptor subtype may exist in diverse cellular tissues throughout the body (Skeberdis,

2004). α-Adrenoceptors include α_{1A}-, α_{1B}-, α_{1D}-, $\alpha_{2A/D}$-, α_{2B}-, α_{2C}-adrenoceptors, and are primarily responsible for constriction of vascular smooth muscle (Guimaraes and Moura, 2001). Epinephrine and norepinephrine, are the primary catecholamines released by sympathetic nerves and adrenal medulla. Epinephrine is virtually equipotent for all adrenoceptor subtypes, unlike norepinephrine, which has higher efficacy at α_1, α_2- and β_1- adrenoceptors (Van Zwieten, 1992). Catecholamines activate β-adrenoceptors on the sinoatrial node, atrioventricular node, Purkinje fibers and cardiac myocytes. Although β_1-adrenoceptors predominate in the heart and stimulation causes tachycardia and increases in atrio-ventricular conduction and contractility, there is now evidence that β_2-adrenoceptors are located on cardiac myocytes and are involved in contractility. α-Adrenoceptors also have the potential to mediate increases in cardiac contractility, however, they elicit a much weaker response that β–adrenoceptors (Van Zwieten, 1992).

Catecholamines regulate vascular smooth muscle tone by activating constrictor α_1- and α_2-adrenoceptors and dilator β-adrenoceptors (Nielson et al., 1992; Parkinson et al., 1992). It was originally thought that vasodilation was mediated exclusively by β_2-adrenoceptors. However, it is now evident that β_1- and β_2-adrenoceptors play vital roles in regulating vascular tone (O'Donnell & Wanstall, 1985; Nakane et al., 1988; Purdy et al., 1988; Taira et al., 1977). β_3-adrenoceptors are also found in blood vessels where they mediate vasodilation (Cohen et al., 1976; Molenaar et al., 1988; Rohrer, 1999), although the physiological relevance of these receptors is unknown since catecholamines are weak agonists of these receptors (Molenaar et al., 1988; Rohrer, 1999).

β_1-, β_2- and β_3-adrenoceptors activate Gs-coupled proteins, which stimulate adenylyl cyclase, and the resulting cAMP activates cAMP dependent protein kinases (Katzung et al., 2002). α_1-adrenoceptors activate $G_{o,q}$-coupled proteins, which activates the phosphoinositide cascade via the release of IP_3 and DAG in the plasma membrane. Calcium is subsequently released from intracellular stores on the endoplasmic reticulum and by voltage sensitive calcium channels, which result in calcium/calmodulin activated myosin/actin contraction (Katzung et al., 2002). α_2-Adrenoceptors are coupled to G_i-coupled proteins that inhibit adenylyl cyclase resulting in a variety of effects including enhanced vascular contraction (Katzung et al., 2002). Specifically, the activated alpha-G_i subunit causes an increase in vascular tone by diminishing G_s protein-mediated vasorelaxation, which is dependent upon the activation of adenylyl cyclase.

CHOLESTEROL-INDUCED CHANGES IN CARDIOVASCULAR FUNCTION

Hypercholesterolemia, which is clinically manifested as a total plasma cholesterol level of 200 mg/dL or greater (Saini et al., 2004), is a major risk factor for cardiovascular disease (Pekkanen et al., 1990; Klag et al., 1993; McKenney, 2002). Elevated plasma cholesterol levels in humans contribute to the development of atherosclerosis and subsequent coronary heart disease, which is a major cause of death in developed countries (Braunwald, 1997; Fruchart and Duriez, 1998, Murray and Lopez, 1997). Atherosclerosis exerts its cardiovascular effects indirectly via the formation of oxidized-LDL plaques in coronary arteries (Saini et al., 2004). On the contrary, elevated cholesterol levels directly contribute to cardiovascular dysfunction by altering membrane fluidity, enzymatic activities and cation transporters in endothelial cells, vascular smooth muscle cells and cardiomyocytes void of atherosclerotic lesions (Saini et al., 2004).

Optimal cellular function depends on the ratio of cholesterol/phospholipid (C:P) in cellular membranes, which is the determinant of membrane fluidity (Presti, 1985). Upon cholesterol supplementation, the cardiac sarcolemma becomes permeable to various electrolytes and non-electrolytes (Mcelhaney et al., 1973; Madden et al., 1981; Schmidt et al., 1998). Elevated dietary cholesterol increases myocardial permeability of Na^{+} and Ca^{2+} in rabbits (Pfeiffer et al., 1978; Kutscherskij et al., 1984). In addition, cellular homeostatic mechanisms which regulate Na^{2+}-Ca^{2+} exchange and Ca^{2+}-dependent K^{+} channels change substantially during fluctuations in membrane cholesterol content (Kutryk and Pierce, 1988; Bolotina et al., 1989; Jeremy and McCarron, 2000). Moreover, augmentation of the sarcoplasmic reticulum with cholesterol inhibits Ca^{2+} pump ATPase activity, which results in abnormal Ca^{2+} homeostasis and subsequent reductions in contractile activity during heart failure (Madden et al., 1981; Schmidt et al., 1998).

In the absence of atherosclerotic lesions, hypercholesterolemia causes disruption of epithelial function in a multitude of species including humans, however, perturbation of the vascular endothelium may be the initial cardiovascular insult in the progression of atherosclerosis (Yamamoto et al., 1988; Zeiher et al., 1991; Creager et al., 1990). Moreover, the mechanisms by which increased concentrations of circulating plasma cholesterol cause endothelial dysfunction are currently unknown. Potential mechanisms of endothelial dysfunction in hypercholesterolemic humans are significant reduction of NO synthesis, disruptions in receptor mediated processes that regulate NO release, and decreased bioavailabilty of NO/endothelium-derived relaxing factor in coronary vessels (Drexler and Hornig, 1999). NO further potentiates its vascular effects by having an inverse effect on vascular endothelin synthesis which may increase their vasoconstrictive effects during hypercholesterolemia (Lerman, 1992).

In humans, hypercholesterolemia is associated with alterations in endothelium dependent relaxation, increased systemic arterial stiffness, and increased pulse pressures (Creager et al., 1990; Roman et al., 2000; Wilkinson et al., 2002). Decreases in diastolic pressure reduce the driving force that is necessary for the filling of coronary arteries during diastole, while increased systolic pressure increase the workload of the heart. In addition, reductions in aortic capacity due to increased aortic stiffness during cardiac ejection limits blood availability for coronary perfusion during diastole. These factors account for the finding that large artery stiffness predicts ischemic threshold in individuals with coronary artery disease (Kingwell et al., 2002).

LEFT VENTRICULAR REMODELING AND HYPERCHOLESTEROLEMIA

The process in which neurohormonal, mechanical, and genetic factors regulate ventricular size, shape, and function is known as left ventricular remodeling (Pfeffer and Braunwald, 1990; Rouleau et al., 1993). Normal physiological growth is dependent on left ventricular remodeling, however, remodeling may also occur during pathological process such as hypercholesterolemia, myocardial infarction, cardiomyopathy, hypertension, or valvular heart disease (Celentano et al., 2001; Martin et al., 2000). Similar to hypertensive patients, hypercholesterolemic individuals may exhibit one of four left ventricular geometric patterns (Celentano et al., 2001; Krumholz et al., 1995). As identified by Ganau et al., (1992), these geometric patterns are concentric remodeling with normal left ventricular mass and increased relative wall thickness; concentric hypertrophy with increased left ventricular mass and increased relative wall thickness; eccentric hypertrophy with increased left ventricular mass and normal relative wall thickness; and normal left ventricular geometry.

Hypercholesterolemia is associated with concentric remodeling of the left ventricle in normotensive patients (Celentano et al., 2001). This geometric change in ventricular structure is likely to impact mortality and morbidity in hypercholesterolemic patients since a similar left ventricular remodeling in hypertensive patients is correlated with increased mortality and morbidity (Devereux et al., 1994; Verdecchia et al., 1999), reduced coronary flow reserve (Shafer et al., 2002), diastolic dysfunction (Qu et al., 2001) and altered systolic function (Sadler et al., 1997). Moreover, hypertensive post-menopausal women with increased total cholesterol and LDL had impaired diastolic function but not left ventricular mass index (Palmeiro et al., 2002). Likewise, an increased left ventricular mass index in untreated hypertensives is not associated with increased total or LDL cholesterol levels (Schillaci et al., 2001). The prognostic importance of left ventricular geometry in hypercholesterolemia is unknown, however, further analysis of the mechanisms by which hypercholesterolemia affects the structure and function of the cardiovascular system will form the basis for the development of novel therapeutic strategies to ameliorate hypercholesterolemia-induced cardiovascular disorders.

Reorganization of existing structures of the heart into a more concentric shape by the inward migration of the layers of the heart without affecting cardiac function is a potential mechanism of left ventricular remodeling. Bishopric et al. (2001) proposed that concentric remodeling occurs via apoptotic removal of epicardial tissue concurrently with synthesis of endocardial tissue. In rabbits, hypercholesterolemia induces apoptosis of aortic valves (Rajamannan et al., 2001). The hearts of spontaneously hypertensive rats experience selective epicardial apoptosis during regression of hypertrophy after treatment with ACE inhibitors, angiotensin II receptor blockers, calcium channel blockers or β-adrenoceptor blockers (Tea et al., 1999).

Growth of the endocardial myocardium may occur via myocyte hypertrophy and/or myocyte proliferation. Previously, the heart was considered to be a terminally differentiated organ, and recent evidence supports the intrinsic capacity of the adult myocardium to undergo cell replication (Anversa and Kajstura, 1998; Soonpaa and Field, 1998; Sonnenblick and Anversa, 1999; Pasumarthi and Field, 2002). Healthy unstressed hearts experience apoptosis and proliferation at a lower rate than most biological tissues (Kajstura et al., 2000), whereas in pathological disease states (Beltrami et al., 2001) and in several strains of transgenic mice, the rate of apoptosis (Fortuno et al., 1998) and proliferation (Moravec et al., 2002) are elevated.

Nitric oxide regulates cardiac morphology. Mice deficient in the endothelial NOS (eNOS) gene develop cardiac hypertrophy whereas those lacking eNOS and neuronal NOS (nNOS) genes develop concentric remodeling (Barouch et al., 2003). This evidence supports the idea that hypercholesterolemia induces concentric remodeling via reduced expression of both NOS isoforms, which subsequently cause a marked reduction in NO bioavailability. The ability of eNOS and nNOS to differentially regulate excitation-contraction coupling in the heart may be due to the spatially distinct distribution of eNOS with caveolae in the plasma membrane and nNOS in the sarcoplasmic reticulum (Hare, 2003).

Schwemmer et al. (2000) determined that hypercholesterolemic guinea pigs have reduced eNOS mRNA in cardiac tissue, which further supports the concept that cholesterol affects cardiac morphology and function via down-regulation of NOS expression. Cardiac calveolin-1 levels, an allosteric inhibitor of $eNOS_3$, are substantially increased in hypercholesterolemic apo $E^{-/-}$ mice, therefore leading to a functional deficiency of eNOS activity (Pelat et al., 2003). In addition,

cultured endothelial cells incubated with serum from hypercholesterolemic patients increases calveolin levels and reduces NO production (Feron et al., 1999).

GUINEA PIGS AS A MODEL OF HUMAN HYPERCHOLESTEROLEMIA

There is now compelling evidence that guinea pigs (*Cavia porcellus*) and humans have similar lipid metabolism profiles, therefore identifying the guinea pig as the most apt small animal model for examining the effects of hypercholesterolemia on human cardiovascular function (Fernandez, 2001). In comparison to rats (Swann et al., 1975; McNamara et al., 1982), humans (Reihner et al., 1990) and guinea pigs (McNamara, 1984) have moderate rates of hepatic sterologenesis and catabolism (Fernandez, 1995b; Reihner et al., 1991) which may be enzymatically modulated by diet, drug treatment and gender. Homeostatic control of cholesterol synthesis has been studied in humans via liver biopsy samples from patients receiving normal cholesterol diets (363 to 904 mg of cholesterol per day), low cholesterol diets (103 mg of cholesterol per day) or high cholesterol diets (3-4 g cholesterol per day) for 3 days (Bhattathiry and Shiperstein, 1963). Bhattathiry and Shiperstein (1963) determined that humans respond to dietary cholesterol by a hepatic feedback mechanism that regulates cholesterol synthesis that is independent of plasma cholesterol levels.

Alterations in hepatic cholesterol metabolism via dietary modification of hepatic free cholesterol (FC) may decrease cholesterol synthesis in guinea pigs, by down-regulation of HMG-CoA reductase. Lin et al. (1992) found that guinea pigs ingesting dietary cholesterol equivalent to an absorbed amount of half of the daily synthesis rate have significant down regulation of HMG-CoA reductase activity. However, dietary cholesterol has no effect on the enzymatic activity of

the rate-limiting enzyme of bile acid synthesis, Cyp7 in guinea pigs (Fernandez, 1995a).

In contrast to other animal models but similar to humans, guinea pigs have moderate rates of cholesterol esterification via the intracellular enzyme acyl coenzyme A cholesterol acyltransferase (ACAT) in various tissues, including the liver. Unique to the liver, humans have higher concentrations of free than esterified cholesterol (Angelin et al., 1992). Guinea pigs also share this similarity by distributing 3.1 ± 0.3 mg/g of their hepatic cholesterol in the free form, while only 0.27 ± 0.10 is in the esterified form (Fernandez et al., 1995a). However, hepatic ACAT activity may be modified by consumption of high dietary cholesterol (Sun et al., 1999). Sun et al. (1999) determined that guinea pigs consuming increased concentrations of high dietary cholesterol had parallel increases in hepatic ACAT activity and microsomal FC. Also, alterations in microsomal lipid composition with FC/phosphatidylcholine (PC) treatment resulted in ACAT activities that were correlated to the FC-to-PC molar ratio. These findings therefore suggest and confirm the findings of other investigators that FC or FC/PC is a major regulator of ACAT activity (Vidal-Quintanar et al., 1997).

Analogous to humans, guinea pigs intravascularly process plasma lipoproteins via multiple pathways. Cholesterol ester transfer protein (CETP) mediates the transport of cholesteryl esters from high density lipoproteins (HDL) towards triglyceride-rich lipoproteins in plasma, therefore resulting in decreased HDL associated cholesterol along with decreased HDL particle size (Ha and Barter, 1982; Hesler, 1988; Tall, 1993). Likewise, lecithin-cholesterol acyl-transferase (LCAT), catalyzes the conversion of cholesterol to form cholesteryl esters in the plasma compartment (Douglas and Pownell, 1991). In guinea pigs consuming low cholesterol diets,

nascent VLDL has negligible amounts of cholesteryl ester (CE) (Abdel-Fattah et al., 1995), while mature VLDL particles contain ~10% CE (Fernandez et al., 1998). As seen in humans, guinea pig VLDL composition resembles that of LDL, therefore suggesting that guinea pig CE in VLDL emanates primarily from LCAT activity (Barter et al., 1977). In addition to LCAT activity and CETP-mediated CE transfer, ACAT activity may be instrumental to the formation of CE in VLDL (Fernandez and McNamara, 1994).

Lipoprotein lipase (LPL) and hepatic lipase which enzymatically hydrolyze circulating triglyceride-rich lipoproteins, corresponds to two thirds and one third of post-heparin lipase activity in humans, respectively (Olivecrona and Bengsston-Olivecrona, 1993). Up-regulation of hepatic lipase in guinea pigs occurs in the presence of a high cholesterol diet, however, identical to humans, HL activity is lower than LPL activity (Heller, 1983; Yin and Olivecrona, 1999).

Guinea pigs fed 0.08, 0.17 or 0.33% dietary cholesterol, which is equivalent to half, one time and two times the endogenous cholesterol synthesis rate in guinea pigs, exhibited dose dependent increases in plasma cholesterol associated with the LDL fraction which were independent of dietary fat concentrations (Lin et al., 1992, 1994, 1995). Maximal suppression of HMG-CoA reductase activity was observed at all concentrations, along with dose dependent increases in free and esterified cholesterol. In agreement with these studies, as dietary concentrations of cholesterol increased, hepatic LDL receptor number decreased (Lin et al., 1994). However, in light of current evidence to support the effects of dietary cholesterol on guinea pigs (Fernandez et al., 1990b) and humans (McNamara et al., 1987), cholesterol toxicity is highly individualized due to variances in the sensitivity of response.

Resembling humans, experimental evidence supports the idea that guinea pigs are the most appropriate animal model to study the role of vitamin C in hepatic lipid metabolism due to their dietary requirement for ascorbic acid (Turley et al., 1976). Substantial evidence from epidemiological studies have shown that decreased ascorbic acid concentrations lead to decreased plasma cholesterol levels (Cerna and Ginter, 1978) and a higher risk for coronary heart disease (Gey et al., 1993). Montano et al., (1998) determined that inadequate dietary intake of vitamin C in guinea pigs results in hypercholesterolemia.

Guinea pigs with dietary ascorbic acid deficiency experience reductions in Cyp7 activity (Greene et al., 1985; Holloway et al., 1981). Vitamin C deficiency has also been associated with reductions in the active form of HMG-CoA reductase in correlation with higher concentrations of cholesteryl esters (Montano et al., 1998). These data suggest that guinea pigs consuming marginal amounts of dietary vitamin C undergo a twofold homeostatic response in the liver that maintains free cholesterol pools by suppressing cholesterol synthesis and increasing hepatic cholesterol stores (Fernandez, 2001).

The most outstanding similarity between human and guinea pig cholesterol metabolism is that both species carry the majority of their cholesterol in the LDL fraction, therefore resulting in high LDL to HDL ratios (Fernandez and McNamara, 1989). In addition, mechanistically, guinea pigs regulate cholesterol and lipoprotein metabolism in response to dietary factors, which is similar to results found in clinical studies (Fernandez et al., 1990a). Therefore in comparison to other animal models, the guinea pig is the most applicable small animal model to study the effects of human hypercholesterolemia (Fernandez et al., 2001).

SUMMARY

Hypercholesterolemia is a major factor responsible for human cardiovascular diseases. This dissertation addresses the mechanisms by which a high-cholesterol diet affects cardiovascular function in the guinea pig. We chose the guinea pig because it is the best animal model to study the effects of hypercholesterolemia on cardiovascular function (see Fernandez et al., 2001).

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CHAPTER 3

CHOLESTEROL-INDUCED CARDIAC CONCENTRIC REMODELING[1]

[1]Owen JR, Barton MH, Munday JS, Graves JE, Lewis SJ. To be submitted to *Journal of Clinical Investigation*.

ABSTRACT

In humans, hypercholesterolemia is an important risk factor for cardiovascular disease and may contribute to cardiac concentric remodeling in normotensive patients. Concentric remodeling of the left ventricle in hypertensive individuals contributes to increased mortality and morbidity, reduced coronary flow reserve and altered systolic and diastolic function. Our laboratory has developed a normotensive small animal model of human hypercholesterolemia, the cholesterol-fed guinea pig, which develops concentric remodeling of the left ventricle. In order to assess hypercholesterolemia-induced alterations in cardiac geometry, we compared echocardiographic measurements of left ventricular (**LV**) mass and the wall thickness to chamber dimension ratio (relative wall thickness, **RWT**) along with gross morphology, in control and cholesterol-fed guinea pigs (1% cholesterol diet for 13 weeks). Echocardiography determined the percentage increase in LV wall thickness in hypercholesterolemic animals as calculated by two methods was 30% and 37% for RWT1 and RWT2, respectively. However, left ventricular mass and fractional shortening was similar in both groups. Gross morphology determined that cholesterol-fed guinea pigs had smaller LV chambers, LV wall volumes, and total LV volumes, whereas minimum LV wall thickness, maximum LV wall thickness and mean LV wall thickness were similar in both groups. In conclusion, our results provide evidence that guinea pigs consuming a 1% cholesterol diet for 13 weeks develop cardiac concentric remodeling characterized by an increase in relative wall thickness without an increase in left ventricular mass.

Key words: hypercholesterolemia, concentric remodeling, echocardiography

INTRODUCTION

Hypercholesterolemia contributes to the progression of a myriad of cardiovascular abnormalities which affect millions of Americans each year (see Saini et al., 2004). Numerous clinical studies have provided evidence that elevated dietary cholesterol intake significantly contributes to the development of atherosclerosis and subsequent myocardial ischemia, especially in subjects with other significant risk factors such as hypertension and diabetes (Saini et al., 2004). However, it is becoming increasingly evident that increased serum cholesterol levels in normotensive patients may contribute to the development of altered left ventricular geometry and other abnormalities in the absence of atherosclerotic lesions (Celentano et al., 2001). Left ventricular geometry in hypertensive patients is prognostic of increased mortality and morbidity (Devereux et al., 1994; Verdecchia et al., 1999). It would seem that the development of an appropriate small animal model to study the effects of hypercholesterolemia on cardiovascular structure and function is a logical step towards understanding the effects of hypercholestermia on cardiac and vascular function in humans.

Left ventricular remodeling is a normal part of the cardiac maturity process and occurs during growth into adult-hood (Celentano et al., 2001; Martin et al., 2000). This remodeling allows for the growth of the heart and expansion of the atrial and ventricular chambers. In essence, the heart grows “out-ward”, via the loss of inner cardiac muscle layers and the generation of outer cardiac muscle layers (Celentano et al., 2001; Martin et al., 2000). Left ventricular remodeling also occurs in adult humans as a result of pathophysiology associated with disease states such as cardiomyopathy, hypertension, and valvular heart disease and in hypercholesterolemic subjects and those that have suffered a myocardial infarction (Celentano et al., 2001; Martin et al., 2000).

Hypercholesterolemic individuals may exhibit one of four left ventricular geometric patterns (see Celentano et al., 2001; Krumholz et al., 1995). These patterns are:

1. Normal left ventricular geometry.
2. Concentric remodeling with normal left ventricular mass and increased relative wall thickness.
3. Concentric hypertrophy with increased left ventricular mass and increased relative wall thickness.
4. Eccentric hypertrophy with increased left ventricular mass and normal relative wall thickness.

In a recent review, Fernandez et al. (2001) provided compelling evidence that the guinea pig is the most suitable small animal model to study the processes by which hypercholesterolemia causes cardiovascular dysfunction in humans. Similar to humans, guinea pigs transport most of their cholesterol in the form of LDL, have similar LDL to HDL ratios, have similar rates of hepatic cholesterol and catabolism and metabolism and are dependent on dietary vitamin C (Fernandez et al., 2001).

The **objective** of this study was to use echocardiographic analyses and histological techniques to characterize left ventricular structure in control guinea pigs (i.e., those on normal diets) and in guinea pigs consuming a 1% cholesterol diet for 13 weeks. The results of these studies will be vital for understanding the findings of parallel studies designed to determine the mechanisms involved in cholesterol-induced alterations in cardiac structure.

MATERIALS AND METHODS

Animals, Treatments, Drugs and Chemicals

All studies were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. The protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. Male Hartley guinea pigs weighing 400-450 g were fed control diets or control diets supplemented with 1% cholesterol (Research Diets, Inc.) for 13 weeks.

Blood Cholesterol Determinations

Guinea pigs were anesthetized with ketamine (120 mg/kg, iv)-acepromazine (12 mg/kg, iv) and a catheter was placed in the carotid artery for measurements of mean arterial blood pressure and heart rate and for collection of 1 ml of blood for lipid analyses. The guinea pigs were allowed 3 days to recover from surgery and blood was drawn at 13 weeks of the dietary regimen. To extract serum, 1 ml of blood was transferred to a 1.5 ml polypropylene tube and centrifuged for 4 min at 3000 rpm. Serum samples from each animal were taken to measure glucose, total cholesterol and HDL cholesterol. A 35 µl aliquot of serum was loaded onto a Cholestech LDX System cassette to measure total cholesterol, HDL cholesterol, triglycerides and glucose (Allain et al., 1974; Roeschlau et al., 1974). Once the cassettes were loaded, the serum flowed to the right side of the cassette where the low density lipoproteins (LDL and VLDL) were precipitated out with dextran sulfate and magnesium acetate precipitating agent. Hydrolyzation of cholesterol esters in the filtrate via cholesterol esterase results in free cholesterol and the corresponding fatty acid. Cholesterol oxidase in addition to oxygen, oxidizes free cholesterol to cholest-4-ene-3-one and hydrogen peroxide. Horseradish peroxidase then reacts with 4-aminoantipyrine and N-Ethyl-N-

sulfohydroxypropyl-m-toluidine sodium salt (TOOS) to form a purple colored quinoneimine dye proportional to total cholesterol and HDL cholesterol concentrations of the sample.

Echocardiography

The control and cholesterol-fed guinea pigs were lightly anesthetized with diazepam (2.5 mg/kg, ip)-ketamine (50 mg/kg, ip). The anesthetic mixture was designed to sedate the guinea pigs to allow for echocardiography since the echocardiography procedure was not painful in any way. Echocardiography on these guinea pigs was performed by Dr. Michelle Barton (Department of Large Animal Medicine, College of Veterinary Medicine) (Jenni et al., 2000; Moore and Barton, 2003; Barton et al., 2003, 2004). Dr. Barton performed these analyses with the help of an ATL 5000 ultrasound unit (Philips, WA) equipped with an electrocardiogram and an 8.5 MHz transducer. The right para-sternal border of the guinea pigs was shaved and the heart imaged in left lateral recumbency. The most reliably obtained view, the 2 dimensional 4-chamber long axis view, was used for measurements of the left ventricle. A diagrammatic view of the 2-D 4-chamber long axis view is shown in **Figure 3.1**. This diagram shows the relative position of the heart chambers and valves. An example of an echocardiogram taken from a control guinea pig at end diastole at the onset of the QRS complex is shown in **Figure 3.2**. The relative positioning of the left and right ventricles and mitral valve are shown.

Echocardiographic Analyses

Interventricular septum thickness during diastole (**IVSd**), internal diameter of the left ventricle during diastole (**LVIDd**), and posterior wall thickness during diastole (**PWTd**) was measured at onset of the QRS complex immediately ventral to the mitral valve. The internal diameter of the

left ventricle during systole (**LVIDs**) was taken at this location when the distance between the interventricular septum and posterior wall was at its nadir. The mean of 3 determinations of inter-ventricular septum thickness during diastole, internal diameter of the left ventricle during diastole, and posterior wall thickness during diastole were used to determine relative wall thickness (**RWT**) and left ventricular mass. Left ventricular mass (gm) was assessed by the formula, Left ventricular mass = 1.04 x [(internal diameter of left ventricle during diastole + posterior wall thickness during diastole + interventricular septum thickness during diastole)^3 – (internal diameter of left ventricle during diastole)^3]. This method accurately reflects left ventricular mass at necropsy (Devereux et al., 1986; Litwin et al., 1994). Relative wall thickness (**RWT**) was calculated by 2 methods, namely, **RWT1** = (interventricular septum thickness during diastole plus posterior wall thickness during diastole)/internal diameter of left ventricle during diastole (Sundstrom et al., 2000; Schafer et al., 2002) and **RWT2** = 2 x (posterior wall thickness during diastole)/internal diameter of left ventricle during diastole (Roman et al., 2000). Fractional shortening (**FS**) was calculated as [(internal diameter of the left ventricle during diastole minus internal diameter of left ventricle during systole)/(internal diameter of the left ventricle during diastole)] x 100. The short-hand notations for the formulae are:

$$\textbf{RWT1} = (IVSd + PWTd)/LVIDd \qquad \textbf{RWT2} = (2 \times PWTd)/PWTd$$

$$\textbf{FS} = (LVIDs/LVIDd) \times 100$$

Morphology

Guinea pigs were fully anesthetized with ketamine (120 mg/kg, ip)-acepromazine (12 mg/kg, ip). The hearts were arrested in diastole via an injection of KCl (1ml of 2 meq) given via a catheter

that was placed in a jugular vein after anesthesia was induced. This procedure was performed to standardize the hearts for the morphological analyses. The hearts were excised and fixed in 5% formalin in phosphate buffered saline for 12 hours. Longitudinal sections (4-6 μm) were cut using a Cryostat and embedded in paraffin and stained using a standard histological solution of a mixture of hematoxylin and eosin. To ensure that all of the sections were taken from a consistent location in the heart, the sections were examined to ensure that neither attachment of the large papillary muscle to the left ventricular wall nor valve cusps were visible in the sections. The analyses of the tissue sections were performed by Dr. John Munday (Department of Pathology, College of Veterinary Medicine) (Munday and Prahl, 2002; Munday and Thompson, 2003; Munday et al., 2003, 2004). Ventricular cross sections were digitized by a Nikon Supercool Scan 4000 ED digital scanner (Nikon Corp., Japan). Left ventricular lumen area, ventricular area (ventricular myocardium plus ventricular lumen), and mean ventricular thickness and individual cell size were measured using Image-Pro Plus image analysis software (Cybernetics, USA).

Drugs and Reagents

Ketamine HCl and acepromazine maleate were obtained from Animal Health (Fort Dodge, IA). All other reagents were from Sigma (St. Louis, MO).

Statistical Analysis

The data are shown as mean ± S.E.M. and were analyzed by repeated-measures analysis of variance (ANOVA) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means using the error mean square terms from the ANOVAs (Winer, 1971; Wallenstein et al., 1980). A value of $P < 0.05$ denoted statistical significance.

RESULTS

Blood Cholesterol Levels

Total plasma cholesterol levels in conscious control (n=8) and 1% cholesterol-fed (n=8) guinea pigs are summarized in **Figure 3.3**. As can be seen, the cholesterol-fed guinea pigs had markedly higher total cholesterol levels (5.63 ± 0.32 mmol/L) than control animals (1.62 ± 0.10 mmol/L).

Resting heart rate and mean arterial blood pressure levels

Resting mean arterial blood pressure and heart rates in the conscious guinea pigs immediately before the blood samples were taken are summarized in **Figure 3.4**. As can be seen, resting cardiovascular parameters were similar in control and cholesterol-fed guinea pigs.

Heart and body weights

Heart weights, body weights and heart weight to body weight ratios of control and cholesterol-fed guinea pigs are summarized in **Figure 3.5**. As can be seen, the heart weights of cholesterol-fed guinea pigs were slightly (9%) but not significantly lower than the heart weights of control guinea pigs. The body weights of the cholesterol-fed guinea pigs were significantly lower (22%) than those of the control guinea pigs. Taken together, the heart weight to body weight ratios of control and cholesterol-fed guinea pigs were similar to one another.

Cholesterol-Induced Cardiac Concentric Remodeling

Guinea pigs fed a 1% cholesterol diet for 13 weeks developed cardiac concentric remodeling, which was characterized by an increase in relative wall thickness, due mainly to a reduction in the size of the ventricular chamber. At typical example of an echocardiogram from a control and

a cholesterol-fed guinea pig is shown in **Figure 3.6**. As can be seen, the left ventricular chamber volume (darker line) in the cholesterol-fed guinea pig was less than that in the control guinea pig. In contrast, the left ventricular wall thickness of the cholesterol-fed guinea pig was similar to that of the control guinea pig. Relative wall thicknesses (**RWT1** and **RWT2**), fractional shortening (**FS**) and left ventricular mass (**LV mass**) values of control and cholesterol-fed guinea pigs are summarized in **Figure 3.7**. As can be seen, relative wall thicknesses were higher in the cholesterol-fed guinea pigs than in the control animals. RWT1 and RWT2 were 30% and 37% higher in the cholesterol-fed guinea pigs, respectively. Fractional shortening and left ventricular mass indices were similar in the control and cholesterol-fed guinea pigs.

Morphological determination of cholesterol-induced cardiac remodeling

An example of the cross-sectional appearance of hearts taken from a control and a cholesterol-fed guinea pig is shown in **Figure 3.8**. The heart of the cholesterol-fed animal underwent cardiac concentric remodeling as it had a smaller left ventricular chamber but similar ventricular wall thickness to that of the control heart. The left ventricular chamber volumes, left ventricular wall volumes and total left ventricular volumes of control and cholesterol-fed animals are summarized in **Figure 3.9**. The cholesterol-fed guinea pigs had substantially smaller left ventricular chamber volumes compared to control animals. However, as shown in **Figure 3.10**, minimum left ventricular wall thickness, maximum left ventricular wall thickness and mean left ventricular wall thickness was similar in both groups. Therefore, it is apparent that cardiac concentric remodeling in the hypercholesterolemic guinea pigs was due to a reduction in left ventricular chamber size that was not associated with an increase in left ventricular wall thickness

DISCUSSION

These studies demonstrated that guinea pigs receiving a 1% cholesterol diet for 13 weeks had markedly elevated blood levels of cholesterol (i.e., they were hypercholesteremic). An important finding was that the resting heart rate and mean arterial blood pressure values of the cholesterol-fed guinea pigs were not different from those of the control guinea pigs. Other studies in our laboratory have found that these cardiovascular parameters in cholesterol-fed guinea pigs are also similar to controls at 2, 4, 8 and 10 weeks of feeding (Lewis, Owen and Graves, unpublished observations). These findings are very important since they provide evidence that concentric remodeling of the left ventricle in cholesterol-fed guinea pigs was not due to changes in mean arterial blood pressure. Moreover, the lack of obvious hypertrophy in the hearts of cholesterol-fed guinea pigs is also consistent with the probability that the concentric remodeling of the left ventricle occurred in the absence of hypertension (i.e., elevations in mean arterial blood pressure did not occur in between the times we actually recorded resting cardiovascular parameters).

As mentioned, these studies also provide compelling evidence that the cholesterol-fed guinea pigs developed pronounced concentric remodeling of the left ventricle. Numerous studies in humans and in experimental animals have validated the use and accuracy of two-dimensional echocardiography to assess left ventricular geometry and mass (Jugdutt et al., 1992; Troy et al., 1972; Devereux et al., 1977; Savage et al., 1979; Ditchey et al., 1981; Valdez et al., 1979). Since echocardiography and post-mortem data provided evidence for a similar type of left ventricular remodeling (i.e., concentric remodeling in the absence of hypertrophy) in hypercholesterolemic guinea pigs, the current study suggests that echocardiography accurately determined the changes in left ventricular structure in the cholesterol-fed guinea pigs.

Echocardiography determined that relative left ventricular wall thickness was increased, whereas left ventricular mass was unchanged in the cholesterol-fed animals. This pattern of changes is characteristic of concentric remodeling (Bishopric et al., 2001). Interestingly, the wet weights of the hearts taken from cholesterol-fed animals were slightly lower (9%) than the control animals. However, it should be stated that the heart weight to body weight ratios were similar in the cholesterol-fed and control animals (the body weights of cholesterol-fed guinea pigs were also approximately 22% less than the control animals). Most importantly, fractional shortening, a determinant of cardiac contractility, was similar in both groups. This finding clearly suggests that the intrinsic efficiency of cardiac muscle was not obviously compromised in the cholesterol-fed guinea pigs. Moreover, it is evident that cardiac contractility can be maintained during the significant structural changes that occur during development of concentric remodeling of the left ventricle during ingestion of a high cholesterol diet. As will be discussed in other chapters, the above findings are consistent with the findings of our pharmacological studies that intrinsic function (including intracellular signaling pathways linked to β-adrenoceptors in pacemaker and muscle cells) is not greatly compromised whereas β-adrenoceptor function is markedly down-regulated in cholesterol-fed guinea pigs.

The purpose of concentric remodeling of the left ventricle is not fully understood (Bishopric et al., 2001) although it could be conjectured that this geometric change serves to maintain cardiac output as efficiently as possible. The precise mechanisms and temporal sequence of events that elicit concentric remodeling of the left ventricle have not been fully established (Bishopric et al., 2001). However, it is clear that this geometric change must involve substantial reorganization of the existing structures of the heart into a more concentric shape. This would involve the inward

migration of the layers of the heart without necessarily altering the efficiency of cardiac function. Concentric remodeling may occur via apoptotic removal of epicardial tissue concurrently with the synthesis of new endocardial tissue (Bishopric et al., 2001). The hearts of Spontaneously Hypertensive rats undergo selective epicardial apoptosis during the regression of hypertrophy upon treatment with angiotensin concerting enzyme (ACE) inhibitors, angiotensin II receptor blockers, calcium channel blockers or β-adrenoceptor blockers (Tea et al., 1999).

The growth of the endocardial myocardium may occur via myocyte hypertrophy and/or myocyte proliferation, however, in our animals there were no morphological signs of hypertrophy. It has been previously hypothesized that terminal diferentiation of the heart occurs during cardiac development, however, recent evidence supports the intrinsic capacity of the adult myocardium to undergo cell replication (Anversa and Kajstura, 1998; Soonpaa and Field, 1998; Sonnenblick and Anversa, 1999; Pasumarthi and Field, 2002). Healthy unstressed hearts experience apoptosis and proliferation at a lower rate than most biological tissues (Kajstura et al., 2000), whereas in pathological disease states (Beltrami et al., 2001) and in several strains of transgenic mice, the rate of apoptosis (Fortuno et al., 1998) and proliferation (Moravec et al., 2002) are elevated.

Fluctuations in the expression of nitric oxide synthase isoforms regulate cardiac morphology. Barouch et al. (2003) determined that mice deficient in the endothelial nitric oxide synthase gene developed cardiac hypertrophy whereas those lacking endothelial and neuronal nitric oxide synthase genes developed concentric remodeling. Evidence that hypercholesterolemic guinea pigs have reduced endothelial nitric oxide synthase mRNA in cardiac tissue, further supports the concept that cholesterol alters cardiac morphology and function via down regulation of nitric

oxide synthase expression (Schwemmer et al., 2000). Caveolin-1, a potent allosteric inhibitor of endothelial nitric oxide synthase, is substantially increased in hypercholesterolemic apo $E^{-/-}$ mice. This increase in caveolin-1 could be a crucial link between dysfunctional lipid metabolism and endothelial nitric oxide synthase deficiency (Pelat et al., 2003).

SUMMARY

In conclusion, guinea pigs fed a 13 week high cholesterol diet developed hypercholesteremia and pronounced cardiac concentric remodeling of the left ventricle. Since this cardiac abnormality parallels those described in human patients, further studies are warranted to provide insight into understanding the mechanisms by which cholesterol initiates changes in cardiac structure and function, along with relating these mechanisms to pharmacological therapies which can be used by individuals stricken with hypercholesterolemia.

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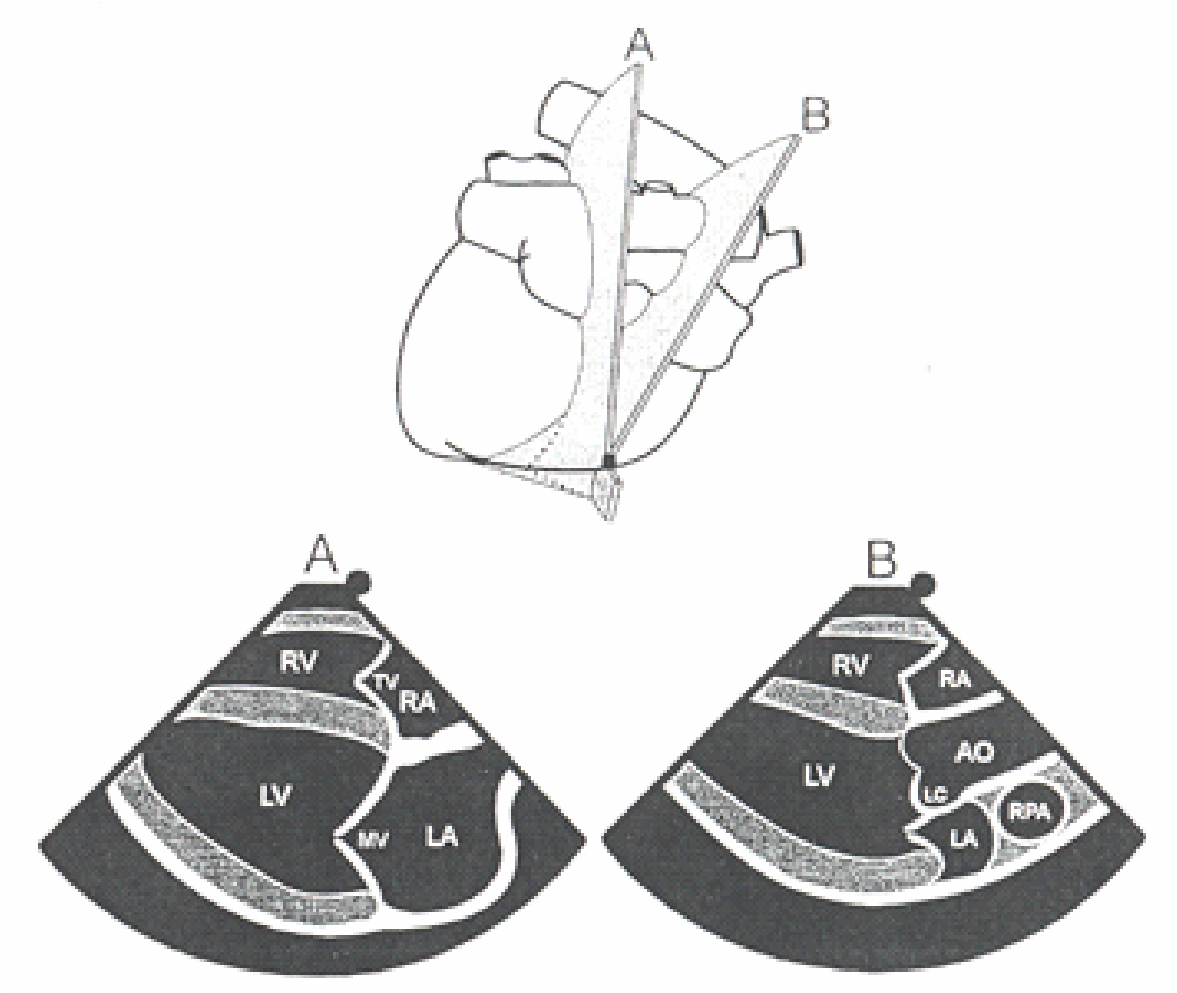


Figure 3.1: Diagrammatic representation of the 2D 4-chamber axis view. RV=right ventricle, TV=tricuspid valve, RA= right atrium, LV=left ventricle, MV=mitral valve, LA=left atrium

Control guinea pig

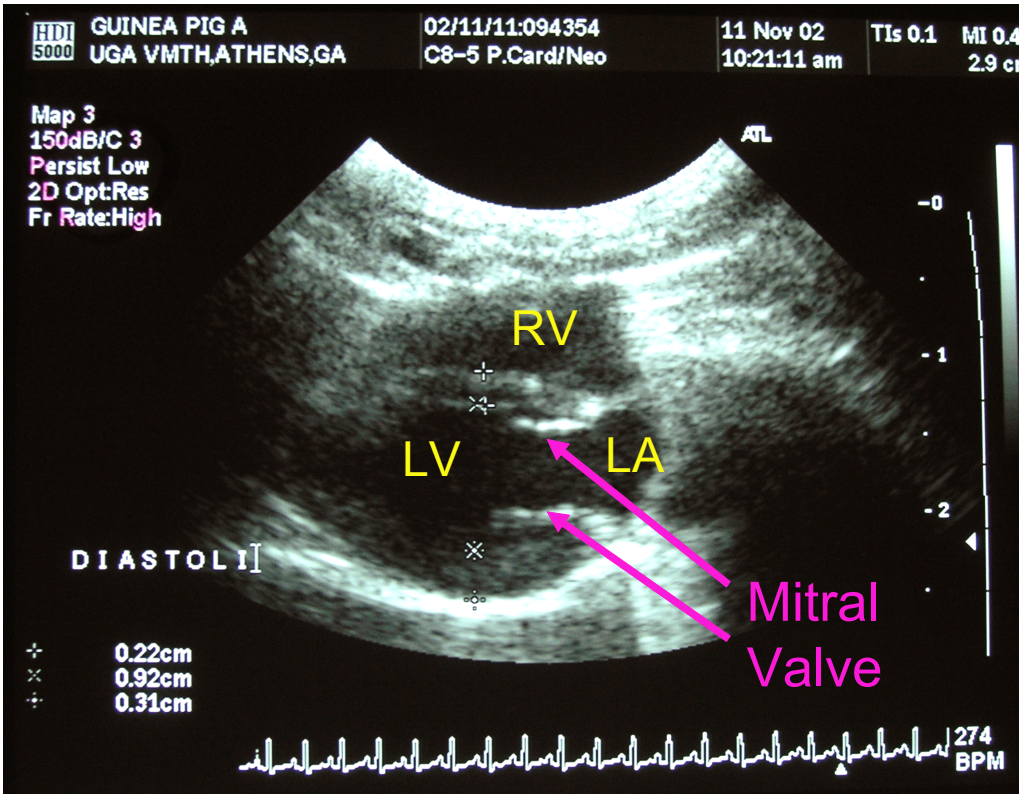


Figure 3.2: Echocardiogram of a healthy (control) guinea pig. Echocardiogram is shown at end diastole at onset of QRS. LV=left ventricle, RV=right ventricle, LA =left atrium. RA=right atrium.

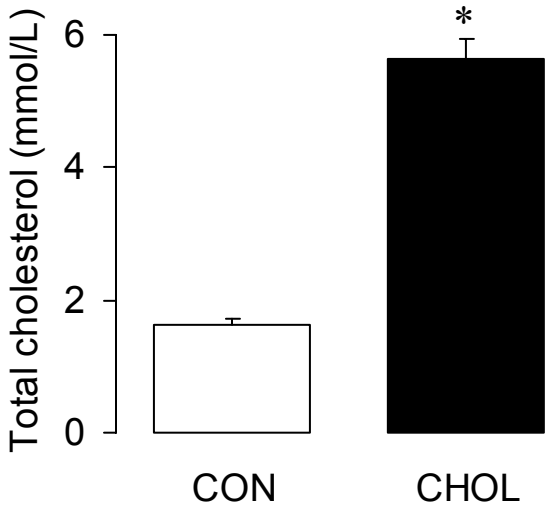


Figure 3.3. Total blood cholesterol levels in control (n=8) and cholesterol-fed (n=8) guinea pigs (1% cholesterol for 13 weeks). The data are shown as mean ± SEM. **P* <0.05, cholesterol-fed versus control.

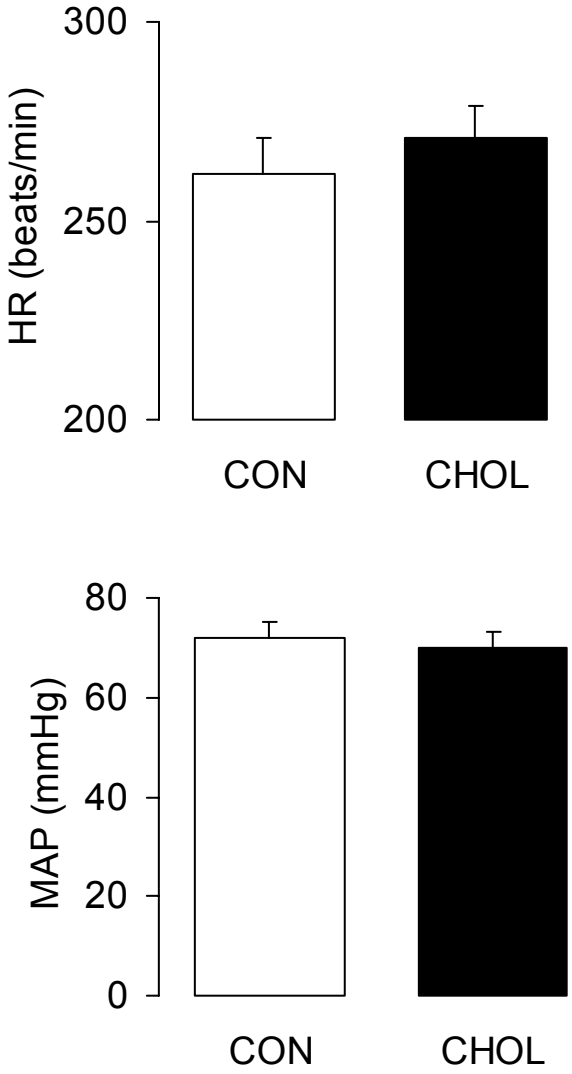


Figure 3.4. Resting heart rate (HR) and mean arterial pressure (MAP) values in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. Note that there were no differences between control and cholesterol-fed guinea pigs at the $P < 0.05$ level.

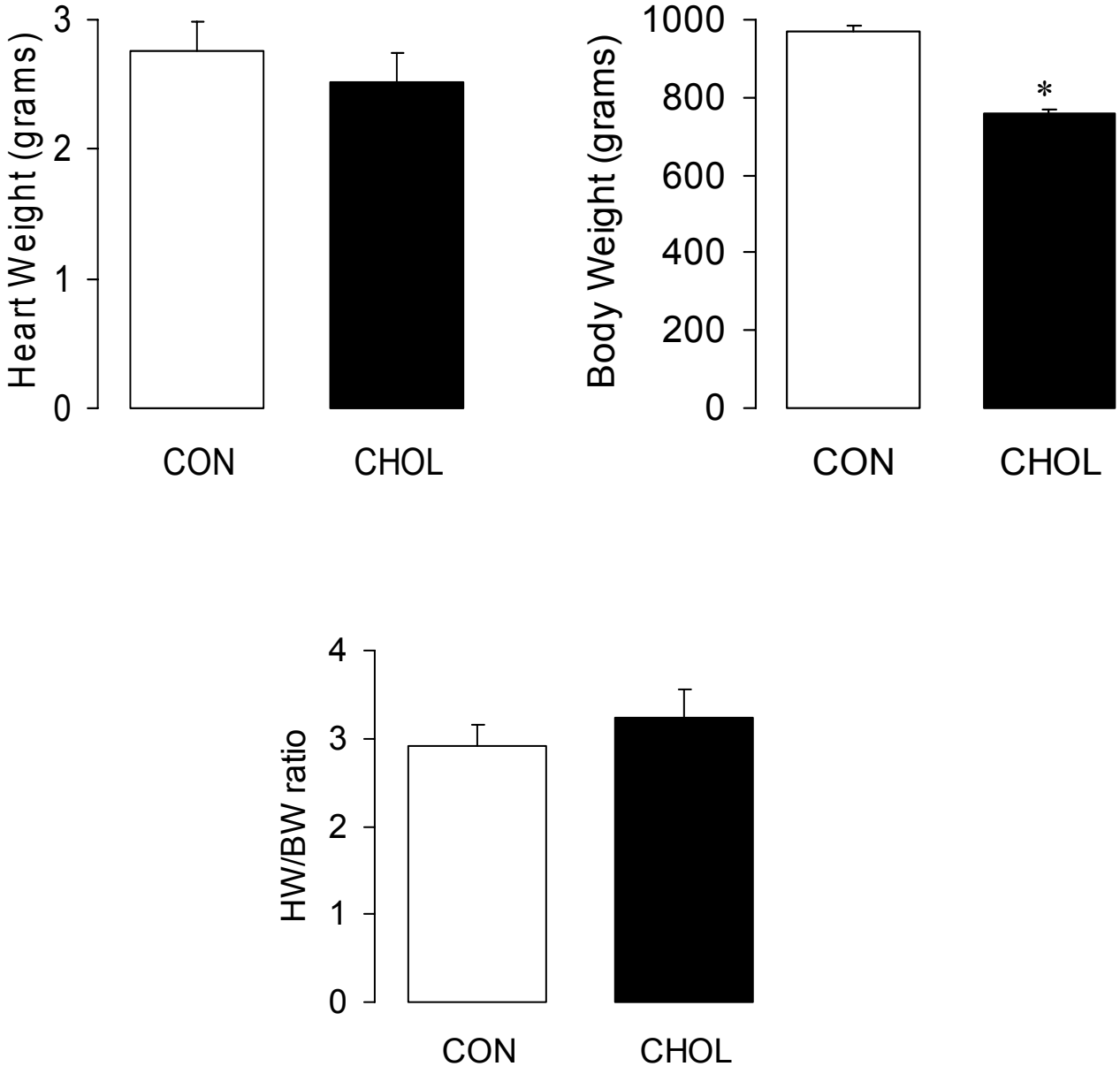
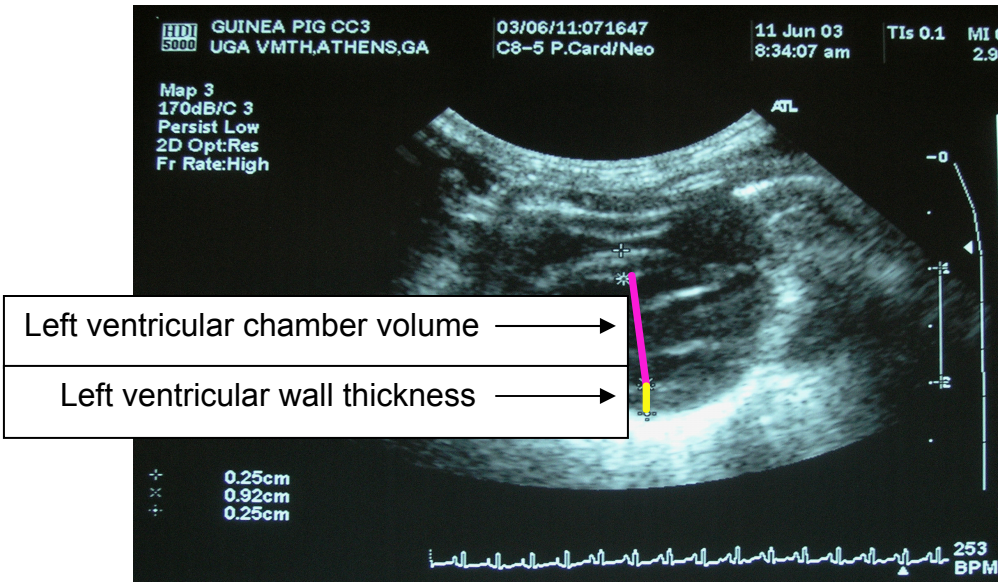


Figure 3.5. Heart weights, body weights and heart weight to body weight ratios of control (n=8) and cholesterol-fed (n=8) guinea pigs (1% cholesterol for 13 weeks). The data are shown as mean ± SEM. **P* <0.05, cholesterol-fed versus control.

Control guinea pig



Cholesterol-fed guinea pig

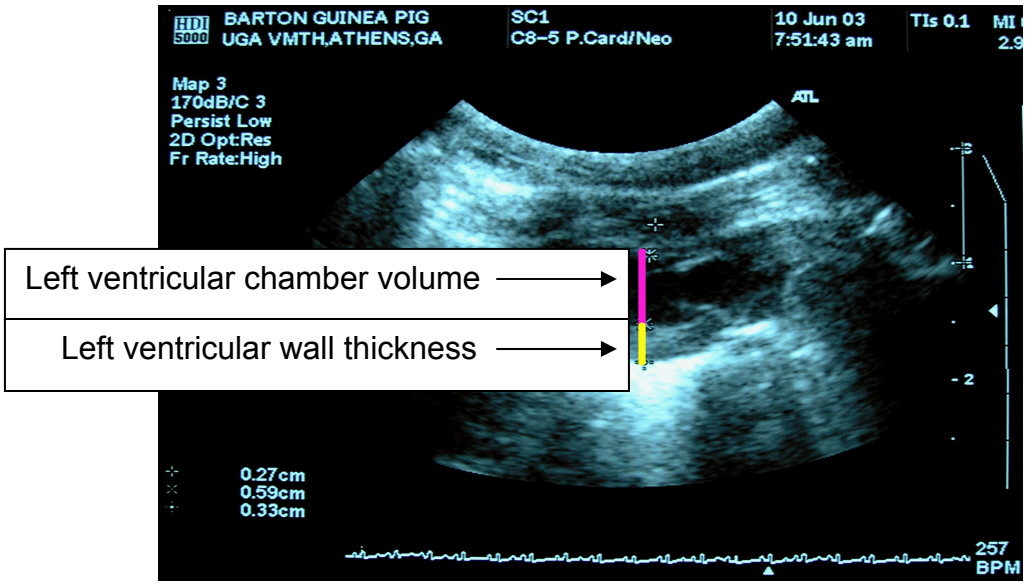


Figure 3.6. Echocardiograms from a control and a cholesterol-fed guinea pig. Darker line = Left ventricular chamber volume. Lighter line = left ventricular wall thickness. **Note** that the left ventricular chamber volume was less in the cholesterol-fed guinea pig whereas the left ventricular wall thickness was similar in the control and cholesterol-fed guinea pigs.

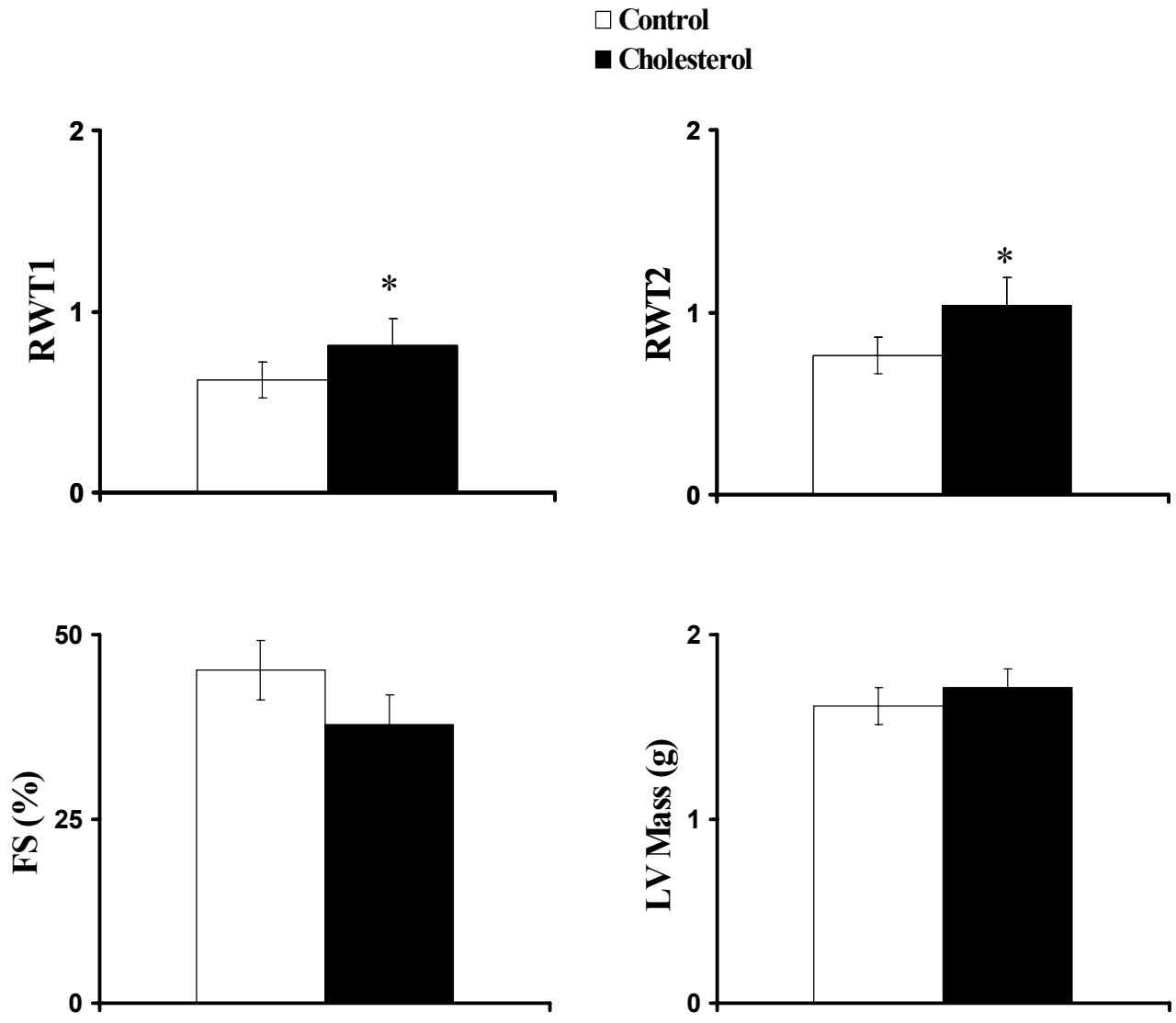


Figure 3.7. A summary of the changes in relative ventricular wall thickness (RWT1 and RWT2), fractional shortening (FS), and left ventricular (LV) mass in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *P < 0.05, cholesterol-fed versus control.

Figure 3.8. Illustration of cardiac gross morphology in a hypercholesterolemic guinea pig (left) and a control guinea pig (right). **Note** that the ventricular chamber in the heart from the cholesterol-fed guinea pig was substantially smaller than that form the control guinea pig.

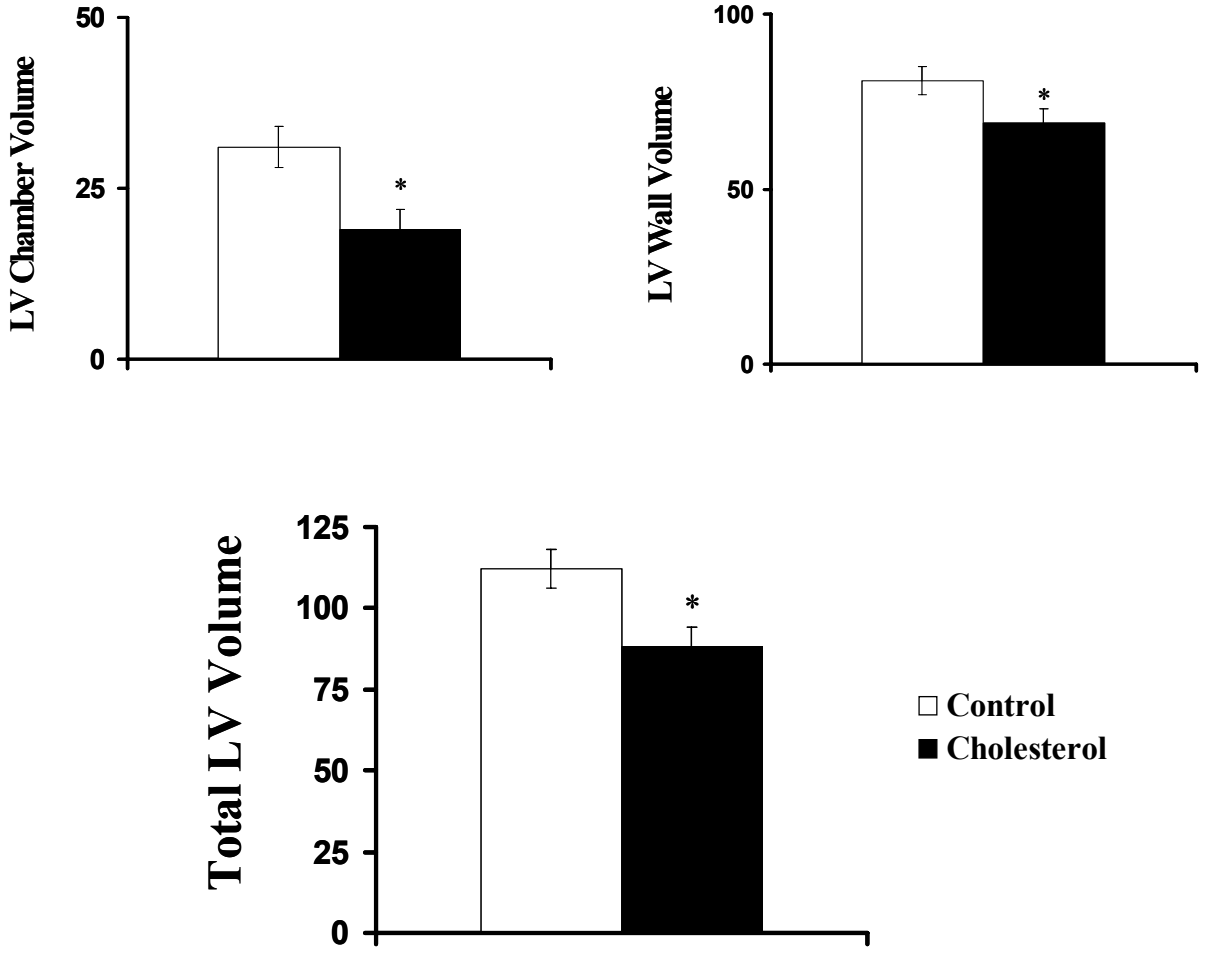


Figure 3.9. A summary of the changes in left ventricular chamber volume, left ventricular wall volume and total left ventricular volume in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. * $P < 0.05$, cholesterol versus control.

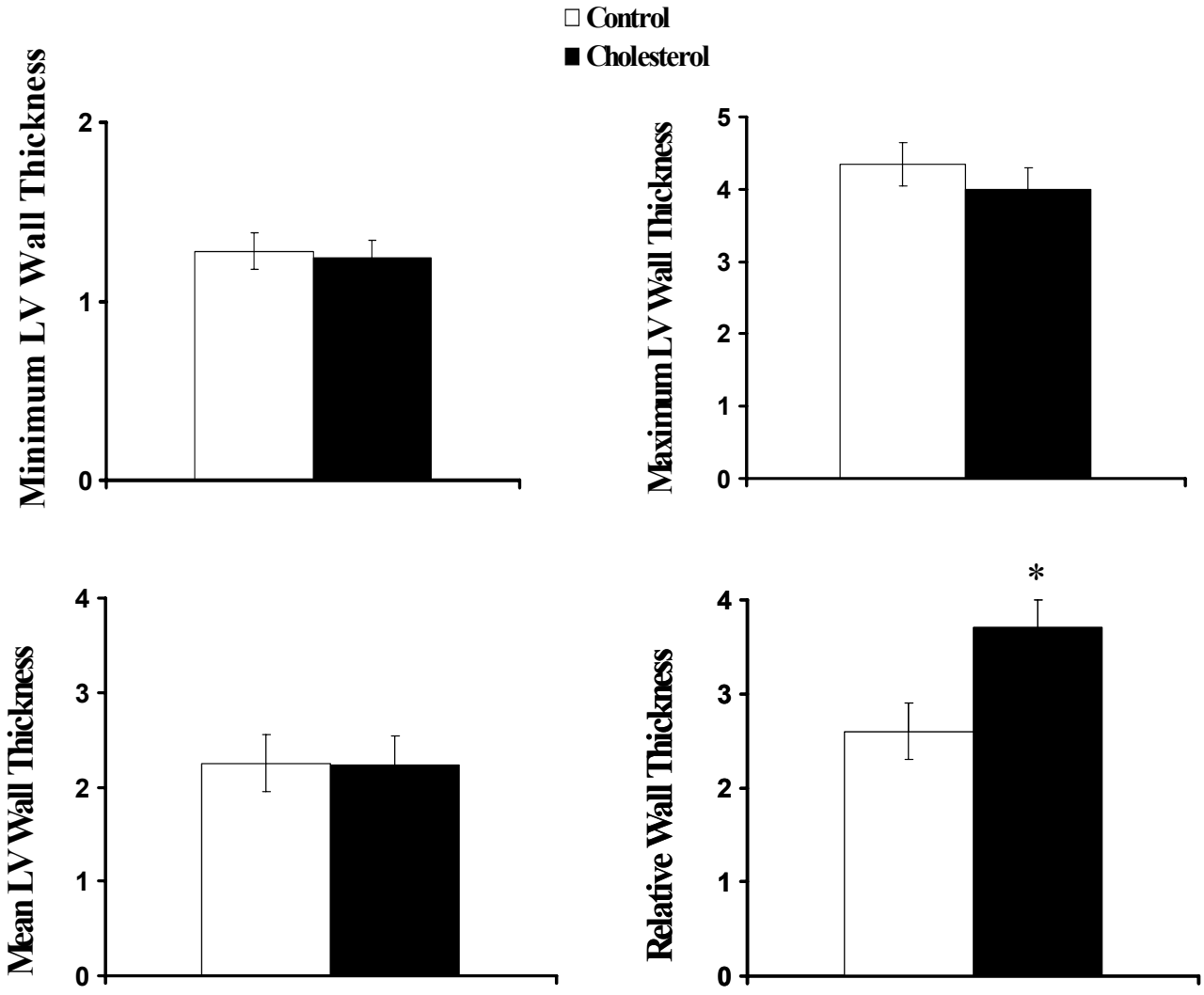


Figure 3.10. A summary of the changes in minimum, maximum, mean and relative left ventricular wall thickness in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

CHAPTER 4

EFFECTS OF HIGH CHOLESTEROL DIET ON BARORECEPTOR REFLEX FUNCTION IN CONSCIOUS GUINEA PIGS[1]

[1]Owen JR, Barton MH, Munday JS, Graves JE, Lewis SJ. To be submitted to *American Journal of Physiology*.

ABSTRACT

The objective of this study was to evaluate baroreceptor heart rate reflex function in conscious control and cholesterol-fed (1% cholesterol for 13 weeks) guinea pigs. The cholesterol-fed animals developed concentric cardiac remodeling although they were normotensive and devoid of classic signs of atherosclerosis. Baroreflex-mediated changes in heart rate were obtained in response to bolus intravenous injections of the pressor agent, phenylephrine, which is an α_1-adrenoceptor agonist, and injections of the depressor agent, sodium nitroprusside, which is a nitric oxide donor. The data were subjected to exponential curve fitting analyses to yield baroreflex parameters. Cholesterol-fed guinea pigs had elevated plasma total cholesterol levels in comparison to controls (1.54 ± 0.12 mM versus 5.40 ± 0.60 mM, $P < 0.05$). Baroreceptor reflex parameters were substantially affected in the cholesterol-fed guinea pigs. The sensitivity (Gain) of the baroreflex was markedly reduced in cholesterol-fed as opposed to control animals (2.63 ± 0.14 versus 4.08 ± 0.20 beats/min per mmHg, $P < 0.05$). The range of the heart rate response (upper minus lower plateau values) was also substantially diminished in the cholesterol-fed as compared to the control animals (69 ± 6 versus 178 ± 11 beats/min, $P < 0.05$). In conclusion, the sensitivity and range of the baroreflex was substantially diminished in cholesterol-fed guinea pigs. These findings tentatively suggest that high dietary consumption of cholesterol disrupts signal transduction mechanisms associated with autonomic control of cardiovascular function.

Key words: autonomic nervous system, baroreceptor heart rate reflex, cholesterol, cardiac concentric remodeling

INTRODUCTION

The sympathetic and parasympathetic nervous systems play an essential role in regulating vascular tone and cardiac performance (Spyer, 1981; Brody et al., 1991; Barres et al., 1992). The baroreceptor reflex plays a vital role in maintaining moment to moment firing of these autonomic nerves and regulates the pattern of sympathetic and parasympathetic discharge to the heart and blood vessels (see Spyer, 1981; Brody et al., 1991; Barres et al., 1992). The loss of baroreceptor reflex function results in elevations in the level and lability of arterial blood pressure, and directly contributes to cardiac remodeling (see Barres et al., 1992). Although the cholesterol-fed guinea pigs have normal mean arterial blood pressures and heart rates, a change in the pattern and efficacy of sympathetic and perhaps parasympathetic drive may play a vital role in the expression of cardiac remodeling observed in these guinea pigs. Moreover, there is compelling evidence that hypercholesterolemia disrupts baroreceptor reflex function via impairment of baroreflex sensitivities in humans and animals (Piccirillo et al., 2001; Wronski et al., 2002). More specifically, these studies provide evidence that hypercholesterolemia impaired the ability of baroreceptor afferents to respond to fluctuations in arterial blood pressure (Piccirillo et al., 2001; Wronski et al., 2002).

It is essential to determine baroreceptor reflex function in cholesterol-fed guinea pigs to obtain a more comprehensive understanding about the mechanisms by which hypercholesterolemia affects cardiovascular performance. As such, the specific aims of this study were to determine baroreceptor reflex-mediated changes in heart rate in conscious male guinea pigs fed control or 1% cholesterol diet for 13 weeks, and to evaluate the relative changes in parasympathetic and sympathetic effector mechanisms.

METHODS

Guinea Pigs and Surgical Procedures

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All experiments were carried out in accordance with protocols approved by the University of Georgia Institutional Animal Care and Use Committee. Male Hartley guinea pigs weighing 400-450 g were fed control or 1% cholesterol diets (Research Diets, Inc.) for 13 weeks. During week 12 of the feeding regimen, guinea pigs were surgically prepared to inject drugs and measure hemodynamic parameters. The guinea pigs were anesthetized with a mixture of ketamine (120 mg/kg, i.p.) and acepromazine (12 mg/kg, i.p.). A catheter (PE-50) was implanted into a carotid artery to continuously record mean, pulsatile and diastolic arterial blood pressure, and heart rate. Another catheter (PE-50) was implanted into a jugular vein to inject drugs. The catheters were then tunneled subcutaneously and exteriorized between the scapulae, and the wounds were closed. Standard aseptic techniques were used throughout the entire surgical procedure, followed by a prophylactic triple antibiotic cream, which was applied to the closed wounds. Daily flushing with heparinized saline was used to keep catheters patent.

Protocols

After 13 weeks of the dietary regimen, arterial catheters were connected to an ADI instruments PowerLab-coupled pressure transducer data acquisition and analysis system. Systolic, diastolic, pulse and mean arterial blood pressures and heart rates were continuously recorded and displayed. Control (n=8) and cholesterol-fed (n=8) guinea pigs received bolus injections of the nitric oxide-donor, sodium nitroprusside (1, 2.5, 5, 10, 20 and 48 μg/kg iv), and the maximal

decreases in mean arterial blood pressure and the associated maximal increases in heart rate were recorded. These guinea pigs also received bolus injections of the selective α_1-adrenoceptor agonist, phenylephrine (1, 2.5, 5, 10, 20 and 40 μg/kg iv), and the maximal increases in mean arterial blood pressure and the associated maximal decreases in heart rate were recorded.

Baroreceptor Reflex Analysis

Data collected from the LabView data acquisition system was formatted into individual files that were analyzed by a baroreflex analysis program. Baroreceptor reflex-mediated changes in heart rate in response to changes in mean arterial blood pressure (**BP**) were determined by exponential sigmoidal curve fitting analyses (Head and McCarty, 1987). Non-linear regression using least square techniques were used to obtain the maximum likelihood estimates of parameter values as described in detail previously (Verberne et al., 1987, 1988; Lewis et al., 1989, 1999). Mean arterial blood pressure (**BP**) and heart rate (**HR**) were related by the formulae:

$$\mathbf{HR} = P_1 + \text{Range}/(1+e^{A(\text{MAP-BP}_{50})}$$

$$\mathbf{HR} = P_2$$

$$\boldsymbol{A} = -4.56 \text{ x } (G_{ave}/\text{Range})$$

$$\boldsymbol{T}_U = \text{BP}_{50} - 1.317 \text{ x } (\text{Range}/(4.56 \times G_{ave}))$$

$$\boldsymbol{T}_L = \text{BP}_{50} + 1.317 \text{ x } (\text{Range}/4.56 \times G_{ave})$$

As can be seen in **Figure 4.1,** the principal baroreceptor reflex parameters were derived from sigmoidal curve-fitting analyses. These baroreflex parameters included, average gain/sensitivity

of baroreflex (Gave), mean arterial blood pressure (MAP) at mid-point of barocurve (BP_{50}), MAP at maximal activation of baroafferents (T_U), MAP at unloading of baroafferents (T_L), maximal heart rate (P_U), minimal heart rate (P_L), and range of heart rate responses (Range). In addition, correlation coefficients, which indicate how well the calculated curves approximate the data, were provided by the analysis programs.

Chemicals

Ketamine HCl and acepromazine maleate were obtained from Fort Dodge Animal Health (Fort Dodge, Iowa, USA). All other reagents were obtained from Sigma-Aldrich Chemical Company, Inc. (St. Louis, MO, USA). All drugs were dissolved and injected in sterile saline.

Statistical Analysis

Data is shown as mean ± SEM, and analyzed by one-way or repeated measures analysis of variance (ANOVA) followed by Bonferroni corrections for multiple comparisons between means using the error mean square terms from the ANOVA design (Winer, 1971; Wallenstein et al., 1990). The single SEM value on each dose-response curve were determined by the formula $SEM = (EMS/n)^{1/2}$, where EMS is the error mean square term from the ANOVA, and n is the number of animals per group. These analyses were done with the use of BMDP and Minitab Statistical packages. The level of $P < 0.05$ was considered statistically significant.

RESULTS

Resting hemodynamic parameters

Resting mean arterial pressure (MAP) and heart rate (HR) values and total plasma cholesterol levels in conscious control and cholesterol-fed guinea pigs are summarized in **Figure 4.2**. As can be seen, resting mean arterial pressure and heart rate values in the cholesterol-fed guinea pigs were similar to control animals, whereas total plasma cholesterol levels were substantially higher in the cholesterol-fed animals. The lability of resting mean arterial blood pressure and heart rate in the cholesterol-fed guinea pigs were also similar to the control guinea pigs ($P > 0.05$ for all comparisons, data not shown).

Effects of Cholesterol on Body Weight

Body weights (BW), heart weights (HW) and heart weight/body weight ratios (HW/BW x 100) of control and cholesterol-fed guinea pigs are summarized in **Figure 4.3**. The cholesterol-fed guinea pigs were approximately 200 grams or 20% lighter than the control guinea pigs. The heart weights of the cholesterol-fed guinea pigs were about 250 mg or 9% lighter than the control guinea pigs. Taken together the heart to body weights ratios were similar in both groups.

Baroreceptor reflex responses elicited by cholesterol

The average gain (sensitivity, linear portion of the curve) and range of the baroreceptor heart rate reflex of the hypercholesterolemic guinea pigs were markedly impaired in comparison to control guinea pigs despite both groups having similar resting mean arterial blood pressure and heart rate values. The average gain (sensitivity) of the baroreflex in control and cholesterol-fed guinea pigs is summarized in **Figure 4.4**. As can be seen, the average gain of the baroreflex was significantly

decreased in cholesterol-fed animals. The maximal and minimal heart rate plateaus and the heart rate range of control and cholesterol-fed guinea pigs are summarized in **Figure 4.5**. As can be seen, the minimal heart rate plateau was higher in cholesterol-fed guinea pigs than control guinea pigs. In addition, the maximal heart rate plateau was lower in cholesterol-fed than in control guinea pigs. Accordingly, the range of the heart rate response was dramatically reduced in the cholesterol fed animals as compared to the controls.

Mean arterial blood pressure (MAP) values at the midpoint of the baroreceptor reflex curve (BP_{50}; i.e., linear portion of the baroreceptor heart rate reflex curve) in control and cholesterol-fed guinea pigs are summarized in **Figure 4.6**. As can be seen, mean arterial blood pressure values at the midpoint of the baroreceptor reflex curve were similar in control and cholesterol-fed guinea pigs.

Mean arterial blood pressure levels at which the baroafferents were fully unloaded (Lower mean arterial blood pressure threshold, T_L) and mean arterial blood pressure levels at which the baroafferents maximally activated (Upper mean arterial blood pressure threshold, T_U) in control and cholesterol-fed guinea pigs are summarized in **Figure 4.7**. As can be seen, mean arterial blood pressure levels at which the baroafferents were fully unloaded were similar in control and cholesterol-fed animals. In addition, mean arterial blood pressure values at which the baroafferents were maximally activated were also similar in control and cholesterol-fed animals.

DISCUSSION

This study found that cholesterol-fed guinea pigs had markedly elevated blood cholesterol levels as compared to the control guinea pigs. The levels of plasma cholesterol in the cholesterol-fed guinea pigs would be considered to be severe hypercholesteremia in animals (Fernandez and McNamara, 1989, 1994; Fernandez et al., 1990a,b, 1995, 1997; Fernandez, 2001) and humans (see Fernandez, 2001). This study also demonstrates that the body weights and heart weights of the cholesterol-fed guinea pigs were less than those of control guinea pigs. Taken together, the heart weight to body weight ratios of the cholesterol-fed guinea pigs were similar to those of control guinea pigs. This is consistent with our previous finding that cardiac remodeling in cholesterol-fed guinea pigs consisted of a concentrically remodeled left ventricle. More specifically, it is evident that cardiac hypertrophy did not develop in the cholesterol-fed guinea pigs.

One principal finding of this study was that resting mean arterial blood pressure and heart rate values in the cholesterol-fed guinea pigs were similar to those of the control guinea pigs. These findings are consistent with the absence of cardiac hypertrophy in which elevated mean arterial blood pressure levels are a major cause (see Fernandez, 2001). The normal levels of resting mean arterial blood pressure and heart rate in cholesterol-fed guinea pigs are remarkable considering the marked impairment of the baroreceptor heart rate reflex in these animals (see below).

The other principal finding of this study was that the hypercholesterolemic guinea pigs displayed a marked impairment in the sensitivity and range of the baroreceptor heart rate reflex. The diminished baroreceptor reflex function in the cholesterol-fed guinea pigs could arise from

changes in afferent function, central processing of the reflex and/or autonomic nerve function including changes in the function of cardiac β_1-adrenoceptors and cholinergic muscarinic receptors. The tachycardia in response to a depressor response is due almost equally to the withdrawal of vagal drive and the activation of sympathetic drive (see Barres et al., 1992). In addition, the bradycardia in response to a pressor response is due almost equally to the activation of vagal drive and the withdrawal of sympathetic drive (see Barres et al., 1992). The finding that the baroreceptor reflex-mediated increases and decreases in heart rate were markedly diminished in the cholesterol-fed guinea pigs argues that baroafferents are impaired in these animals although it is certainly possible that efferent-mediated changes in heart rate are primarily responsible for the diminished baroreflex activity (see below).

Elevations in the level and lability of arterial blood pressure are indicative of diminished baroreceptor reflex function (see Barres et al., 1992). In view of our evidence that mean arterial blood pressure and heart rate were not labile in the cholesterol-fed guinea pigs, it could be argued that a loss of afferent and/or central processing of the baroreceptor reflex may not be involved in the diminished baroreceptor reflex activity in cholesterol-fed guinea pigs. More specifically, the loss of heart rate response in the cholesterol-fed guinea pigs may be due to impaired vagal/sympathetic effector mechanisms including (1) reduced release of acetylcholine (from post-ganglionic vagal efferents) and norepinephrine (from post-ganglionic sympathetic efferents), and/or (2) down-regulation of cardiac muscarinic receptors and β-adrenoceptors.

Additional studies to be described later in this dissertation provide evidence that afferent/central processing of the baroreceptor reflex was markedly impaired in anesthetized cholesterol-fed

guinea pigs. Moreover, other studies to be described in this dissertation provide compelling evidence that vagal efferent mechanisms (including the status of cholinergic muscarinic receptors) are not down-regulated whereas sympathetic efferent mechanisms (and especially β_1-adrenoceptor function) are markedly down-regulated in cholesterol-fed guinea pigs.

SUMMARY

The present study provides clear evidence that the baroreceptor heart rate reflex is markedly impaired in cholesterol-fed guinea pigs. Despite this marked impairment of baroreceptor reflex function, the cholesterol-fed guinea pigs were normotensive and did not display elevated lability of arterial blood pressure. Although definitive studies are lacking at present, we speculate that the diminished baroreflex activity may contribute to the development of concentric cardiac remodeling in the cholesterol-fed guinea pigs. Later sections in this dissertation will detail the findings of mechanistic studies regarding the status of $G_{o,q}$ protein-coupled cholinergic muscarinic receptors and G_s protein-coupled β-adrenoceptors, in cardiac pacemaker cells (sinoaortic and atrioventricular nodal cells and Purkinje fibres) (Spyer, 1981; Brody et al., 1991).

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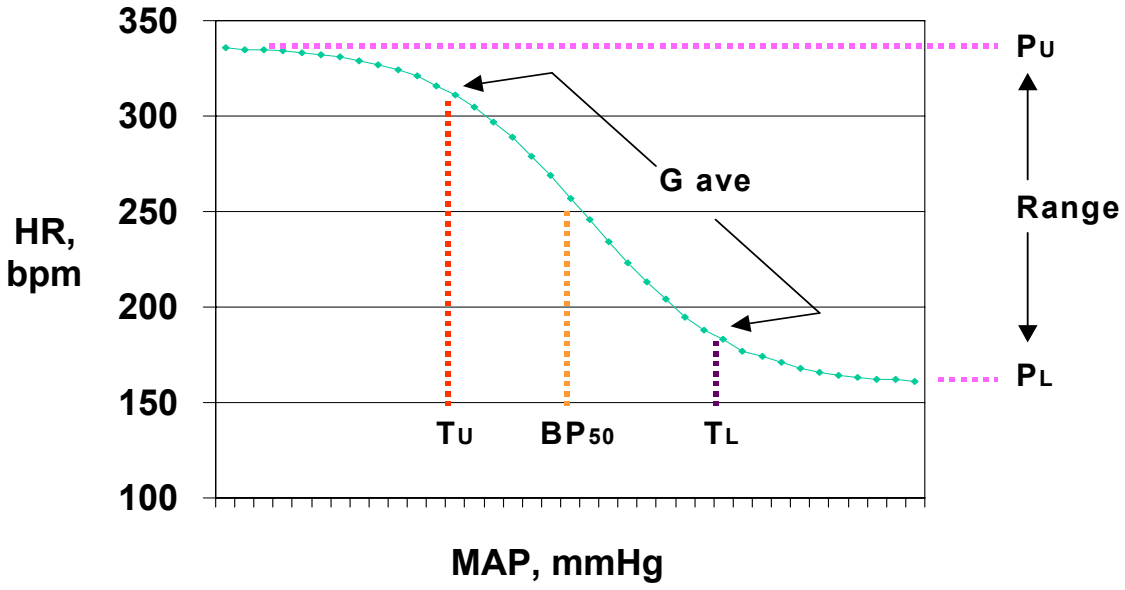

Gave = Average gain/sensitivity of baroreflex

BP_{50} = Map at mid-point of barocurve

T_U = MAP at maximal activation of baroafferents

T_L = MAP at unloading of baroafferents

P_U = Maximal heart rate

P_L = Minimal heart rate

Range = Range of heart rate responses

Figure 4.1. Baroreceptor reflex parameters arising from sigmoidal curve-fitting analyses.

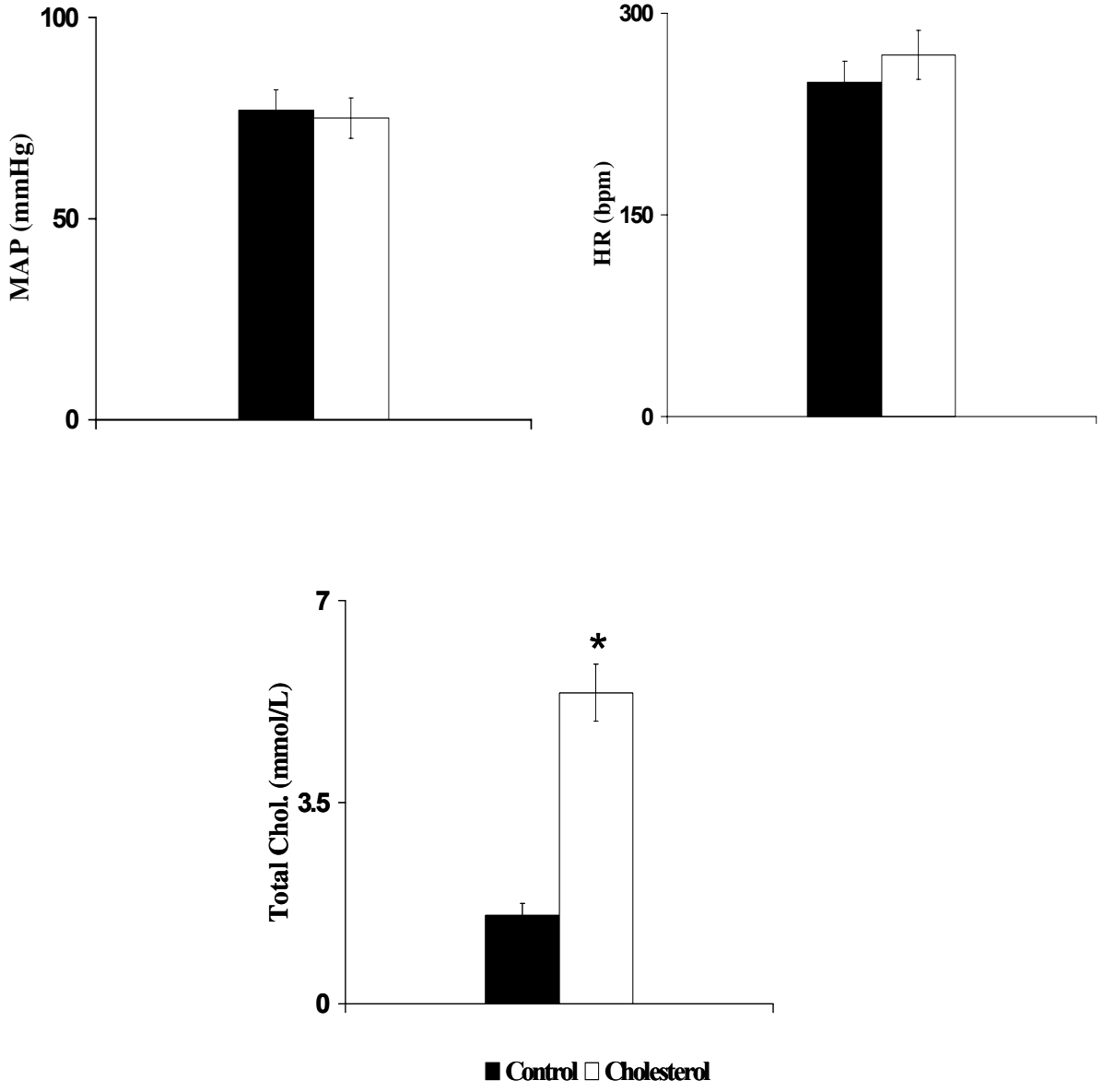

Figure 4.2. Resting mean arterial pressure (MAP), heart rate (HR) and total plasma cholesterol levels in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

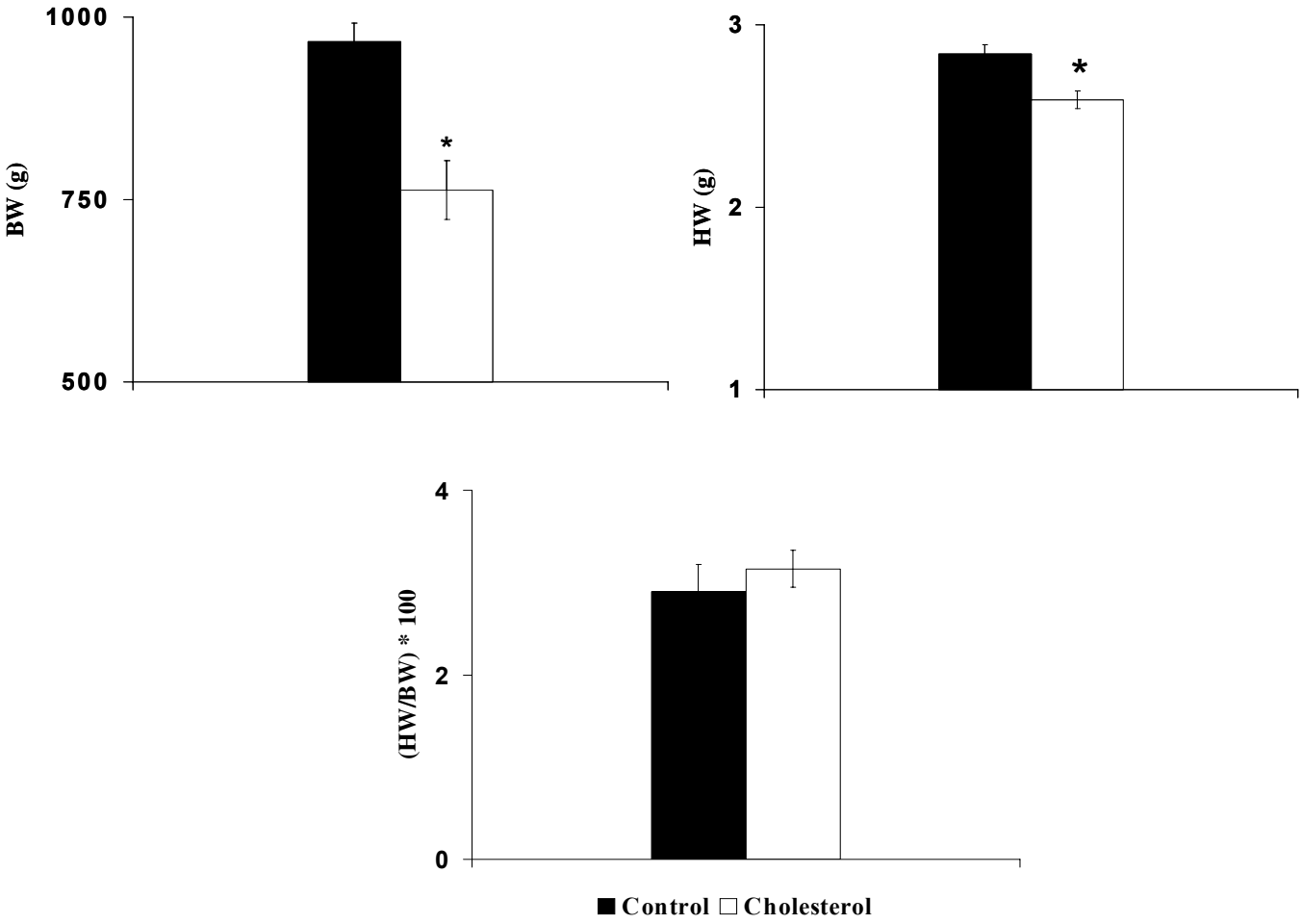

Figure 4.3. Body weights (BW), heart weights (HW) and heart weight/body weight ratios (HW/BW x 100) in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

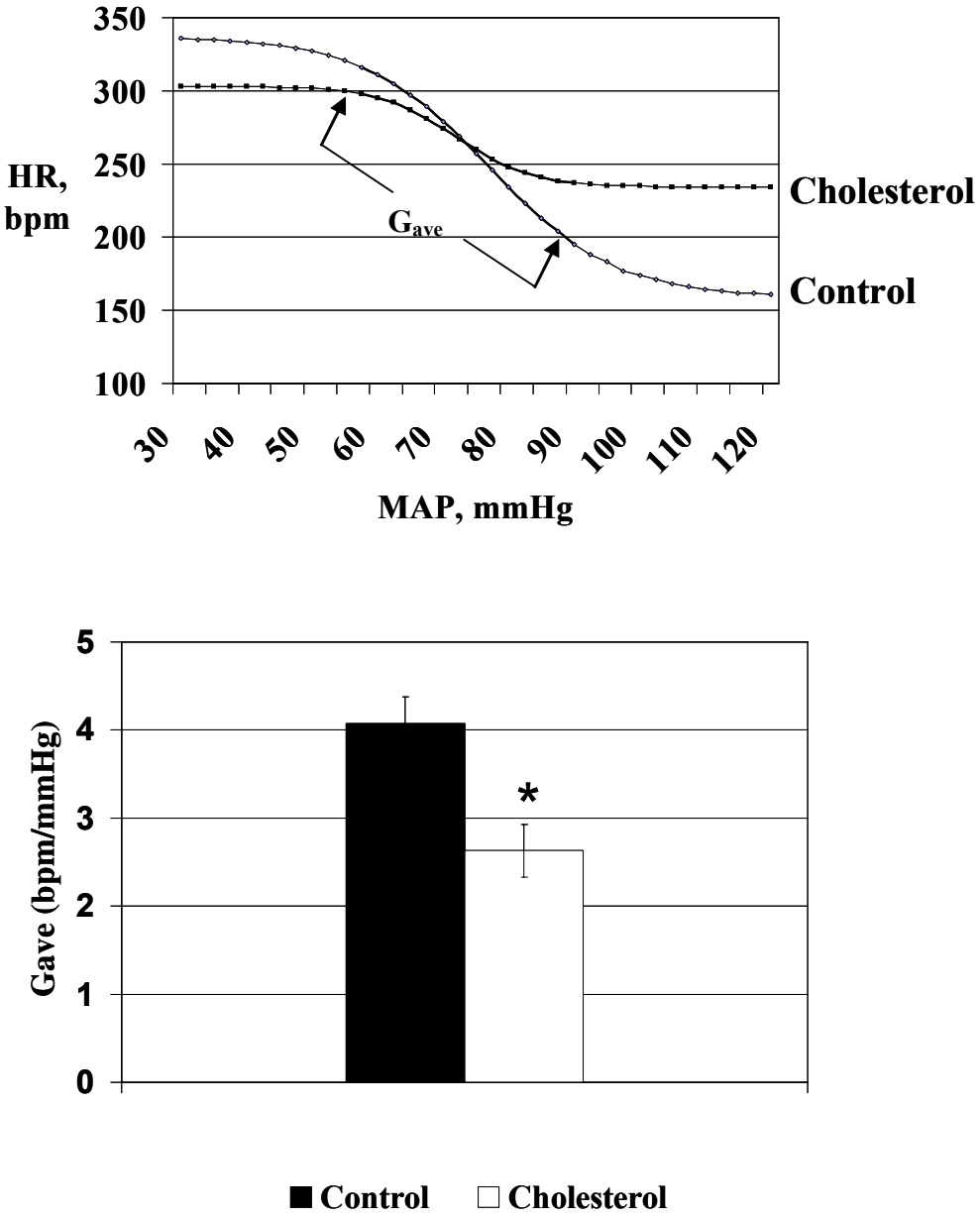

Figure 4.4. Average gain/sensitivity of baroreflex (G_{ave}) in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are mean ± SEM. *$P < 0.05$, cholesterol versus control.

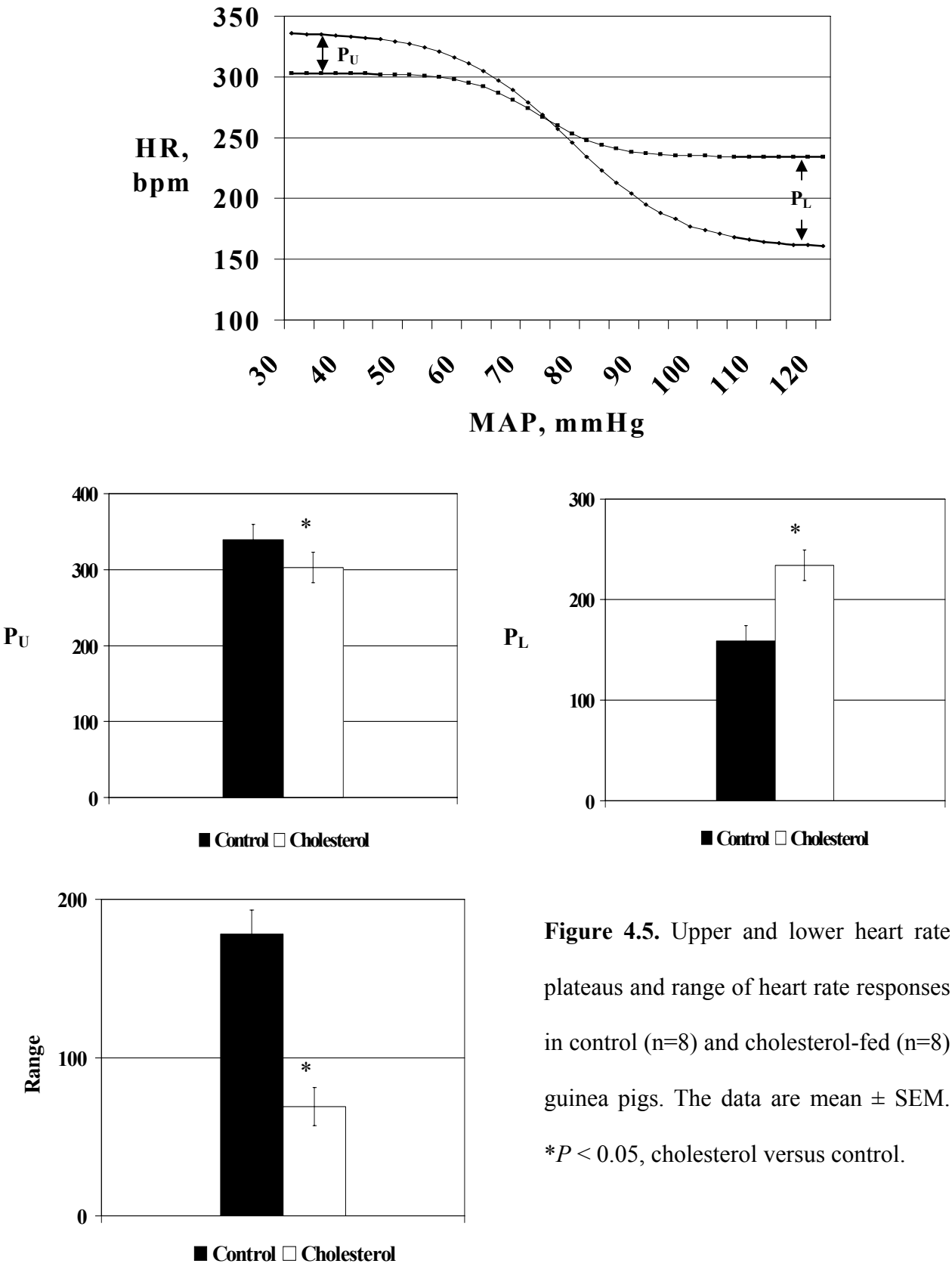

Figure 4.5. Upper and lower heart rate plateaus and range of heart rate responses in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are mean ± SEM. **P* < 0.05, cholesterol versus control.

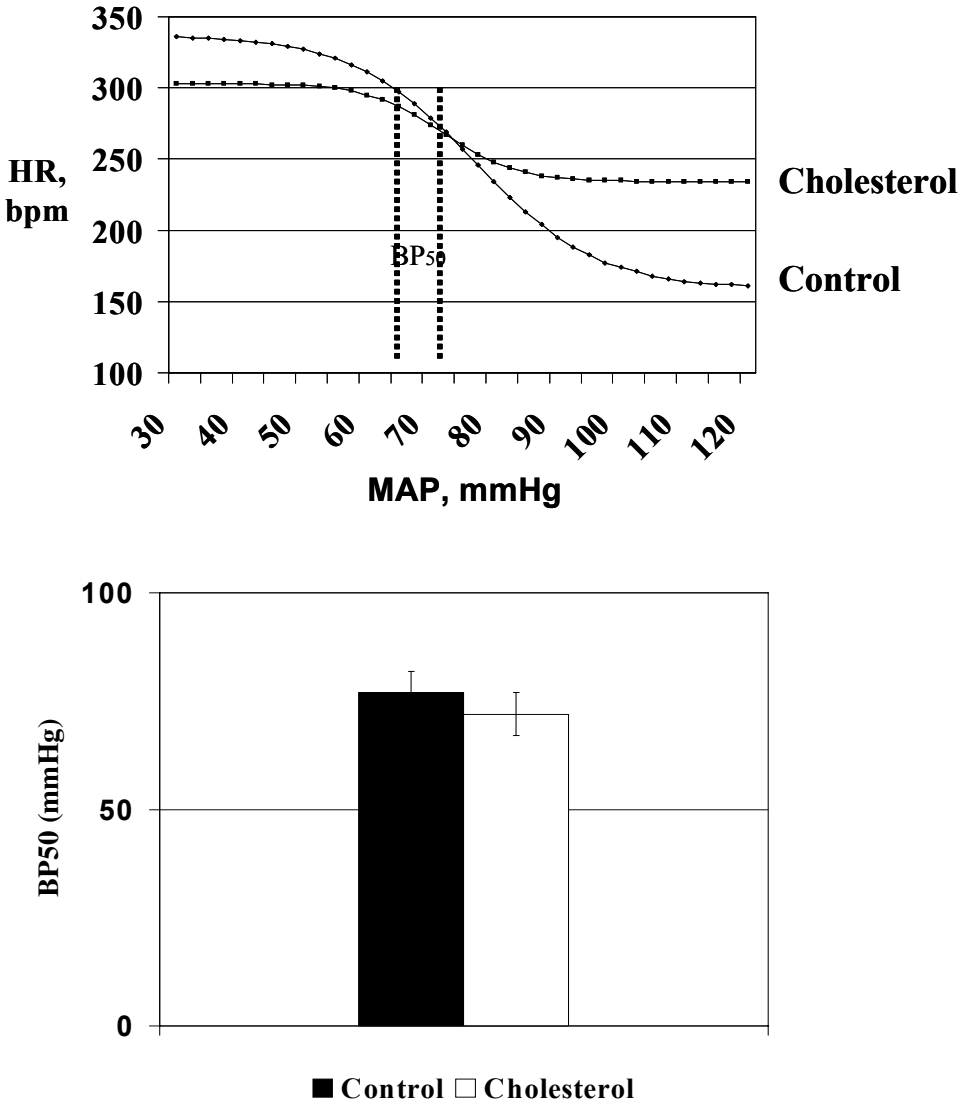

Figure 4.6. Mean arterial blood pressure at midpoint of barocurve (BP_{50}) in control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are mean ± SEM. There were no differences in BP_{50} values of control and cholesterol-fed guinea pigs at the $P < 0.05$ level.

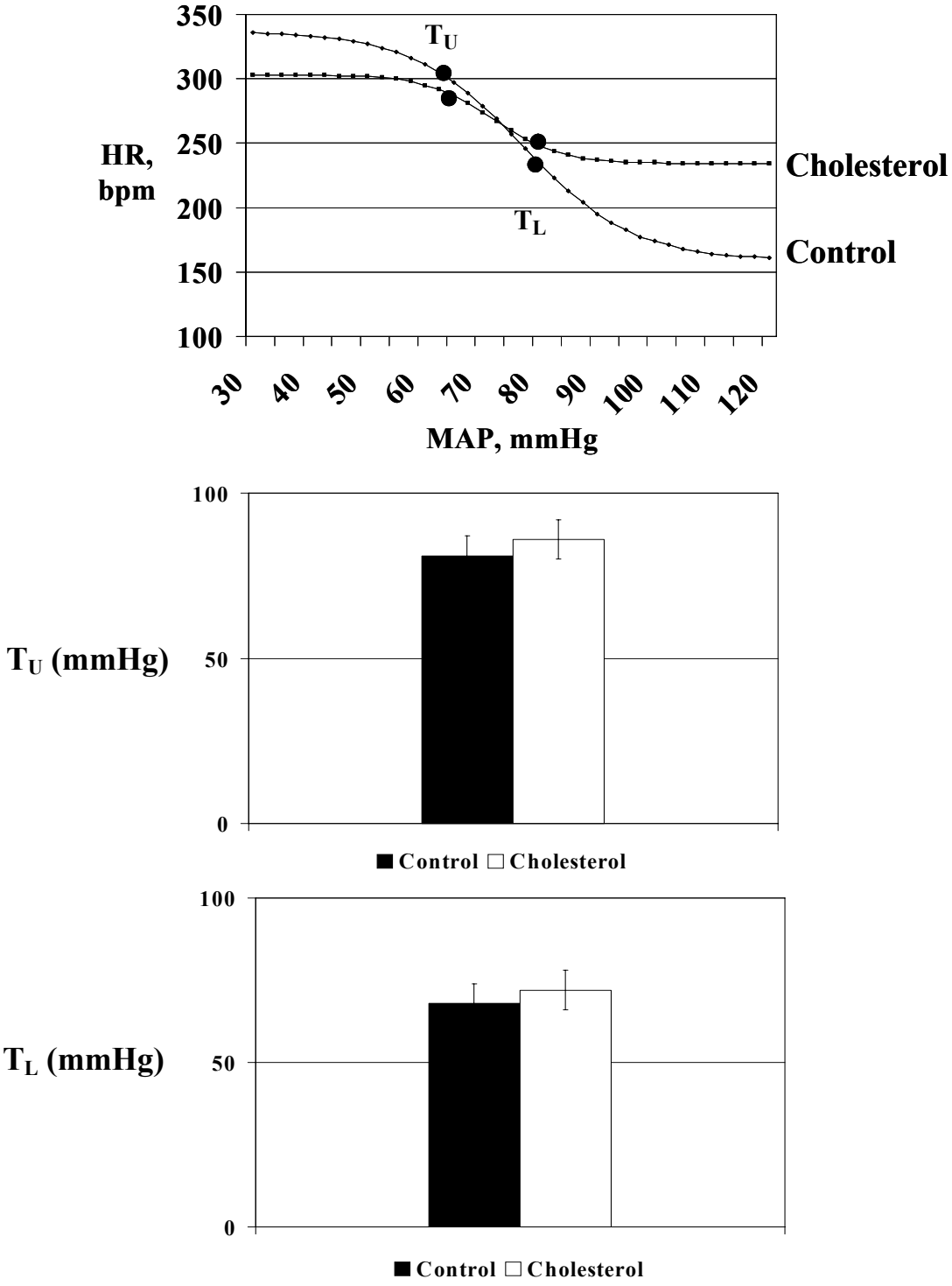

Figure 4.7. Upper (T_U) and lower (T_L) blood pressure thresholds in control (n=8) and cholesterol-fed (n=8) guinea pigs. Data are mean ± SEM. There were no differences in T_U or T_L between control and cholesterol-fed animals ($P > 0.05$ for both comparisons).

CHAPTER 5

MECHANISMS INVOLVED IN CHOLESTEROL-INDUCED DISRUPTION OF CARDIOVASCULAR FUNCTION[1]

[1]Owen JR, Barton MH, Munday JS, Graves JE, Lewis SJ. To be submitted to *American Journal of Physiology*.

ABSTRACT

We found that male Hartley guinea pigs fed a high cholesterol (1%) diet for 13 weeks developed cardiac concentric remodeling although they showed no signs of atherosclerosis. Although the cholesterol-fed guinea pigs were normotensive, they had markedly reduced baroreceptor reflex function. The loss of baroreflex function could involve a multiplicity of mechanisms including a loss of central processing of the afferent information. The aim of this study was to perform a series of mechanistic studies to address the possible mechanisms involved in the apparent loss of baroreflex function in cholesterol-fed guinea pigs. The first study involved direct electrical stimulation of baroafferent fibers in the aortic depressor nerve and direct electrical stimulation of the efferent vagus. The main findings were that (1) electrical stimulation of the aortic depressor nerve elicited frequency-dependent reductions in heart rate and mean arterial blood pressure in anesthetized control guinea pigs that were due to vagal afferent-mediated activation of cardiac muscarinic receptors, (2) electrical stimulation of the aortic depressor nerve elicited markedly smaller responses in cholesterol-fed as compared to control guinea pigs, (3) electrical stimulation of the efferent vagus elicited robust responses in control and in cholesterol-fed guinea pigs. Taken together, these findings suggest that the central processing of baroafferent input is altered in cholesterol-fed guinea pigs. The second study established that the increases in heart rate and cardiac contractility elicited by electrical stimulation of the stellate ganglion were markedly diminished in cholesterol-fed guinea pigs. Subsequent pharmacological experiments provided evidence that the loss of response to neurogenically-derived catecholamines was due to a down-regulation of cardiac β-adrenoceptor signaling rather than disturbances in intracellular signaling cascades elicited by activation of Gs protein-coupled receptors. Finally, our pharmacological studies established that intrinsic heart rate (resting heart rate in the absence of sympathetic and

parasympathetic input was substantially elevated in cholesterol-fed guinea pigs. This suggests that a high cholesterol diet alters the intrinsic activity of pacemaker cells by as yet undetermined mechanisms.

Key words: autonomic nervous system, baroafferents, intrinsic heart rate

INTRODUCTION

Hypercholesterolemia disrupts baroreceptor reflex function in humans and animals (Piccirillo et al., 2001; Wronski et al., 2002). Our initial studies demonstrated that the sensitivity and range of the baroreflex were attenuated in cholesterol-fed guinea pigs exhibiting cardiac concentric remodeling. Alterations in baroreflex function are prognostic of cardiac (Minami and Head, 1993; Lantelme et al., 1998; De Ferrari et al., 1992) and vascular abnormalties (Lantelme et al., 1994). Moreover, dogs that develop ventricular fibrillation after myocardial infarction have a lower baroreceptor reflex sensitivity compared to their healthy counterparts (De Ferrari et al., 1992). Hypercholesterolemia alters baroreflex function in aging individuals, which may be attributed to circulating levels of LDL cholesterol eliciting endothelial dysfunction. Specifically, the loss of endothelium-derived relaxing factors in arteries supplied by baroreceptor afferent nerve terminals may be less responsive to changes in arterial blood pressure (Piccirillo et al., 2001). Similarly, Lantelme et al (1994) reported that hypertensive patients with no signs of cardiac hypertrophy displayed dysfunctional baroreflex activity which was vascular in origin.

The baroreceptor reflex regulates the moment to moment status of the cardiovascular system (Abboud et al., 1976; Spyer, 1981; Machado et al., 1994). Baroreceptor afferents innervate the aortic arch via the aortic depressor nerves and carotid sinus nerves. Baroafferents innervating the aortic arch have their cell bodies in the nodose ganglia and the central terminals are located in the nucleus tractus solitaii (NTS) (Abboud et al., 1976; Spyer, 1981). Baroafferents innervating the carotid sinus have their cell bodies in the petrosal ganglia and the central terminals are also located in the NTS (Spyer, 1981). The baroreceptor reflex controls hemodynamic status mainly by changing the activity of the sympathetic nerves innervating the heart (pacemaker cells and

cardiac muscle) and vasculature, and parasympathetic nerves innervating the heart (pacemaker cells). Under sustained hypotension, the baroreflex also elicits the release of angiotensin II from juxtaglomerular cells in the kidney and catecholamines from adrenal medulla cells (Abboud et al., 1976; Spyer, 1981). Post-ganglionic sympathetic nerves elicit their cardiovascular effects mainly by activating α-adrenoceptors (principally vascular smooth muscle) and β-adrenoceptors (heart and vascular smooth muscle) (Abboud et al., 1976; Spyer, 1981).

Hypercholesterolemia may affect baroreflex activity by altering (1) signal transduction pathways in afferent terminals, (2) the distensibility of the aortic arch/carotid sinus, (3) central processing of afferent input, and/or (4) signal transduction pathways in cardiac pacemaker and muscle cells. Cholinergic muscarinic receptors and α- and β-adrenoceptors are G-protein coupled receptors that are localized in cholesterol-rich lipid rafts in cardiac pacemaker cells and myocytes (Insel et al., 2005). As such, increased blood cholesterol levels may affect vagal/sympathetic responses by disrupting muscarinic and/or β-adrenoceptor function.

Elucidation of the mechanisms involved in cholesterol-induced baroreflex dysfunction may yield vital data which will form the basis of studies designed to develop novel therapeutic strategies to ameliorate the cardiovascular abnormalities arising from hypercholesterolemia. The **aim** of this study was to address some of the mechanisms that may be responsible for the apparent loss of baroreflex function in cholesterol-treated guinea pigs. In these studies, control and cholesterol-fed guinea pigs received episodes of electrical stimulation of an aortic depressor nerve, efferent vagus or stellate ganglion and various pharmacological agents to elucidate the mechanisms involved in the etiology of hypercholesterolemia induced baroreflex dysfunction.

MATERIALS AND METHODS

Guinea Pigs, Anesthesia and Catheterization Procedures

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996 and in accordance with protocols approved by the University of Georgia Institutional Animal Care and Use Committee. Male Hartley guinea pigs (400-450 g) were fed control or 1% cholesterol diets (Research Diets, Inc.) for 13 weeks. For studies in conscious guinea pigs, the animals were anesthetized with a mixture of ketamine (120 mg/kg, ip) and acepromazine (12 mg/kg, ip) at week 12. A catheter (PE-50) was implanted into the left carotid artery to continuously record mean, pulsatile and diastolic arterial blood pressure, and heart rate. Another catheter (PE-50) was implanted into left jugular vein to inject drugs. In some guinea pigs another catheter (PE-50) was also placed into the left ventricle via the left carotid artery to measure the rate of left ventricular pressure development (dP/dT). The catheters were then tunneled subcutaneously and exteriorized between the scapulae, and the wounds were closed. Standard aseptic techniques were used throughout the entire surgical procedure, followed by a prophylactic triple antibiotic cream, which was applied to the closed wounds. Daily flushing with heparinized saline was used to keep catheters patent. These animals were tested 1 week later (i.e., at 13 weeks of treatment.).

For studies in anesthetized animals, the guinea pigs were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) at week 13 of control or cholesterol feeding. The catheters described above were implanted and one of the following surgeries described on the next page was also performed. On the day of the experiment, the arterial and intra-cardiac catheters were connected to a LabView data acquisition system to continuously monitor cardiovascular parameters.

Electrical Stimulation of the Aortic Depressor Nerve in Anesthetized Guinea Pigs

A midline incision was made on the ventral surface of the neck and the left omohyoid muscle was cut and reflected to expose the left aortic depressor nerve. A 4-6 mm length of the nerve, beginning from its point of emergence from the superior laryngeal nerve, was isolated under a dissecting microscope. The nerve was placed on a bipolar platinum electrode with an inter-electrode distance of 1 mm. The nerve and electrode were raised above the surrounding tissue and bathed in warm mineral oil. Square-wave electrical stimuli (8V, 0.5 msec) were applied to the ADN at 1, 2.5, 5, and 10 Hz for 15 sec. The electrical stimuli were delivered by a Grass S-4 stimulator through an isolation unit. The individual stimuli were given 5-10 min apart in order to allow the responses elicited by a stimulus to subside completely before another was given. These methods were based on those used by our laboratory in anesthetized rats (see Ohta et al., 1992; Possas and Lewis, 1997).

Electrical Stimulation of the Efferent Vagus in Anesthetized Guinea Pigs

The left cervical vagus was isolated and cut, and a bipolar platinum electrode was placed on the peripheral end of the vagus projecting to the heart (Lewis et al., 1989). The vagus and electrodes were immersed in mineral oil. The responses elicited by electrical stimulation of the efferent vagus (1-10 Hz, 10 V, 10 ms for 10 sec) were determined.

Electrical Stimulation of the Stellate Ganglion in Anesthetized Guinea Pigs

The rats were placed on a small animal ventilator (Harvard) and a careful incision was made between the second and third ribs to isolate the left stellate ganglion. The ventilator ensured proper respiration in case the pleural membrane was damaged. The ganglion (usually exterior to

the inner pleural membrane) was isolated and all connections other than the trunk projecting to the heart were surgically transected. A bipolar platinum electrode was placed on the ganglion and the ganglion and electrodes were immersed in mineral oil. Any damaged membranes were sutured in order to seal the pleural cavity and the guinea pigs were weaned off the respirator. The responses elicited by direct electrical stimulation of the stellate ganglion (1-10 Hz, 10 V, 10 ms for 10 sec) were determined before and after various drug treatments.

Protocol 1 – Aortic Depressor Nerve Experiments

There were 8 guinea pigs in each of the groups in all protocols. The reductions in heart rate and mean arterial blood pressure elicited by episodes of electrical stimulation of the aortic depressor nerve (1-10 Hz, 8V, 0.5 msec for 15 sec) were determined in control and cholesterol-fed guinea pigs before and beginning 15 min after administration of saline or the selective β_1-adrenoceptor antagonist, atenolol (1 mg/kg, iv). The reductions in heart rate and mean arterial blood pressure elicited by episodes of electrical stimulation of the aortic depressor nerve (1-10 Hz, 8V, 0.5 msec for 15 sec) were determined in control and cholesterol-fed guinea pigs before and beginning 15 min after administration of the muscarinic receptor antagonist, methyl-atropine (1 mg/kg, iv). The individual electrical stimuli were given 5-10 min apart to allow the responses elicited by a stimulus to subside completely before another was given.

Protocol 2 - Efferent Vagus Experiments

The reductions in heart rate and mean arterial blood pressure elicited by episodes of electrical stimulation of the efferent vagus (1-10 Hz for 10 sec) were determined in control and in cholesterol-fed guinea pigs. The individual electrical stimuli were given as in Protocol 1.

Protocol 3 - Stellate Ganglion Experiments

The increases in heart rate, rate of left ventricular pressure development (dP/dT) and mean arterial blood pressure elicited by brief episodes of direct electrical stimulation of the stellate ganglion (1-10 Hz for 10 sec) were determined before and after a bolus injection of the selective β_1-adrenoceptor antagonist, atenolol (1 mg/kg, iv). The individual electrical stimuli were given 5-10 min apart in order to allow the responses elicited by a stimulus to subside completely before another was given.

Protocol 4 – Isoproterenol and 8-CPT-cAMP Experiments

The increases in heart rate, rate of left ventricular pressure development (dP/dT), and mean arterial blood pressure elicited by injections of the β_1-, β_2-, and β_3-adrenoceptor agonist, isoproterenol (0.05-0.5 μg/kg, iv) (see Whalen and Lewis, 1999; Whalen et al., 2000), were determined in conscious control and cholesterol-fed guinea pigs. The changes in heart rate, rate of left ventricular pressure development (dP/dT), and mean arterial blood pressure elicited by bolus injections of the direct activator of cAMP-dependent kinase, 8-CPT-cAMP (5-20 μmol/kg, iv) (Whalen et al., 1999), were determined in conscious control and cholesterol-fed guinea pigs. The injections of isoproterenol were given 5-10 min apart in order to allow the responses elicited by each injection to subside completely before another was given. The injections of 8-CPT-cAMP were given 20-30 min apart in order to allow the responses elicited by each injection to subside completely before another was given.

Protocol 5 – Intrinsic Heart Rate

Resting heart rate and mean arterial blood pressure values were determined before and after the

administration of the muscarinic receptor antagonist, methyl-atropine (1 mg/kg, iv) in control and cholesterol-fed guinea pigs. Resting parameters were again determined after the subsequent administration of the selective β_1-adrenoceptor antagonist, atenolol (1mg/kg, iv). In other control and cholesterol-fed guinea pigs, resting heart rate and mean arterial blood pressure values were determined before and after injection of atenolol (1 mg/kg, iv). These parameters were again determined after the subsequent injection of methyl-atropine (1 mg/kg, iv). In these experiments methyl-atropine and atenolol were given 15 min apart in order to properly determine the changes in baseline cardiovascular parameters.

Drugs

Atenolol, isoproterenol and methyl-atropine and were obtained from Sigma (St. Louis, MO, USA). Ketamine, acepromazine, sterile saline and pentobarbital sodium were obtained from Abbott (U.S.A). All drugs were dissolved and diluted for injection in sterile saline.

Statistics

The data are presented as mean ± S.E.M. and were analyzed by repeated-measures analysis of variance (Winer, 1971) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means using the error mean square terms from the repeated measures analysis of variance (see Wallenstein et al., 1980). A value of $P < 0.05$ was taken to denote statistical significance.

RESULTS

Aortic Depressor Nerve Stimulation in Naïve Anesthetized Guinea Pigs

The aortic depressor nerve was stimulated to evaluate whether central processing of baroafferent information is altered in the cholesterol-fed guinea pigs. The electrical stimulation of the aortic depressor nerve was expected to elicit reductions in heart rate due to (1) activation of the efferent vagus, which reduces pacemaker activity by activating muscarinic receptors, and (2) withdrawal of sympathetic nerve activity and hence diminished release of norepinephrine and activation of β-adrenoceptors on pacemaker cells and cardiac muscle (Spyer, 1981). Prior to aortic depressor nerve stimulation, resting heart rates was similar in control and in cholesterol-fed animals (264 ± 10 versus 270 ± 9 beats/min, respectively, $P > 0.05$). Resting mean arterial blood pressures were also similar in control and cholesterol-fed guinea pigs (74 ± 4 versus 73 ± 3 mmHg, respectively, $P > 0.05$). The reductions in heart rate and mean arterial blood pressure elicited by electrical stimulations of the aortic depressor nerve (1-10 kHz) in the control and cholesterol-fed guinea pigs are summarized in **Figure 5.1**. These stimuli elicited frequency-dependent reductions in heart rate and mean arterial blood pressure in the control and cholesterol-fed guinea-pigs. However, the reductions in heart rate and mean arterial pressure were substantially smaller in the cholesterol-fed guinea pigs.

Aortic Depressor Nerve Stimulation in Anesthetized Atenolol-Treated Guinea Pigs

The objective of this study was to determine the role of sympathetic withdrawal on the responses elicited by aortic depressor nerve stimulation in control and cholesterol-fed guinea pigs. The injection of atenolol (1mg/kg, iv) to the above control guinea pigs lowered resting heart rate from 266 ± 9 to 228 ± 8 beats/min (-38 ± 4 beats/min, $P < 0.05$). Atenolol also lowered resting heart

rate in the above cholesterol-fed animals from 272 ± 9 to 264 ± 7 beats/min (-8 ± 2 beats/min, $P < 0.05$). However, the atenolol-induced decrease in heart rate was substantially smaller in the cholesterol-fed than the control guinea pigs ($P < 0.05$). Atenolol did not affect resting mean arterial blood pressure in control (74 ± 4 versus 74 ± 3 mmHg, pre and post values, respectively, $P > 0.05$) or cholesterol-fed guinea pigs (73 ± 3 versus 72 ± 3 mmHg, pre and post values, respectively, $P > 0.05$). The reductions in heart rate and mean arterial blood pressure elicited by electrical stimulations of the aortic depressor nerve (1-10 kHz) in the atenolol-treated control and cholesterol-fed guinea pigs are summarized in **Figure 5.2**. These electrical stimuli elicited frequency-dependent reductions in heart rate and mean arterial blood pressure in the control and cholesterol-fed guinea-pigs. The reductions in heart rate and mean arterial pressure were substantially smaller in the cholesterol-fed guinea pigs. The responses in the atenolol-treated control and cholesterol-fed guinea pigs were very similar to those in the naïve animals ($P > 0.05$, for all comparisons).

Aortic Depressor Nerve Stimulation in Anesthetized Methyl-Atropine-Treated Guinea Pigs

The objective of this study was to determine the role of vagal efferent (acetylcholine)-induced activation of cardiac muscarinic receptors in the responses elicited by aortic depressor nerve stimulation in control and cholesterol-fed guinea pigs. Injection of methyl-atropine (1mg/kg, iv) in control guinea pigs increased resting heart rate from 262 ± 9 to 289 ± 10 beats/min (+27 ± 3 beats/min, $P < 0.05$). Methyl-atropine raised resting heart rate in cholesterol-fed guinea pigs from 266 ± 9 to 319 ± 10 beats/min (+53 ± 6 beats/min, $P < 0.05$). The methyl-atropine-induced increase in resting heart rate was greater in the cholesterol-fed than the control guinea pigs ($P < 0.05$). Methyl-atropine did not affect resting mean arterial blood pressure in control (72 ± 3

versus 73 ± 3 mmHg, pre and post values, respectively, $P > 0.05$) or cholesterol-fed animals (74 ± 3 versus 76 ± 3 mmHg, pre and post values, respectively, $P > 0.05$). The reductions in heart rate and mean arterial blood pressure elicited by electrical stimulation of the aortic depressor nerve (1-10 kHz) prior to injection of methyl-atropine were identical to those shown in **Figure 5.1** (data not shown). As can be seen in **Figure 5.3**, electrical stimulation of the aortic depressor nerve elicited negligible responses after administration of methyl-atropine.

Efferent Vagal Stimulation

The objective of this study was to address the cardiovascular changes elicited by direct electrical stimulation of the efferent vagus in control and cholesterol-fed guinea pigs. Prior to stimulation of the transected portion of the vagus projecting to the heart, resting heart rates were similar in control and cholesterol-fed animals (270 ± 8 versus 265 ± 7 beats/min, respectively, $P > 0.05$). Resting mean arterial blood pressures were also similar in control and cholesterol-fed guinea pigs (72 ± 4 versus 70 ± 3 mmHg, respectively, $P > 0.05$). The reductions in heart rate and mean arterial blood pressure elicited by electrical stimulation of the efferent vagus nerve (1-10 kHz) in control and cholesterol-fed guinea pigs are summarized in **Figure 5.4**. These electrical stimuli elicited frequency-dependent reductions in heart rate and mean arterial blood pressure in the control and cholesterol-fed guinea-pigs. The reductions in heart rate and mean arterial pressure were similar in the control and cholesterol-fed guinea pigs.

Stellate Ganglion Stimulation

The objective of this study was to address the cardiovascular changes elicited by direct electrical stimulation of the stellate ganglion in control and cholesterol-fed guinea pigs. This ganglion

provides the bulk of post-ganglionic sympathetic innervation of the heart (see Spyer, 1981). Prior to stimulation of the left stellate ganglion, resting heart rates was similar in the control and cholesterol-fed animals (273 ± 8 versus 271 ± 6 beats/min, respectively, $P > 0.05$). Resting mean arterial blood pressures were also similar in control and cholesterol-fed guinea pigs (70 ± 4 versus 69 ± 3 mmHg, respectively, $P > 0.05$). However, the rate of developed pressure in the left ventricle (dP/dT) was substantially smaller in the cholesterol-fed than in the control guinea pigs (2568 ± 286 versus 4067 ± 312 mmHg/sec, $P < 0.05$). The increases in heart rate, left ventricular dP/dT, and mean arterial blood pressure elicited by electrical stimulation of the stellate ganglion (1-10 kHz) in control and cholesterol-fed guinea pigs are summarized in **Figure 5.5**. These electrical stimuli elicited frequency-dependent increases in heart rate, dP/dT and mean arterial blood pressure in control and cholesterol-fed guinea-pigs. The responses were markedly smaller in the cholesterol-fed animals. The percent changes in heart rate, dP/dT and mean arterial blood pressure in control and cholesterol-fed guinea-pigs are summarized in **Figure 5.6**. The percent increases in these parameters were also substantially less pronounced in cholesterol-fed guinea pigs that in the control animals. The responses elicited by stellate stimulation were completely abolished by atenolol ($P < 0.05$, for all comparisons, data not shown).

Cardiovascular Responses Elicited by Isoproterenol in Conscious Guinea Pigs

The objective of this study was to determine the cardiovascular responses elicited by systemic injections of isoproterenol, which activates Gs protein-coupled β_1-, β_2-, and β_3-adrenoceptors (see Whalen and Lewis, 1999; Whalen et al., 2000). Activation of these β-adrenoceptors leads to the activation of adenylate cyclase, which converts adenosine triphosphate (ATP) to cyclic adenoseine monophosphate (cAMP). In turn, cAMP activates cAMP-dependent protein kinase

(PKA) (see Whalen and Lewis, 1999; Whalen et al., 2000). Resting heart rates were similar in control and in cholesterol-fed animals (263 ± 8 versus 266 ± 9 beats/min, respectively, $P > 0.05$). Resting mean arterial blood pressures were also similar in control and cholesterol-fed animals (74 ± 4 versus 77 ± 3 mmHg, respectively, $P > 0.05$). However, the rate of developed left ventricular pressure (dP/dT) was substantially smaller in cholesterol-fed than in control animals (2746 ± 223 versus 4311 ± 298 mmHg/sec, $P < 0.05$). The arithmetic and percent increases in heart rate and rate of left ventricular pressure development (dP/dT), and the falls in mean arterial blood pressure elicited by injections of isoproterenol (0.05-0.5 μg/kg, iv) are summarized in **Figure 5.7** and **Figure 5.8**, respectively. Isoproterenol elicited dose-dependent increases in heart rate, dP/dT, and falls in mean arterial blood pressure in control guinea pigs. These responses were markedly smaller in the cholesterol-fed guinea pigs.

Cardiovascular Effects Elicited by 8CPT-cAMP, a Direct Activator of PKA

Isoproterenol activates Gs protein-coupled β_1-, β_2-, and β_3-adrenoceptors (Whalen and Lewis, 1999; Whalen et al., 2000). The loss of responsiveness to isoproterenol in the cholesterol-fed guinea pigs may be due to the down-regulation of β-adrenoceptors in heart and vascular smooth muscle cells. However, the loss of response to isoproterenol may also involve diminished cAMP-signaling in these cells. The objective of this study was to determine the cardiovascular responses elicited by injections of 8-chlorophenylthio-cAMP (8-CPT-cAMP), which is a membrane-permeable direct activator of PKA (Whalen et al., 1999). Prior to the injection of 8-CPT-cAMP, resting heart rates were similar in control and in cholesterol-fed animals (265 ± 8 versus 269 ± 9 beats/min, respectively, $P > 0.05$). Resting mean arterial blood pressures were also similar in control and cholesterol-fed guinea pigs (72 ± 4 versus 74 ± 3 mmHg, respectively,

$P > 0.05$). The rate of developed pressure in the left ventricle (dP/dT) was substantially smaller in the cholesterol-fed than in the control guinea pigs (2548 ± 213 versus 4404 ± 305 mmHg/sec, $P < 0.05$).

The arithmetic changes in heart rate, dP/dT and mean arterial blood pressure elicited by bolus injections of 8-CPT-cAMP (5-20 μmol/kg, iv) in conscious control and cholesterol-fed guinea pigs are summarized in **Figure 5.9**. 8-CPT-cAMP elicited robust increases in heart rate and dP/dT and reductions in mean arterial blood pressure. 8-CPT-cAMP elicited similar arithmetic increases in heart rate in the cholesterol-fed guinea pigs but smaller arithmetic increases in dP/dT and reductions in mean arterial blood pressure than in the control animals. These findings suggest that PKA-dependent signaling was diminished in cardiac muscle cells but not cardiac pacemaker cells in cholesterol-fed guinea pigs. However, the diminished contractile efficiency of the heart (i.e., reduced baseline dP/dT values) due to concentric cardiac remodeling may have been a key factor in the smaller 8-CPT-cAMP-induced increases in dP/dT. More specifically, there may not be a dysfunction of PKA signaling but rather the smaller responses reflect smaller cardiac efficiency *per se*. In order to address this possibility, we graphed the responses as percent changes (see **Figure 5.10**). As expected the percent changes in HR elicited by the injections of 8-CPT-cAMP were similar in the control and cholesterol-fed guinea pigs. However, the increases in dP/dT elicited by 8-CPT-cAMP were now similar in the control and cholesterol-fed guinea pigs. Again as expected, the percent reductions in mean arterial blood pressure were smaller in cholesterol-fed than in control guinea pigs.

Intrinsic Heart Rate in Conscious Guinea Pigs

Intrinsic heart rate is the resting heart rate after complete autonomic blockade. As such, intrinsic heart rate in normal subjects is determined by the intrinsic properties of the cardiac pacemaker cells. However, true intrinsic rate in subjects suffering from diseases such as hypercholesteremia may be difficult to ascertain because of the actions of known/unknown factors in the blood on pacemaker activity. Nonetheless, it is a valuable exercise to determine intrinsic heart rate in the cholesterol-fed guinea pigs as a starting point for functional studies of isolated hearts. Resting heart rate and mean arterial blood pressure values before and after injection of methyl-atropine (1 mg/kg, iv) and then after the subsequent injection of atenolol (1mg/kg, iv) in control and cholesterol-fed guinea pigs are summarized in the upper panels of **Figure 5.11**. Resting heart rate and mean arterial blood pressure values before and after injection of atenolol (1mg/kg, iv) and then after the subsequent injection of methyl-atropine (1 mg/kg, iv) in other groups of control and cholesterol-fed guinea pigs are summarized in the bottom panels of **Figure 5.11**. Prior to injection of any drug, resting heart rates and mean arterial blood pressures in these four groups of guinea pigs were similar to one another ($P > 0.05$, for all comparisons). It should be noted that administration of atenolol and methyl-atropine alone or in combination did not affect resting mean arterial blood pressures ($P > 0.05$, for all comparisons).

The administration of methyl-atropine produced robust increases in heart rate in the control and cholesterol-fed guinea pigs (see upper panels of **Figure 5.11**). The precise magnitudes of these responses will be described below. The subsequent injection of atenolol caused a reduction in heart rate in both groups. The asterisk on the methyl-atropine + atenolol column for the control and cholesterol-fed guinea pigs signifies that the arithmetic differences from pre values are

significant (see below). The dagger signifies that the post methyl-atropine + atenolol heart rate values in cholesterol-fed guinea pigs were higher than in control guinea pigs. The administration of atenolol produced robust decreases in heart rate in the control guinea pigs but much smaller responses in the cholesterol-fed guinea pigs (see lower panels of **Figure 5.11**). The precise magnitudes of these responses will be described below. Subsequent injection of methyl-atropine caused an increase in heart rate in both groups. The asterisk on the methyl-atropine + atenolol column for the control and cholesterol-fed guinea pigs signifies that the arithmetic differences from pre values are significant (see below). The dagger signifies that the post methyl-atropine + atenolol heart rate values in cholesterol-fed guinea pigs were higher than in control guinea pigs.

The actual changes in heart rate elicited by methyl-atropine + atenolol and atenolol + methyl-atropine in the above control and cholesterol-fed guinea pigs are summarized in **Figure 5.12**. Methyl-atropine elicited increases in heart rate that were greater in cholesterol-fed than in control animals (upper panels of **Figure 5. 12**). Subsequent injection of atenolol reduced heart rate in both groups. Resting heart rates after combined administration of methyl atropine + atenolol in control animals were arithmetically lower than Pre values. In contrast, resting heart rate after combined injection of methyl atropine + atenolol in cholesterol-fed animals were arithmetically higher than the Pre values. Atenolol elicited substantially greater reductions in heart rate in control than cholesterol-fed guinea pigs (lower panels of **Figure 5. 12**). The subsequent injection of methyl-atropine increased heart rate in both groups. Again, resting heart rate values after the combined administration of atenolol + methyl atropine in control guinea pigs were arithmetically lower than Pre values whereas they were arithmetically higher than Pre values in cholesterol-fed guinea pigs.

DISCUSSION

Objective

The previous chapter provided evidence that the activity of the baroreceptor heart rate reflex in conscious cholesterol-fed guinea pigs was substantially reduced as compared to control animals. More specifically the sensitivity (gain) and range (difference between upper and lower heart rate plateaus) of the baroreflex was diminished in cholesterol fed animals. The diminished baroreflex sensitivity could involve a loss of afferent function itself, diminished central processing of the reflex and/or alterations in cardiac muscarinic and β-adrenoceptor signaling processes. However, the diminished range of the baroreceptor reflex in the cholesterol-fed guinea pigs lends tentative support for a loss of afferent function and/or central processing since the diminished range suggests problems with activation and/or withdrawal of both vagal and sympathetic drive. The major **objective** of the studies reported in this chapter was to address the possible mechanisms responsible for the diminished baroreceptor reflex activity in cholesterol-fed guinea pigs.

Aortic Depressor Nerve and Efferent Vagus

We chose to examine the cardiovascular responses elicited by electrical stimulation of the aortic depressor nerve because this nerve carries baroreceptor afferents only (Abboud et al., 1976; Spyer, 1981). This study shows that episodes of direct electrical stimulation of the aortic depressor nerve elicited frequency-dependent reductions in heart rate and mean arterial blood pressure in pentobarbital-anesthetized control guinea pigs. The baroafferent-induced responses were virtually abolished by the muscarinic receptor antagonist, methyl-atropine, whereas they were minimally affected by the β_1-adrenoceptor antagonist, atenolol. These findings suggest that the bradycardia elicited by stimulation of the aortic depressor nerve are mediated principally by

vagal efferent-mediated release of acetylcholine and activation of cardiac muscarinic receptors. In addition, the above findings suggest that the depressor responses elicited by stimulation of the aortic depressor nerve are due primarily to vagal-afferent-mediated reductions in heart rate and cardiac output. The cardiovascular responses elicited by stimulation of the aortic depressor nerve were markedly smaller in the cholesterol-fed guinea pigs. A key additional finding was that the reductions in heart rate and mean arterial blood pressure elicited by direct electrical stimulation of the efferent vagus were not diminished in cholesterol-fed guinea pigs as compared to control guinea pigs. As such, the loss of response to aortic depressor nerve stimulation in the cholesterol-fed guinea pigs cannot be obviously attributed to diminished vagal-efferent function *per se* including the activity of muscarinic receptors on cardiac pacemaker cells.

Taken together, these findings suggest that baroafferent activity and/or central processing of the afferent information is impaired in cholesterol-fed animals. The present study employed direct electrical stimulation of the peripheral axons of baroafferents in the aortic depressor nerve and so any loss of sensitivity of baroafferent terminals to their natural stimuli (i.e., increased pressure-induced stretch of the terminals) was not addressed. We are currently establishing the equipment and protocols that are necessary to record changes in aortic depressor nerve activity in response to increases and decreases in mean arterial blood pressure in control and cholesterol-fed guinea pigs to address the issue as to whether the afferent terminals themselves are dysfunctional in the cholesterol-fed guinea pigs. Nonetheless, there appears to be one compelling conclusion from our studies. Specifically, on the basis of our findings obtained from the aortic depressor nerve experiments, it appears that the central processing of baroafferent information is diminished in cholesterol-fed guinea pigs. Whether this involves altered processing in the nucleus tractus

solitarius (site of termination of baroafferents), altered input from the nucleus tractus solitarius to the dorsal motor nucleus of the vagus and nucleus ambiguus (site of preganglionic vagal motor nerves) (Abboud et al., 1976; Spyer, 1981; Machado et al., 1994), remains to be determined.

Stellate Ganglion

A key component of the baroreceptor reflex-mediated tachycardia and increases in cardiac output in response to falls in mean arterial blood pressure is mediated by cardiac sympathetic nerves, which release norepinephrine to activate β-adrenoceptors on cardiac pacemaker cells and cardiac muscle (Abboud et al., 1976; Spyer, 1981). As such, the diminished tachycardia in conscious cholesterol-fed guinea pigs in response to reductions in mean arterial blood pressure may involve reduced central activation of post-ganglionic sympathetic nerves and/or reduced function of cardiac β-adrenoceptors. In order to address this possibility, we first examined the cardiovascular responses elicited by direct electrical stimulation of a stellate ganglion in control and cholesterol-fed pentobarbital-anesthetized guinea pigs. The stellate ganglia provide the majority of post-ganglionic sympathetic innervation of cardiac structures including pacemaker cells, cardiac muscle and coronary vasculature (Abboud et al., 1976; Spyer, 1981).

The present study demonstrates that electrical stimulation of the stellate ganglion induced robust increases in heart rate, rate of development of left ventricular pressure (dP/dT) and mean arterial blood pressure in pentobarbital-anesthetized control guinea pigs. The increase in mean arterial blood pressure is due to an increase in cardiac output since the stellate ganglion does not project widely to vascular structures other than those in the heart and superior cervical ganglion (Abboud et al., 1976; Spyer, 1981). Our observation that the cardiovascular responses were

virtually eliminated by atenolol, is consistent with these responses being due to norepinephrine-mediated activation of cardiac β-adrenoceptors (Abboud et al., 1976; Whalen et al., 1999). The key finding was that the cardiovascular responses elicited by electrical stimulation of the stellate ganglion were markedly diminished in the cholesterol-fed guinea pigs. The loss of response to this stimulation may include diminished release of neurotransmitter stores of norepinephrine from the sympathetic nerve terminals and/or diminished biological activity of norepinephrine due to the down-regulation of β-adrenoceptor signaling.

β-Adrenoceptors

To address the possibility that β-adrenoceptor signaling is diminished in cholesterol-fed guinea pigs, we determined the cardiovascular responses elicited by isoproterenol, which activates Gs protein-coupled β_1-, β_2-, and β_3-adrenoceptors (see Whalen and Lewis, 1999; Whalen et al., 2000). This study found that systemic injections of isoproterenol elicited pronounced dose-dependent increases in heart rate and rate of development of left ventricular pressure (dP/dT) in conscious control guinea pigs that were accompanied by decreases in mean arterial blood pressure. The cardiac responses elicited by isoproterenol are due to activation of β-adrenoceptors on cardiac pacemaker and muscle cells whereas the reduction in mean arterial blood pressure is due primarily to the direct activation of vasodilator β-adrenoceptors on vascular smooth muscle in resistance arteries (see Whalen and Lewis, 1999; Whalen et al., 2000). We have not performed studies to determine the subtypes of β-adrenoceptors involved in mediating these responses at each dose of isoproterenol. This is an important aim since β_3-adrenoceptors are progressively more activated with increasing doses of isoproterenol in rats (see Whalen and Lewis, 1999; Whalen et al., 2000).

The key finding of this study was that the cardiovascular responses elicited by isoproterenol were markedly diminished in cholesterol-fed guinea pigs. Accordingly, it appears that β-adrenoceptor signaling is diminished in cardiac pacemaker and muscle cells and in the vasculature. However, this study could not address whether the loss of response to isoproterenol was due to the down-regulation of β-adrenoceptors *per se* or the signaling mechanisms recruited by these receptors.

Direct activation of PKA

The above findings made it essential to try and establish whether the loss of response to stellate ganglion stimulation was due to the down-regulation of cardiac β-adrenoceptors and/or their signaling mechanisms. The agonist-induced occupation of β-adrenoceptors leads to Gs protein-mediated activation of adenylate cyclase, which converts ATP to cAMP. In turn, cAMP activates PKA, which elicits its responses by a variety of mechanisms including the phosphorylation of voltage-gated calcium channels (see Whalen and Lewis, 1999; Whalen et al., 2000). In order to attempt to come to terms with the effects of high cholesterol diet on β-adrenoceptor signaling, we determined the cardiovascular responses elicited by the membrane-permeable direct activator of PKA, 8-CPTcAMP, in conscious control and cholesterol-fed guinea pigs. The injection of 8-CPT-cAMP elicited pronounced increases in heart rate and the rate of development of left ventricular pressure (dP/dT) in control guinea pigs that was accompanied by relatively minor reductions in mean arterial blood pressure. These responses are consistent with the expected roles of PKA in the heart and vasculature (see Whalen and Lewis, 1999; Whalen et al., 2000).

One key finding was that the increases in heart rate elicited by 8-CPT-cAMP in cholesterol-fed guinea pigs were virtually identical to those in the control guinea pigs. Moreover, when corrected

for the diminished baseline left ventricular pressure dP/dT, it appears that the increase in cardiac contractility elicited by 8-CPT-cAMP in the cholesterol-fed guinea pigs were very similar to those in the control guinea pigs. Taken together, these findings provide tentative evidence that cAMP-dependent signaling in the heart is not diminished in cholesterol-fed guinea pigs. As such, the reduced ability of isoproterenol to increase heart rate and cardiac contractility in cholesterol-fed guinea pigs may involve four primary mechanisms, namely (1) the down-regulation of β-adrenoceptors (desensitization and/or internalization leading to a decrease in affinity and density of β-adrenoceptors), (2) reduced coupling of β-adrenoceptors to alpha sub-units of Gs proteins, (3) diminished ability of alpha sub-units of Gs proteins to activate adenylate cyclase, and (4) diminished ability of adenylate cyclase to generate cAMP. The precise involvement of these mechanisms must await further studies.

Intrinsic Heart Rate

The administration of the β_1-adrenoceptor antagonist, atenolol, elicited a pronounced bradycardia in control guinea pigs whereas it elicited relatively minor effects on heart rate in cholesterol-fed guinea pigs. This observation is consistent with the above section which provided evidence that β-adrenoceptors are down-regulated in cholesterol-fed guinea pigs. In contrast, the tachycardia elicited by the muscarinic receptor antagonist, methyl-atropine, was greater in cholesterol-fed than in control guinea pigs. This is consistent with the findings reported above that vagal efferent mechanisms including the functional status of cholinergic muscarinic receptors do not appear to be compromised in cholesterol-fed guinea pigs. The augmented tachycardia in response to methyl-atropine in cholesterol-fed guinea pigs may be due to an increased dependence on vagal efferent drive in these animals.

One key observation of this study was that the combined administration of atenolol and methyl-atropine resulted in an intrinsic heart rate value in control guinea pigs that was less than before drug administration (i.e., normal resting heart rate). This finding, which is consistent with that found in rats (Machado and Brody, 1989), suggests that the sympathetic nervous system has a greater impact on resting heart rate than the parasympathetic nervous system in conscious guinea pigs. Of particular importance was the finding that combined administration of atenolol and methyl-atropine resulted in an intrinsic heart rate value that was greater than prior to drug administration in cholesterol-fed guinea pigs. This suggests that the high cholesterol diet caused a change in the intrinsic properties of the cardiac pacemaker cells by mechanisms that need to be elucidated. One important caveat is that blood-borne factors (the identities of which are unknown to us) may contribute to the apparent increase in intrinsic heart rate in cholesterol-fed animals.

SUMMARY

These studies provided evidence that guinea pigs with cholesterol-induced cardiac concentric remodeling have altered autonomic function which involves disruption of central processing of baroafferent function and an apparent down-regulation of β-adrenoceptors. G-protein coupled receptors are co-localized in lipid raft/caveolae microdomains of cardiac myocytes or cardiac membranes (Insel et al., 2005). Therefore, high dietary cholesterol may exert deleterious cardiac and vascular effects by disruption of these microdomains, which are structurally present to facilitate signal transduction pathways. Further mechanistic studies are warranted to determine if cholesterol-induced disruption of lipid rafts within cellular membranes renders G-protein coupled receptors inoperable. The finding that cholesterol-fed guinea pigs had elevated intrinsic heart rate values suggests that hypercholesterolemia may be a primary contributor of pacemaker

cell dysfunction. The complex cardiovascular adaptations observed in these guinea pigs exhibiting left ventricular remodeling may eventually lead to an increased risk of mortality and morbidity , however, elucidating these mechanisms will significantly contribute to the discovery of pharmacological therapies to treat and prevent hypercholesterolemia-induced abnormalities.

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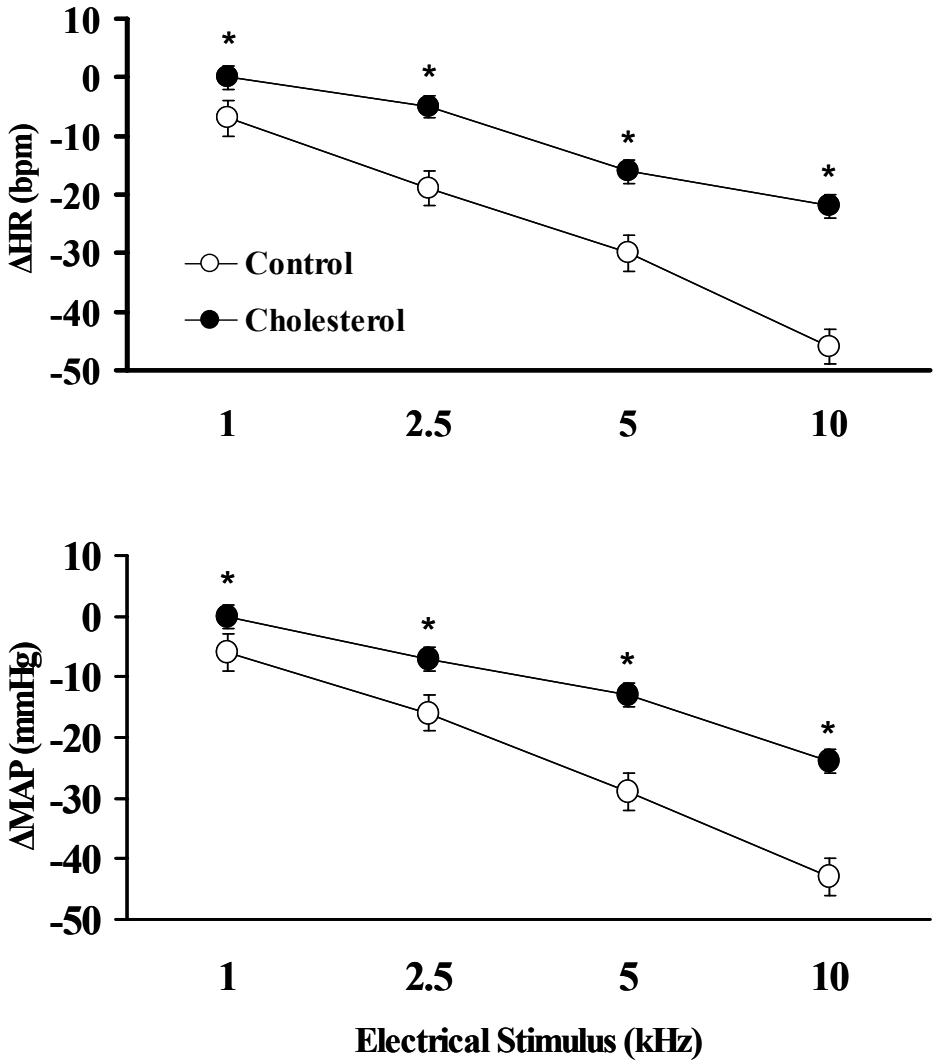

Figure 5.1. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by aortic depressor nerve (ADN) stimulation (1-10 kHz) in anesthetized control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

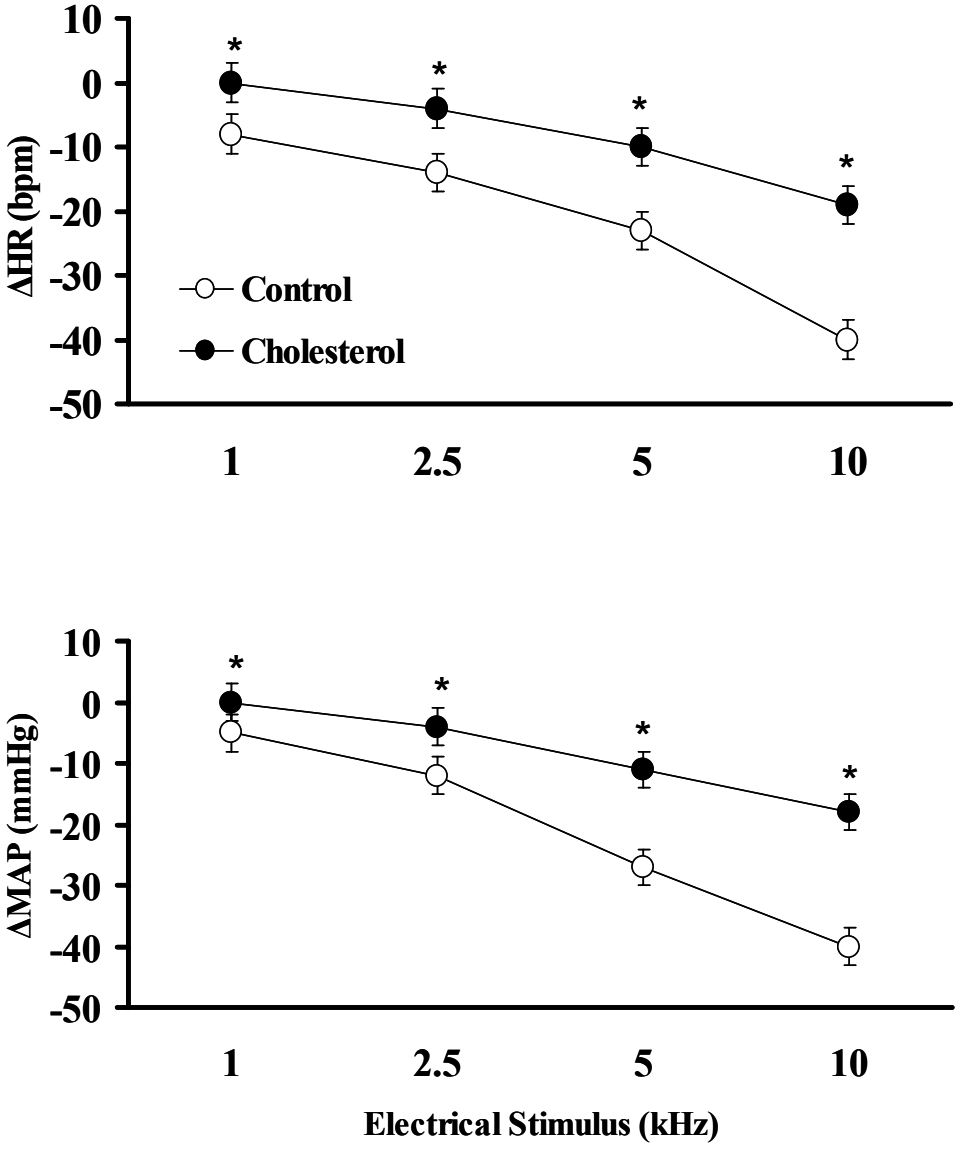

Figure 5.2. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by aortic depressor nerve (ADN) stimulation (1-10 kHz) in the presence of atenolol (ATN 1mg/kg, iv) in anesthetized control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

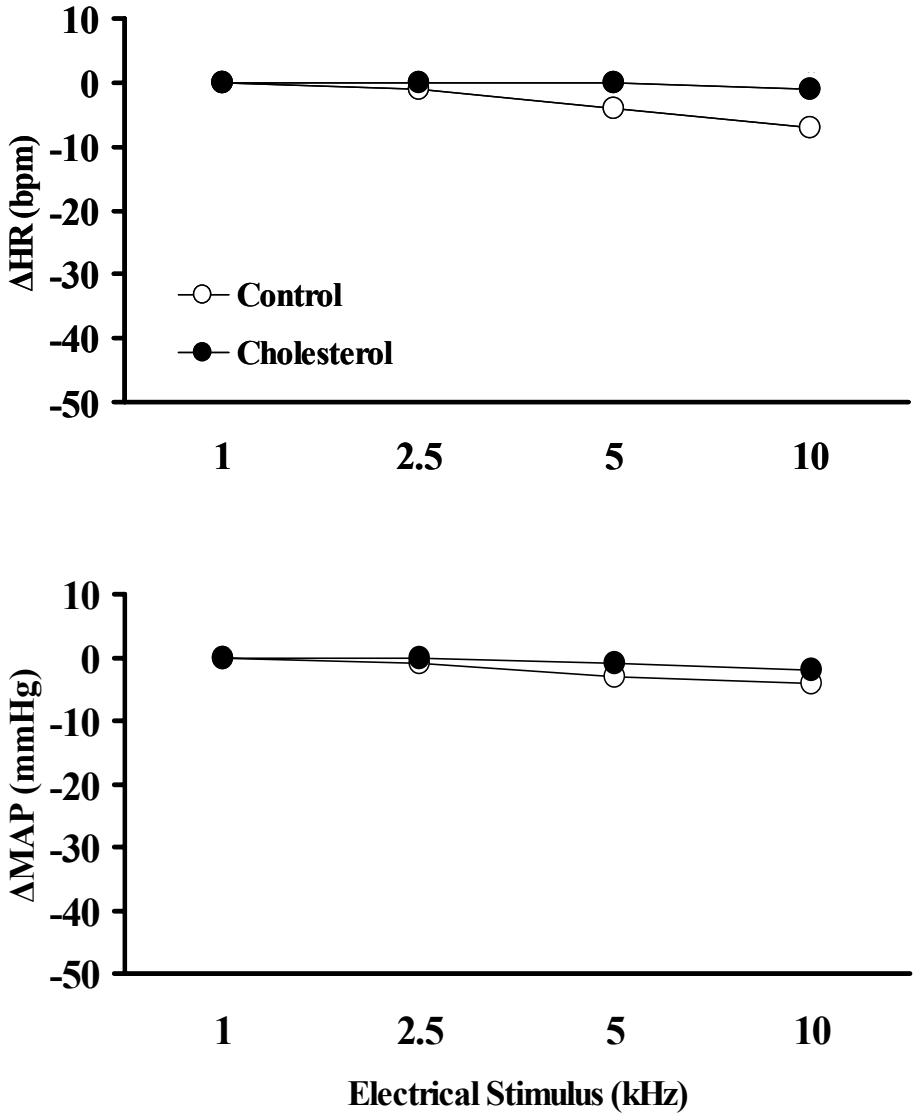

Figure 5.3. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by aortic depressor nerve (ADN) stimulation (1-10 kHz) in the presence of methyl atropine (MAT 1mg/kg, iv) in anesthetized control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

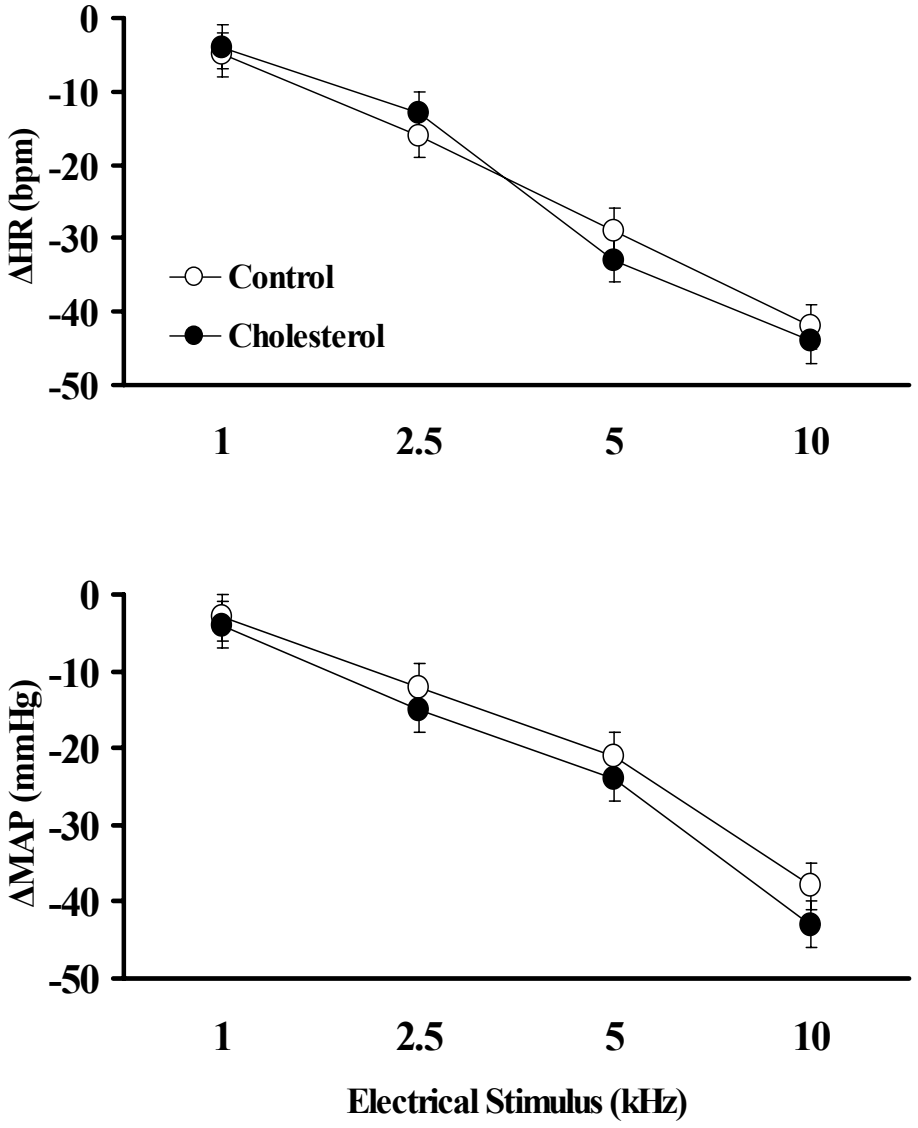

Figure 5.4. A summary of the changes in heart rate (HR) and mean arterial pressure (MAP) elicited by vagal stimulation (1-10 kHz) in anesthetized control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

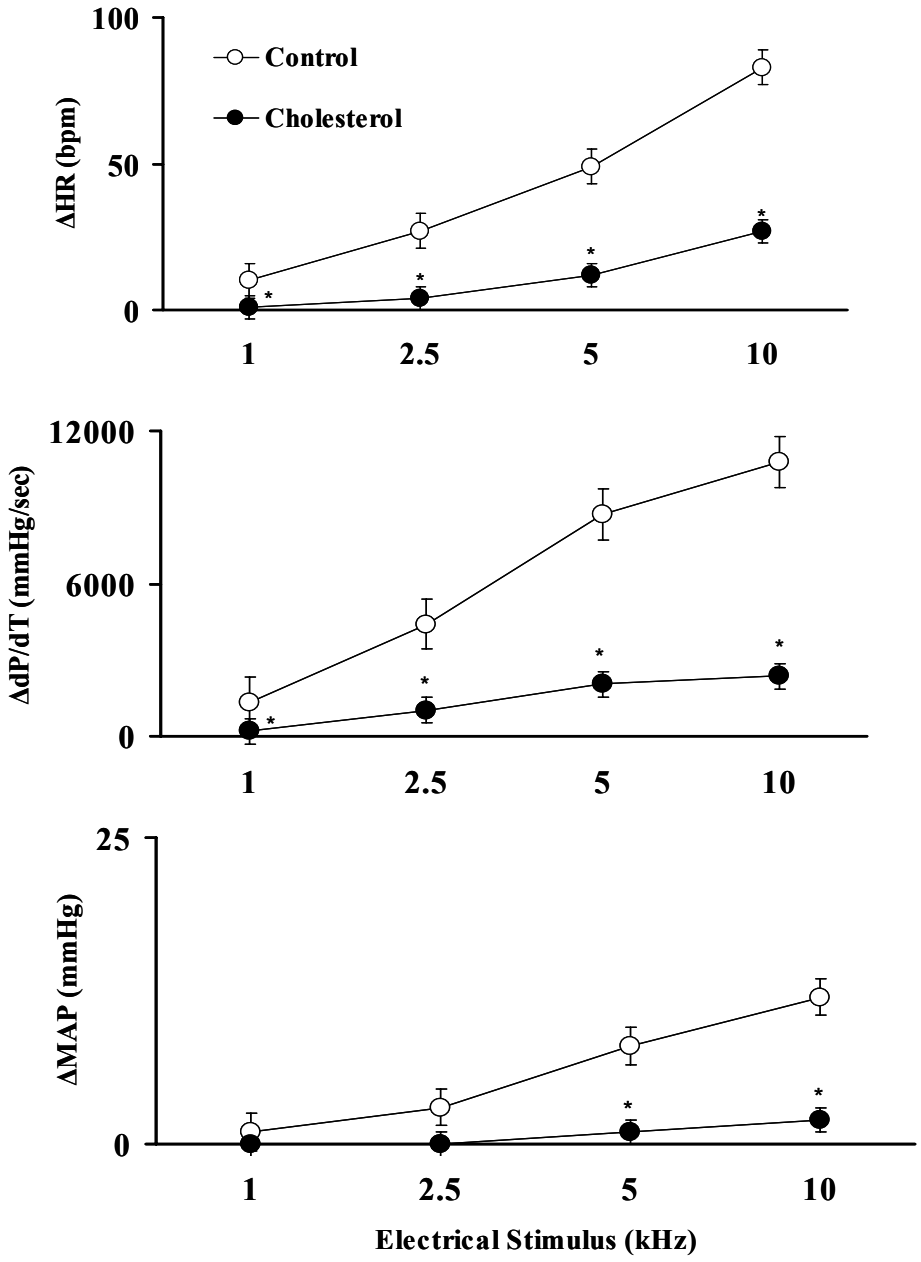

Figure 5.5. A summary of the changes in heart rate (HR), left ventricular pressure (dP/dt), and mean arterial pressure (MAP), elicited by stellate stimulation (1-10 kHz) in anesthetized control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

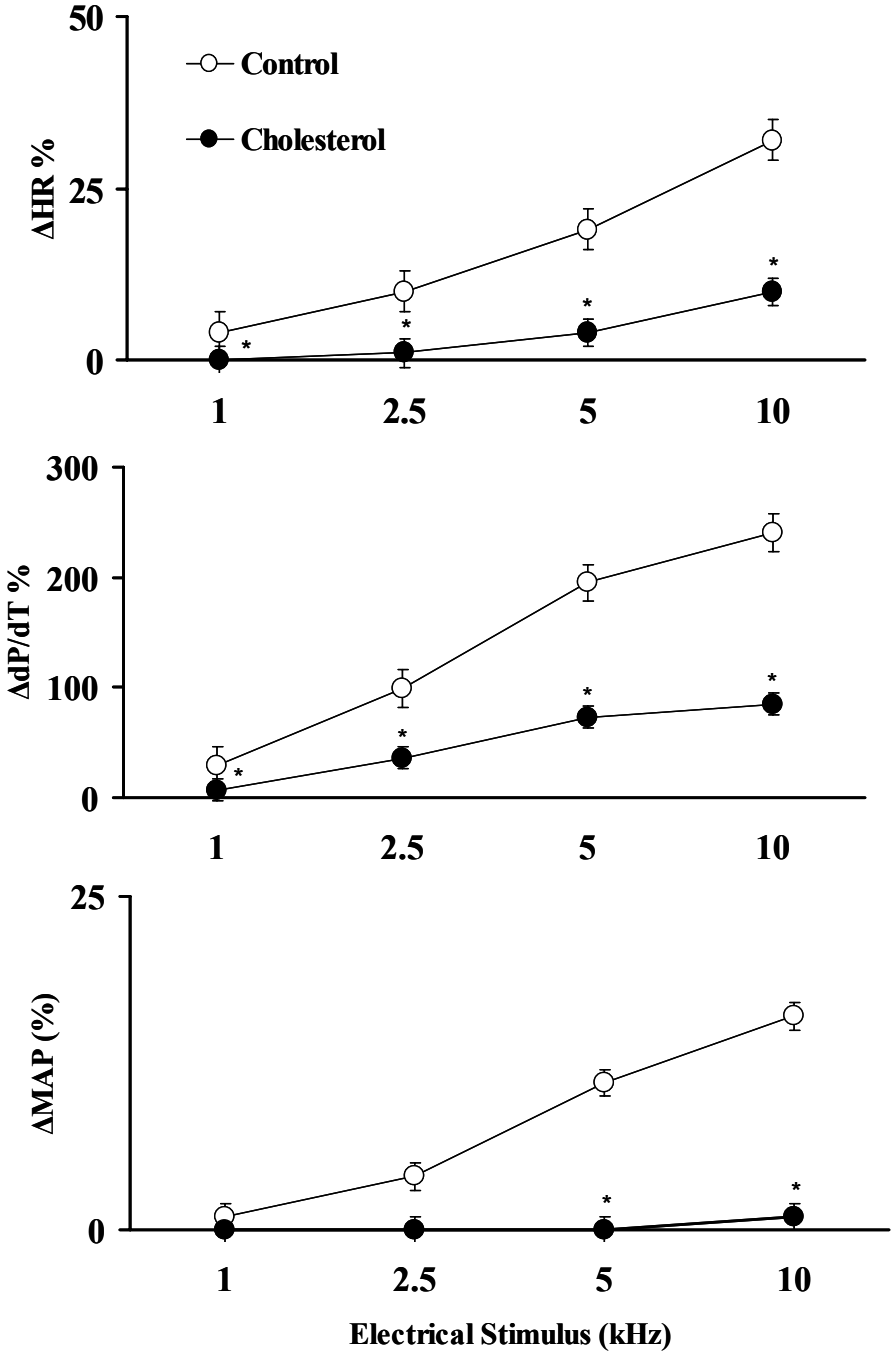

Figure 5.6: A summary of heart rate (HR), left ventricular pressure (dP/dt), and mean arterial pressure (MAP) percentage values, elicited by stellate stimulation (1-10 kHz) in anesthetized control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

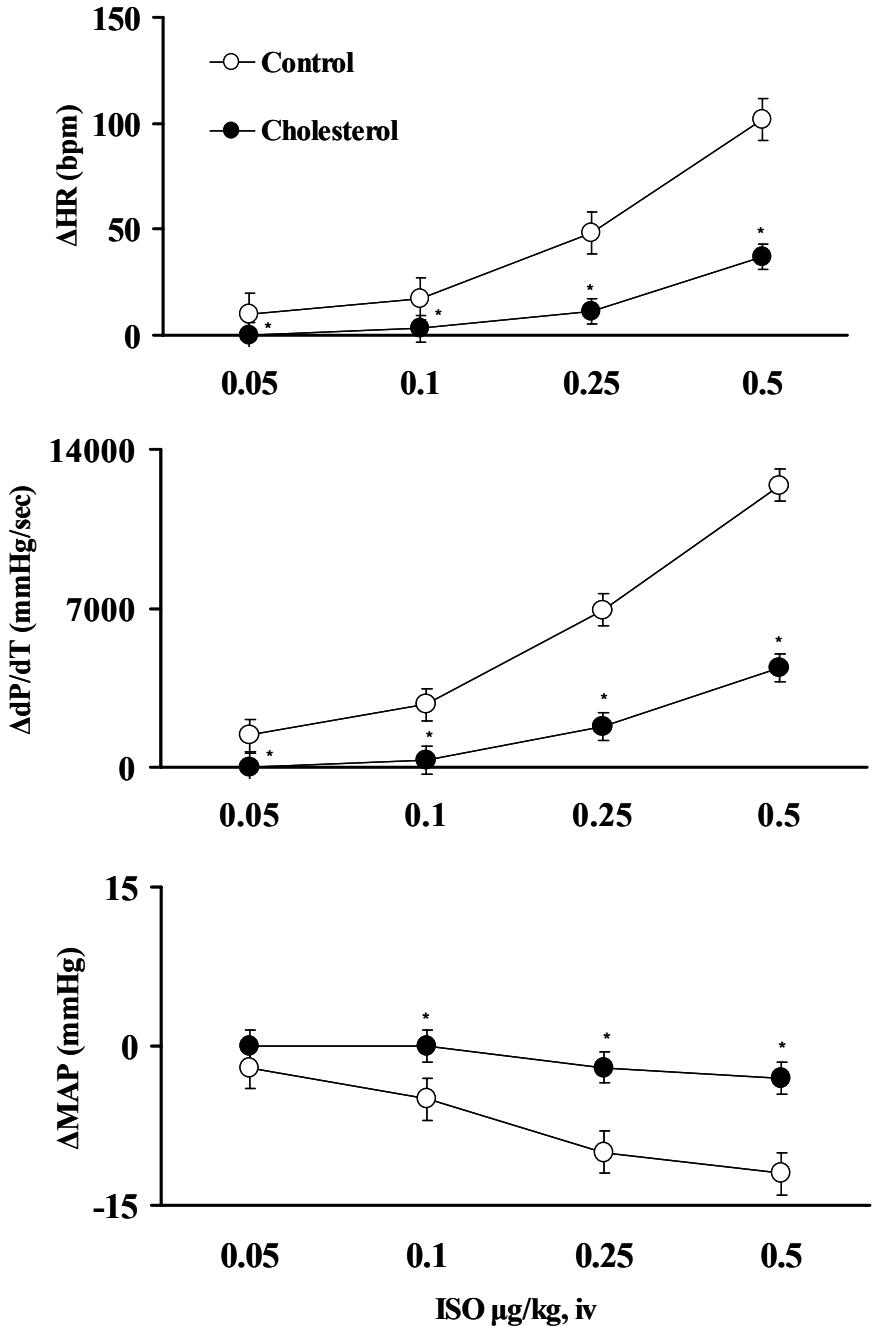

Figure 5.7. A summary of the changes in heart rate (HR), left ventricular pressure (dP/dt), and mean arterial pressure (MAP) elicited by systemic injections of isoproterenol (ISO; 0.05-0.5 µg/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

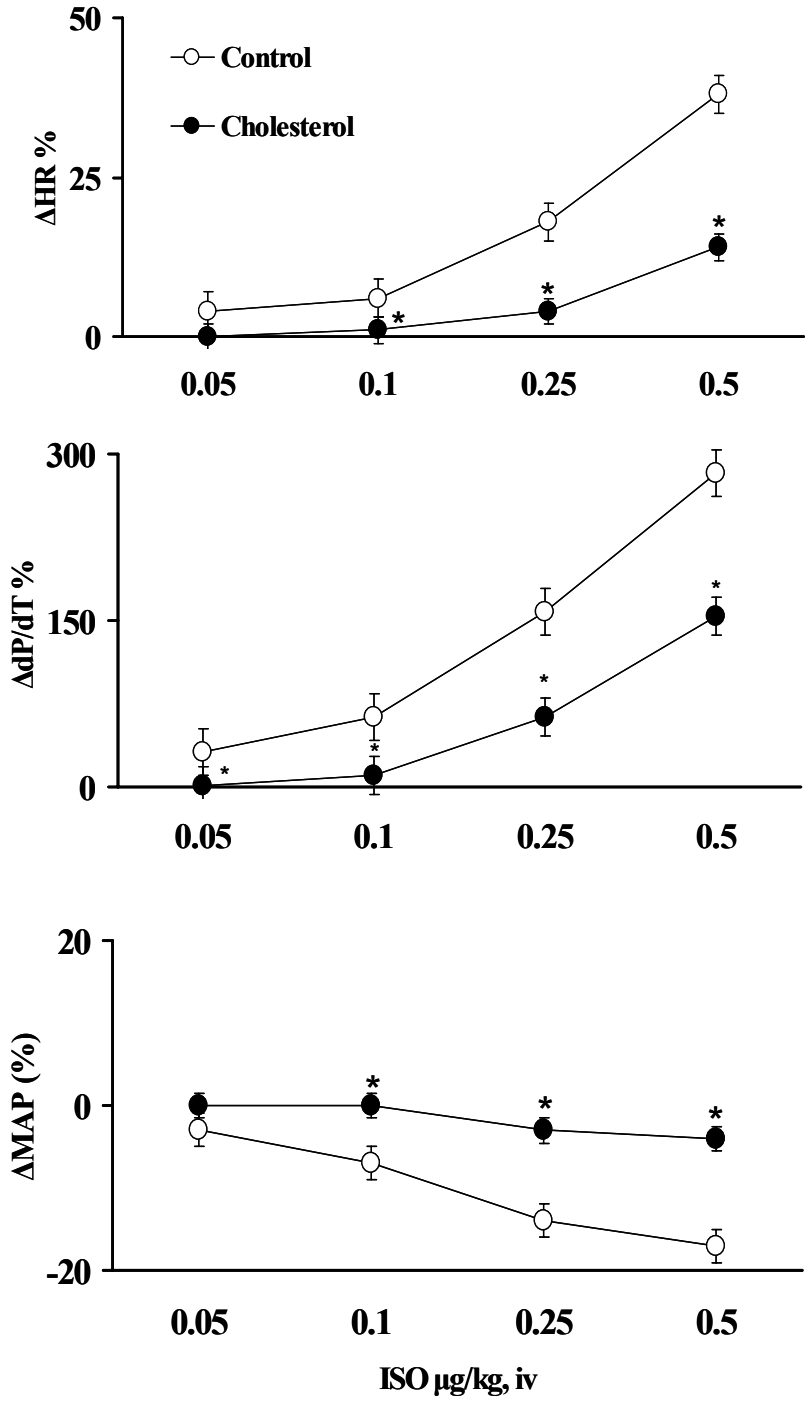

Figure 5.8. A summary of the percent changes in heart rate (HR), rate of left ventricular pressure development (dP/dT), and mean arterial pressure (MAP) elicited by systemic injections of isoproterenol (ISO; 0.05-0.5 µg/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

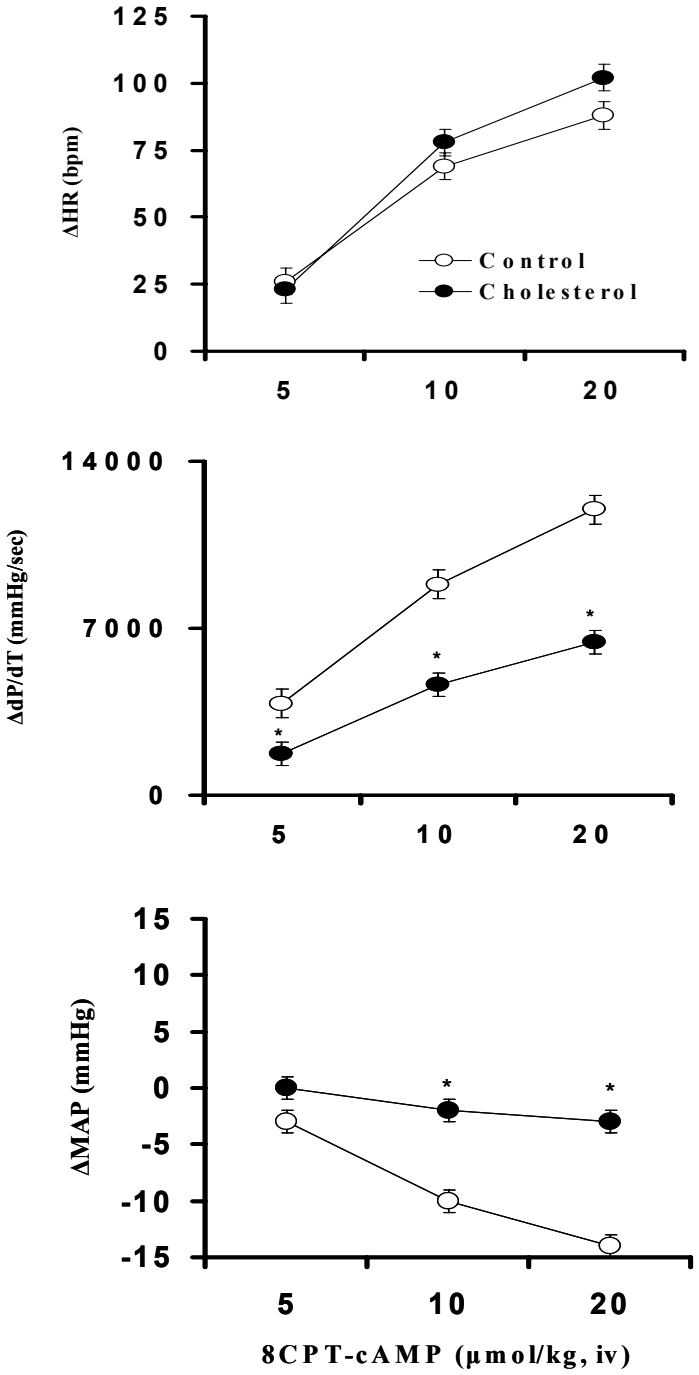

Figure 5.9. A summary of the changes in heart rate (HR), left ventricular pressure (dP/dT), and mean arterial pressure (MAP) elicited by systemic injections of 8CPT-cAMP (5-20 μmol/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

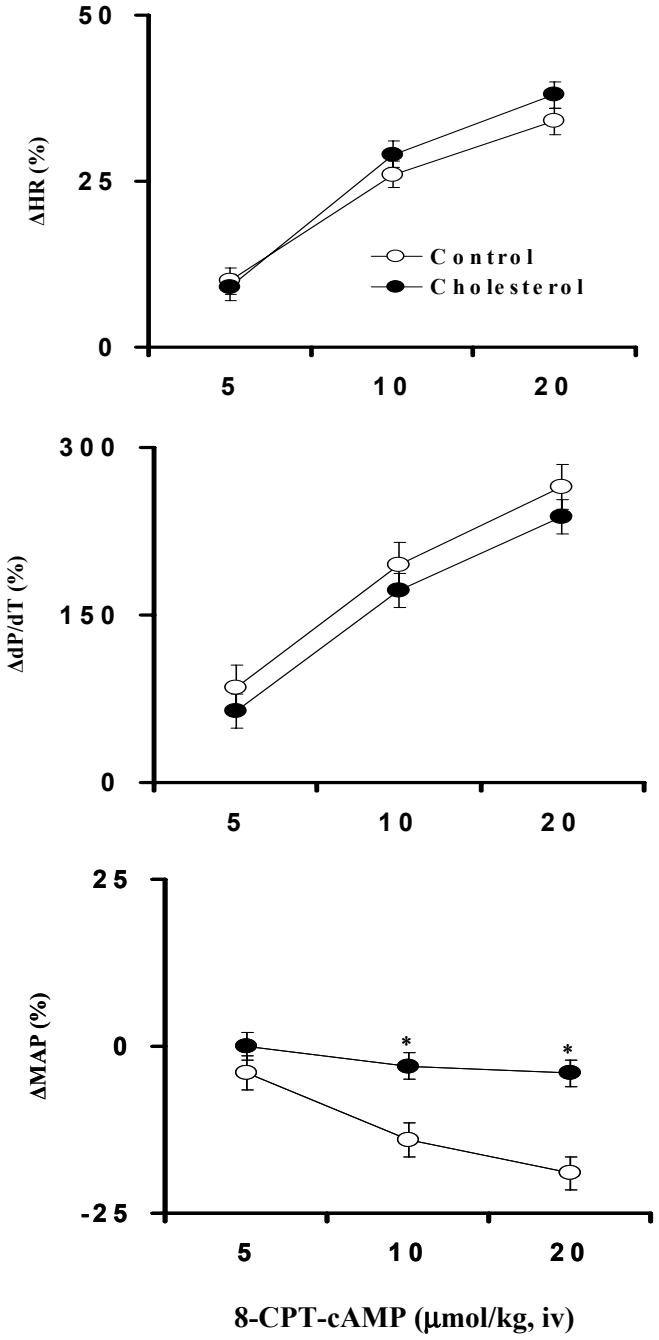

Figure 5.10. A summary of the heart rate (HR), left ventricular pressure (dP/dt), and mean arterial pressure (MAP) percentage values elicited by systemic injections of 8CPT-cAMP (5-20 μmol/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol versus control.

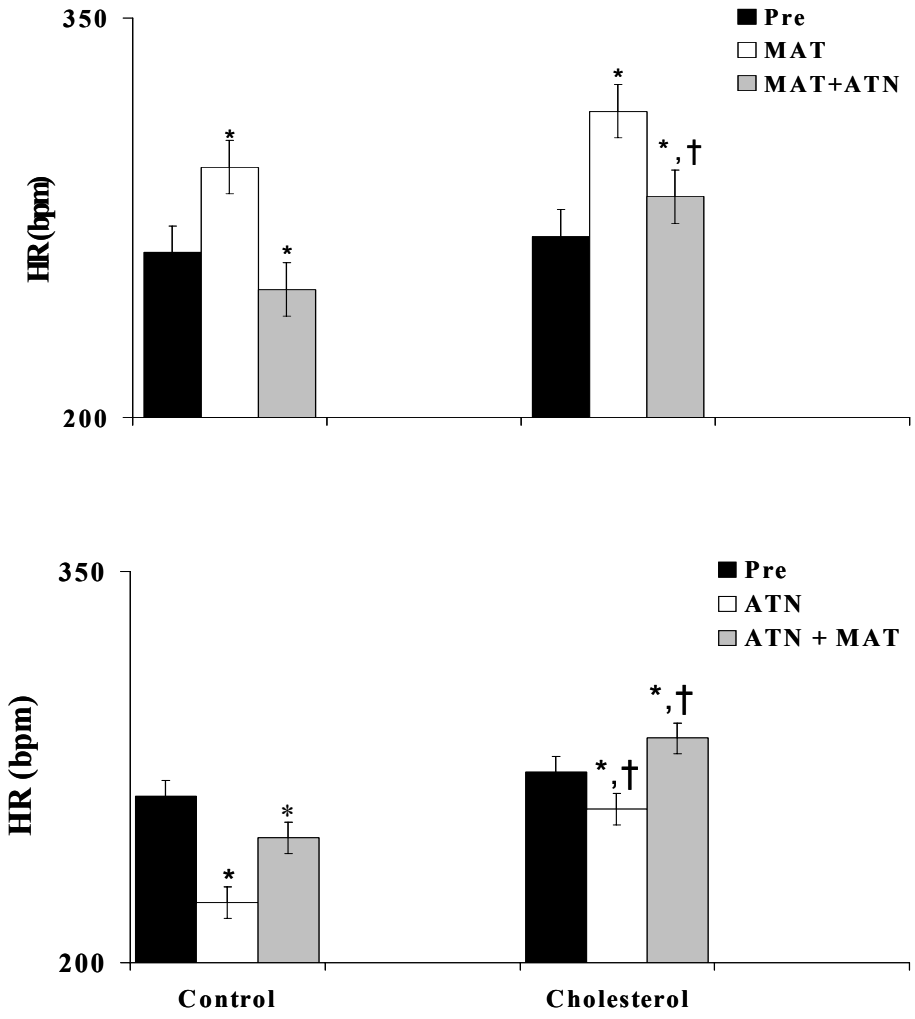

Figure 5.11. A summary of intrinsic heart rate values in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs after administration of methyl atropine (MAT) and atenolol (ATN). The data are shown as mean ± SEM. $^{\dagger}P < 0.05$, cholesterol versus control. $*P < 0.05$, ATN or MAT versus pre-values.

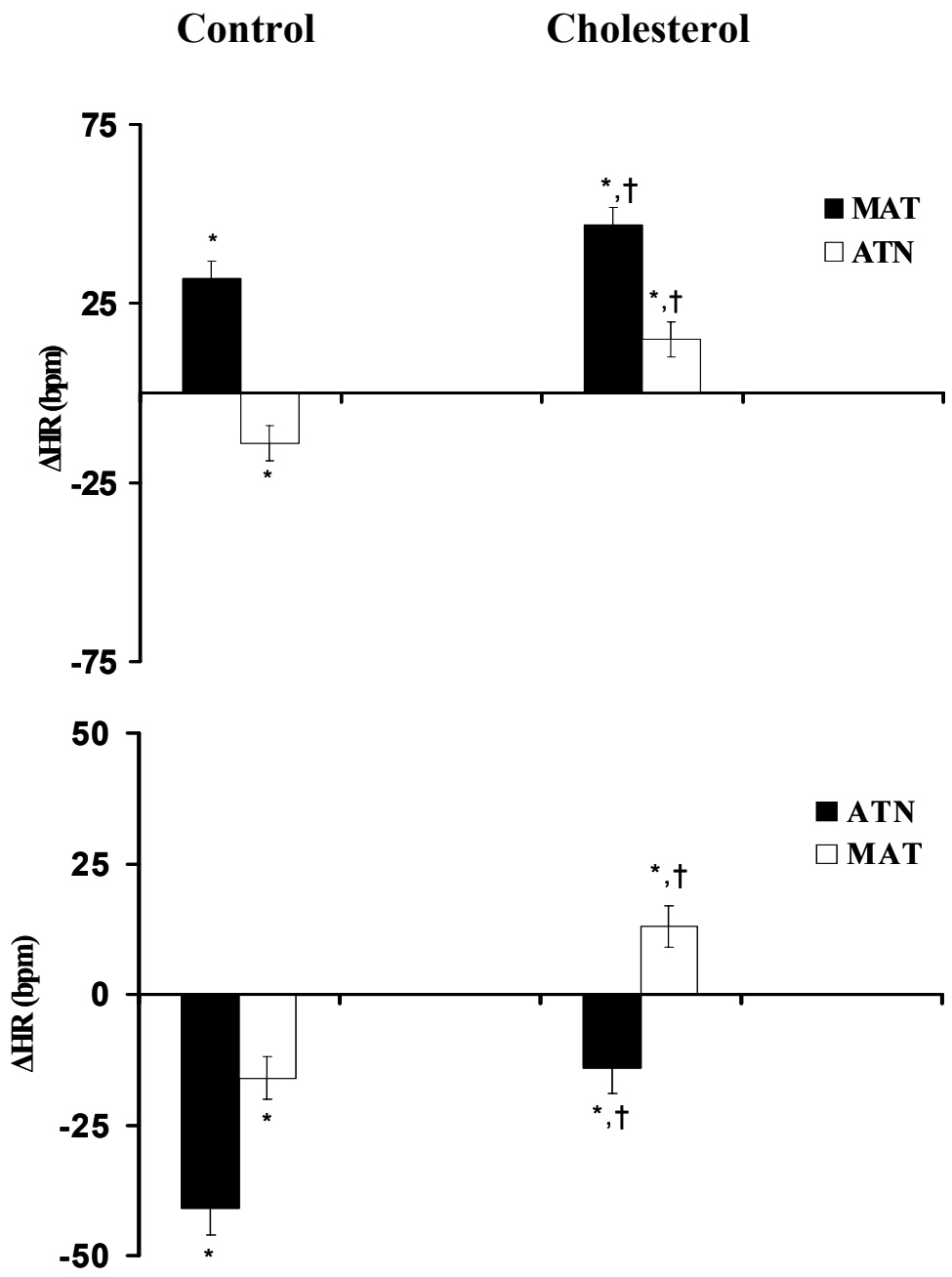

Figure 5.12. A summary of the changes in intrinsic heart rate elicited by systemic injections of methyl atropine (MAT; 1mg/kg, iv) or atenolol (ATN; 1 mg/kg, iv) in conscious control (n=8) or cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. †$P < 0.05$, cholesterol versus control, *$P < 0.05$, ATN or MAT versus pre-values (pre-values not shown).

CHAPTER 6

EFFECTS OF HIGH CHOLESTEROL DIET ON BEZOLD-JARISCH REFLEX FUNCTION IN CONSCIOUS GUINEA PIGS[1]

[1]Owen JR, Barton MH, Munday JS, Graves JE, Lewis SJ. To be submitted to *Neuropharmacology*.

ABSTRACT

The objective of this study was to examine the possibility that hypercholesterolemia interferes with the functional status of 5-HT_3 receptors on cardiopulmonary vagal afferents that mediate the Bezold-Jarisch reflex. Guinea pigs were fed control or high cholesterol (1% cholesterol) diet for 13 weeks. The cholesterol-fed animals were normotensive although they displayed diminished baroreflex activity and clear signs of cardiac concentric remodeling. The Bezold-Jarisch reflex-mediated reductions in heart rate and mean arterial pressure were elicited by systemic injections of the selective 5-HT_3 receptor agonist phenylbiguanide (10-100 μg/kg, iv). The reductions in heart rate and mean arterial pressure elicited by phenylbiguanide in control and cholesterol-fed guinea pigs were markedly attenuated by the selective muscarinic receptor antagonist, methyl-atropine (1 mg/kg, iv), whereas they were minimally affected by the selective β-adrenoceptor antagonist, atenolol (1 mg/kg, iv). This suggests that the Bezold-Jarisch reflex responses in conscious guinea pigs are due primarily to activation of cardiovagal drive.

The Bezold-Jarisch reflex-mediated reductions in heart rate and mean arterial pressure elicited by phenylbiguanide were markedly diminished in the cholesterol-fed guinea pigs. Evidence suggests that this is due to the down-regulation of 5-HT_3 receptors on vagal cardiopulmonary afferents mediating the Bezold-Jarisch reflex rather than to the central and/or efferent processing of the Bezold-Jarisch reflex. The rate of development of tachyphylaxis to the Bezold-Jarisch reflex responses observed upon administration of successive injections of phenylbiguanide was markedly accelerated in cholesterol-fed guinea pigs. On the basis of evidence that tachyphylaxis was observed to the Bezold-Jarisch reflex responses elicited by 5-HT (which also elicits the Bezold-Jarisch reflex via activation of 5-HT_3 receptors) but not L-S-nitrosocysteine (which

elicits the Bezold-Jarisch reflex via activation of stereoselective S-nitrosothiol recognition sites) after administration of the injections of phenylbiguanide, it appears that the development of tachyphylaxis to phenylbiguanide was due to the down-regulation of 5-HT_3 receptors rather than the loss of central and/or efferent processing of the Bezold-Jarisch reflex. Taken together, our findings suggest that the hypercholesterolemia impairs the functional status of 5-HT_3 receptors by mechanisms that are yet to be determined.

Keywords: Bezold-Jarisch reflex, cardiopulmonary afferents, 5-HT_3 receptors, phenylbiguanide

INTRODUCTION

Neural reflex control of circulation is maintained by baroreceptor and cardiopulmonary reflexes (Thoren, 1979; Spyer, 1981; Zanchetti and Mancia, 1991; Aviado and Guevara Aviado, 2001). Changes in cardiac pressure or circulating factors such as 5-hydroxytryptamine (5-HT), stimulate cardiopulmonary receptors in the atria and ventricles which exerts a tonic restraint on cardiac function and contributes to the regulation of pulmonary and systemic arterial blood pressures (Thoren, 1979; Higuchi et al., 1988; Ustinova and Schultz, 1994; Vasquez et al., 1997). The Bezold-Jarisch reflex is a cardiopulmonary vagal afferent-mediated reflex that elicits pronounced vagal efferent-induced bradycardia and hypotension (see Thoren, 1979; Vasquez et al., 1997). The primary purpose of this vital reflex is to defend the pulmonary circulation against sudden pronounced increases in arterial blood pressure.

The Bezold-Jarisch reflex can be induced in by inflating a non-occlusive balloon catheter in the right atrium (Thoren, 1979; Aviado and Guevara Aviado, 2001) and by systemic injections of 5-hydroxytryptamine (5-HT), which stimulates 5-$HT_{3A,3B}$ receptors on vagal cardiopulmonary afferents (Fozard, 1984; Richardson and Engel, 1986; Dabire et al., 1990). 5-$HT_{3A,3B}$ receptors are ligand-gated ion-channels which conduct Na^+ and K^+ ions (Hartig et al., 1990; Julius, 1991; Hartig, 1992; Zifa and Fillion, 1992). Since 5-HT elicits many other hemodynamic responses by activation of 5-$HT_{1A,B,D}$ and 5-$HT_{2A,B}$ receptors in the cardiovascular system, investigators often prefer to inject selective 5-HT_3 receptor agonists such as phenylbiguanide and/or 2-methyl-5HT (Whalen et al., 2000). These agonists elicit the Bezold-Jarisch reflex only since these receptors do not exist on other sub-types of afferent fibers regulating cardiovascular function or in blood vessels or cardiac structures (Richardson and Engel, 1986; Whalen et al., 2000).

Changes in cardiopulmonary afferent function are sensitive indicators of the progression of cardiopulmonary disease processes associated with pulmonary and systemic hypertension, atherosclerosis and hypercholesterolemia (Whalen et al., 2000; Owen et al., 2005). These disease processes directly affect cardiopulmonary afferent function by alterations in metabolic and signal transduction processes (i.e., disposition and function of membrane-associated receptors and ion-channels) in the afferent terminals (Aviado and Guevara Aviado, 2001; Owen et al., 2005). These disease processes can also indirectly alter afferent responses by changing the distensibility of atria and arteries in the cardiopulmonary circulation (see Aviado and Guevara Aviado, 2001).

On the basis of existing experimental and clinical evidence (Zanchetti and Mancia, 1991; Meyrelles et al., 1994, 1996) that links various pathological conditions with alterations in the Bezold-Jarisch reflex, the **first objective** of this study was to determine Bezold-Jarisch reflex function in conscious guinea pigs fed control diet or 1% cholesterol diet for 13 weeks and to establish the relative contribution of the parasympathetic and sympathetic nervous systems in mediating these responses.. The Bezold-Jarisch reflex was elicited by systemic injections of the selective 5-HT_3 receptor agonist, phenylbiguanide. The Bezold-Jarisch reflex responses elicited by phenylbiguanide and 2-methyl-5-HT are subject to pronounced tachyphylaxis (see Whalen et al., 2000; Owen et al., 2005). It appears that this tachyphylaxis is due to the down-regulation of 5-HT_3 receptors on vagal cardiopulmonary afferent terminals rather than the loss of central and/or efferent processing of the Bezold-Jarisch reflex (see Whalen et al., 2000; Owen et al., 2005). In order to establish whether hypercholesterolemia influences the mechanisms regulating receptor status, the **second objective** of this study was to compare the rate of development of tachyphylaxis to phenylbiguanide in control and cholesterol-fed guinea pigs

MATERIALS AND METHODS

Guinea Pigs and Surgical Procedures

All experimental methodologies were carried out in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. Experimental protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. Male Hartley guinea pigs that were on control or 1% cholesterol diet (Research Diets, Inc) for 12 weeks were anesthetized with acepromazine (120 mg/kg, ip)-ketamine (12 mg/kg, ip) and a catheter (PE-50) was placed in a carotid artery to record arterial blood pressure and heart rate. A catheter (PE-50) was also placed in a jugular vein to administer drugs. The catheters were exteriorized, all wounds sutured closed, and catheters kept patent with daily flushing of heparinized saline. Guinea pigs had 6-7 days to recover from surgery, such that the experiments began after completion of 13 weeks of the dietary regimen.

Protocols

Study 1. The Bezold-Jarisch reflex-mediated reductions in mean arterial blood pressure and heart rate elicited by bolus injections of phenylbiguanide (10-100 μg/kg, iv) were determined in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs before and beginning 15 min after administration of the muscarinic receptor antagonist, methyl-atropine (1 mg/kg, iv). The Bezold-Jarisch reflex-mediated reductions in mean arterial blood pressure and heart rate elicited by bolus injections of phenylbiguanide (10-100 μg/kg, iv) were determined in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs before and beginning 15 min after administration of the β_1-adrenoceptor antagonist, atenolol (1 mg/kg, iv). Each injection of phenylbiguanide was given 5 min apart to allow the responses to subside completely before another injection was given.

Study 2. The Bezold-Jarisch reflex reductions in mean arterial blood pressure and heart rate elicited by five injections of phenylbiguanide (100 μg/kg, iv) were determined in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. Bezold-Jarisch reflex responses elicited by 5-HT (20 μg/kg, iv) and L-S-nitrosocysteine (5 μmol/kg, iv) were determined before and after the injections of phenylbiguanide. The above injections were given 5 min apart to allow the responses to subside completely before another injection was given. The injections of 5-HT were given to determine that desensitization of 5-HT_3 receptors elicited by phenylbiguanide caused a loss of response to the natural ligand for these receptors. L-S-nitrosocysteine elicits the Bezold-Jarisch reflex by activating recognition sites on vagal cardiopulmonary afferents, which are distinct from 5-HT_3 receptors (Whalen et al., 2000; Owen et al., 2005). As such, the injections of L-S-nitrosocysteine were given before and after the injections of phenylbiguanide to provide evidence that the use-dependent loss of response to phenylbiguanide was not due to the loss of afferent, central and/or efferent processing of the Bezold-Jarisch reflex (Owen et al., 2005).

Drugs

All drugs were from Sigma (St. Louis, MO) except for ketamine and acepromazine (Abbott (Chicago, IL) and phenylbiguanide (Research Biochemicals, Natick, MA). L-S-nitrosocysteine was synthesized immediately before use as described previously (see Davisson et al., 1996a).

Statistics

Data are shown as mean ± S.E.M. and were analyzed by repeated-measures analysis of variance (Winer, 1971) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means (Wallenstein et al., 1980). A value of $P < 0.05$ denoted significance.

RESULTS

Bezold-Jarisch Reflex Responses Elicited by Phenylbiguanide

Resting heart rate and mean arterial blood pressure values during the period over which the injections of phenylbiguanide were given were similar in the conscious control and cholesterol-fed guinea pigs. Heart rate values in the control and cholesterol-fed guinea pigs were 263 ± 8 and 269 ± 9 beats/min, respectively ($P > 0.05$). Resting mean arterial blood pressure values in the control and cholesterol-fed guinea pigs were 73 ± 3 and 77 ± 4 mmHg, respectively ($P > 0.05$). The Bezold-Jarisch reflex-mediated reductions in heart rate and mean arterial pressure elicited by the bolus injections of phenylbiguanide (10-100 μg/kg, iv) in conscious control and cholesterol-fed guinea pigs are summarized in **Figure 6.1.** As can be seen, phenylbiguanide elicited dose-dependent and pronounced responses in control guinea pigs. These responses were markedly smaller in the cholesterol-fed guinea pigs.

Role of Parasympathetic and Sympathetic Efferents in the Bezold-Jarisch Reflex Responses

A. Parasympathetic

The administration of the muscarinic receptor antagonist, methyl-atropine (1 mg/kg, iv) elicited prompt and sustained increases in heart rate in conscious control and cholesterol-fed guinea pigs whereas it did not affect resting mean arterial blood pressure in either group. Resting heart rate values in control guinea pigs before and after the administration of methyl-atropine were 266 ± 7 and 293 ± 8 beats/min, respectively ($P < 0.05$). Resting heart rate values in cholesterol-fed guinea pigs before and after injection of methyl-atropine were 269 ± 7 and 320 ± 8 beats/min, respectively ($P < 0.05$). The arithmetic increase in heart rate was bigger in cholesterol-fed than in the control animals (+51 ± 5 and +27 ± 3 beats/min, respectively, $P < 0.05$). Resting mean

arterial blood pressure values prior to the administration of methyl-atropine were similar in control and cholesterol-fed guinea pigs (75 ± 3 and 76 ± 9 mmHg, respectively, $P > 0.05$). The administration of methyl-atropine did not affect resting mean arterial blood pressure values in control or cholesterol-fed guinea pigs (+2 ± 2 and -1 ± 2 mmHg, respectively, $P > 0.05$). The Bezold-Jarisch reflex-mediated reductions in heart rate and mean arterial pressure elicited by bolus injections of phenylbiguanide (10-100 μg/kg, iv) in conscious control or cholesterol-fed guinea pigs before and after administration of methyl-atropine (1 mg/kg, iv) are summarized in **Figure 6.2** and **Figure 6.3**, respectively**.** As can be seen, phenylbiguanide induced negligible Bezold-Jarisch reflex responses after administration of the muscarinic receptor antagonist.

B. Sympathetic

The administration of the β_1-adrenoceptor antagonist, atenolol (1 mg/kg, iv), elicited prompt and sustained decreases in heart rate in conscious control and cholesterol-fed guinea pigs whereas it did not affect resting mean arterial blood pressure in either group. Resting heart rate values in control guinea pigs before and after the administration of atenolol were 271 ± 6 and 230 ± 8 beats/min, respectively ($P < 0.05$). Resting heart rate values in cholesterol-fed guinea pigs before and after the administration of methyl-atropine were 268 ± 7 and 254 ± 8 beats/min, respectively ($P < 0.05$). The arithmetic decrease in heart rate was bigger in the control than in the cholesterol-fed animals (-41 ± 4 and -14 ± 3 beats/min, respectively, $P < 0.05$). Resting mean arterial blood pressure values prior to the administration of atenolol were similar in control and cholesterol-fed guinea pigs (74 ± 3 and 72 ± 9 mmHg, respectively, $P > 0.05$). The administration of atenolol did not affect resting mean arterial blood pressure values in control or cholesterol-fed guinea pigs

(+1 ± 2 and +1 ± 2 mmHg, respectively, $P > 0.05$).

The Bezold-Jarisch reflex-mediated falls in heart rate and mean arterial pressure elicited by systemic injection of phenylbiguanide (10-100 μg/kg, iv) in conscious control and cholesterol-fed guinea pigs before and after injection of atenolol (1 mg/kg, iv) are summarized in **Figure 6.4** and **Figure 6.5,** respectively**.** As can be seen, the Bezold-Jarisch reflex responses elicited by phenylbiguanide were virtually identical before and after administration of the β_1-adrenoceptor antagonist in both groups of guinea pigs.

Tachyphylaxis to Phenylbiguanide

Resting heart rate and mean arterial blood pressure values during the time period over which the bolus systemic injections of 5-HT, L-S-nitrosocysteine and phenylbiguanide were administered (see below) were similar in the conscious control and cholesterol-fed guinea pigs. The heart rate values in the control and cholesterol-fed guinea pigs were 265 ± 8 and 263 ± 9 beats/min, respectively ($P > 0.05$). Resting mean arterial blood pressure values in control and cholesterol-fed guinea pigs were 74 ± 3 and 74 ± 4 mmHg, respectively ($P > 0.05$). More specifically, the development of tachyphylaxis to phenylbiguanide did cause any changes in resting heart rate and mean arterial blood pressure ($P > 0.05$ for all comparisons).

The Bezold-Jarisch reflex-induced falls in heart rate and mean arterial pressure elicited by five successive injections of phenylbiguanide (100 μg/kg, iv) in conscious control guinea pigs are summarized in **Figure 6.6.** The Bezold-Jarisch reflex responses elicited by 5-HT (20 μg/kg, iv) and L-S-nitrosocysteine (5 μmol/kg, iv) are also shown. Tachyphylaxis to the phenylbiguanide-

induced responses occurred upon successive injection of this selective 5-HT_3 receptor agonist. The responses elicited by injection 3 were diminished compared to those elicited by injection 1. The developed tachyphylaxis to phenylbiguanide was very pronounced such that injection 5 of phenylbiguanide elicited minor responses only. The Bezold-Jarisch reflex responses elicited by 5-HT were markedly reduced when given after the injections of phenylbiguanide whereas the L-S-nitrosocysteine responses were not affected.

The Bezold-Jarisch reflex-induced reduction in heart rate and mean arterial pressure elicited by five successive injections of phenylbiguanide (100 μg/kg, iv) in conscious cholesterol-fed guinea pigs are shown in **Figure 6.7.** The Bezold-Jarisch reflex responses elicited by bolus injections of 5-HT (20 μg/kg, iv) and L-S-nitrosocysteine (5 μmol/kg, iv) are also shown. Tachyphylaxis to the phenylbiguanide-induced responses were evident with injection 2 and subsequent injections elicited negligible responses. The rate of development of tachyphylaxis to the phenylbiguanide-induced reductions in heart rate and mean arterial blood pressure was considerably faster than in control guinea pigs. The Bezold-Jarisch reflex responses elicited by 5-HT were virtually absent when given after phenylbiguanide whereas the L-S-nitrosocysteine-induced responses were similar before and after the injections of phenylbiguanide.

DISCUSSION

The systemic injections of the selective 5-HT_3 receptor agonist, phenylbiguanide, elicited dose-dependent Bezold-Jarisch reflex-mediated reductions in heart rate and mean arterial blood pressure in conscious control guinea pigs. One principal finding of this study was that the Bezold-Jarisch reflex responses elicited by phenylbiguanide were substantially smaller in the cholesterol-fed guinea pigs. The Bezold-Jarisch reflex responses elicited by phenylbiguanide were virtually abolished by the muscarinic receptor antagonist, methyl-atropine, in the control and cholesterol-fed guinea pigs. In contrast, the Bezold-Jarisch reflex responses elicited by phenylbiguanide were minimally affected by the selective β_1-adrenoceptor antagonist, atenolol. We demonstrated that direct electrical stimulation of the peripherally projecting segment of the transected vagus elicited robust and similar reductions in heart rate and mean arterial blood pressure in anesthetized control and cholesterol-fed guinea pigs (see **Chapter 5**). Moreover, we found that methyl-atropine elicited a greater increase in heart rate in the cholesterol-fed than in the control guinea pigs (this Chapter and **Chapter 5**).

Taken together, the above findings suggest that the diminished Bezold-Jarisch reflex responses elicited by phenylbiguanide in the cholesterol-fed guinea pigs may be due to the down regulation of 5-HT_3 receptors on vagal cardiopulmonary afferents that mediate the Bezold-Jarisch reflex. However, the possibility that vagal cardiopulmonary afferent function and/or central processing of afferent information are compromised in the cholesterol-fed guinea pigs cannot be discounted. It is unlikely that reduced baroreflex activity in the cholesterol-fed guinea pigs (see **Chapter 4**) explains our findings since sinoaortic baroreceptor denervation greatly augments the Bezold-Jarisch reflex in anesthetized rats (Meller et al., 1990).

This study demonstrates that tachyphylaxis to the Bezold-Jarisch reflex-mediated cardiovascular responses elicited by phenylbiguanide readily developed in conscious control and cholesterol-fed guinea pigs. The Bezold-Jarisch reflex responses elicited by 5-HT were markedly attenuated after administration of phenylbiguanide whereas the Bezold-Jarisch reflex responses elicited by L-S-nitrosocysteine were not affected. The loss of response to 5-HT confirms that tachyphylaxis to phenylbiguanide down-regulates the 5-HT_3 receptors by which the natural ligand exerts its effects. It should be noted that the subsequent 5-HT_2 receptor-mediated pressor response elicited by 5-HT was not affected by the development of tachyphylaxis to phenylbiguanide (data not shown). The pressor responses elicited by 5-HT are due primarily to 5-HT_2 receptor-mediated constriction of vascular smooth muscle although direct actions on neurons on the anteroventral third ventricle are also involved (see Muntzel et al., 1996; Whalen et al., 2000). As such, the present findings suggest that the loss of 5-HT_3 receptor function does not impact the functional status of 5-HT_2 receptors in the vasculature.

A vital finding was that the Bezold-Jarisch reflex responses elicited by L-S-nitrosocysteine (see Whalen et al., 2000; Owen et al., 2005) were not diminished after development of tachyphylaxis to the Bezold-Jarisch reflex responses elicited by phenylbiguanide in control or cholesterol-fed guinea pigs. L-S-nitrosocysteine stimulates vagal cardiopulmonary afferents via activation of stereoselective L-S-nitrosocysteine recognition sites that are distinct from 5-HT_3 and 5-HT_2 receptors (see Whalen et al., 2000; Owen et al., 2005). Taken together, these findings suggest that the loss of response to phenylbiguanide and 5-HT is due to the down-regulation of 5-HT_3 receptors rather than the loss of cardiopulmonary afferent function and/or central processing of the Bezold-Jarisch reflex.

The principal finding was that tachyphylaxis to the Bezold-Jarisch reflex responses elicited by phenylbiguanide occurred more rapidly in the cholesterol-fed guinea pigs. It should be noted that the mechanisms responsible for the desensitization of 5-HT_3 receptors have not been determined (see Whalen et al., 2000). However, the accelerated rate of development of tachyphylaxis to phenylbiguanide in cholesterol-fed guinea pigs may be a function of the compromised status of 5-HT_3 receptors on vagal cardiopulmonary afferents. However, it is certainly possible that the dynamic processes involved in "resensitizing" 5-HT_3 receptors (i.e., those processes involved in re-establishing the function of these ion-channel receptors and/or their re-incorporation into the plasma membrane) are compromised in the cholesterol-fed animals.

It should also be noted that co-activation of 5-HT_2 receptors on vagal afferents are essential for the full expression of the Bezold-Jarisch reflex in anesthetized rats (Meller et al., 1991, 1992) and that the Bezold-Jarisch reflex responses elicited by 5-HT are not subject to tachyphylaxis unless 5-HT_2 receptors are blocked (Lacolley at al., 2005a,b). The observation that the Bezold-Jarisch reflex responses elicited by 5-HT were smaller in the cholesterol-fed than in the control animals raises the possibility that 5-HT_2 receptors are down-regulated on vagal cardiopulmonary afferents in the cholesterol-fed animals. One essential experiment that will be performed in the future pertains to the issue as to whether tachyphylaxis to 5-HT occurs in the absence of 5-HT_2 receptor blockade in cholesterol-fed guinea pigs. Taken together, the present finding raise the possibility that hypercholesterolemia impairs the functional status of 5-HT_3 receptors perhaps by interfering with desensitization, down-regulation and resensitization processes. Future studies that address the mechanisms by which hypercholesterolemia affects the functional status of ion-channel and G protein-coupled receptors are certainly warranted.

SUMMARY

In conclusion, our studies demonstrated that the Bezold-Jarisch reflex responses elicited by phenylbiguanide were markedly attenuated in guinea pigs consuming a high cholesterol diet for 13 weeks. Our findings tentatively suggest that hypercholesterolemia impairs the functional status of 5-HT3 ion-channel receptors. Future mechanistic studies are warranted to determine the precise affects of hypercholesterolemia in the Bezold-Jarisch reflex arc (e.g., affects on synthesis and release of neurotransmitters within the brain) and whether hypercholesterolemia affects the functional status of other ion-channel receptors (e.g., nicotinic cholinergic receptors and N-methyl-D-aspartate receptors) and G protein coupled receptors (e.g., β-adrenoceptors).

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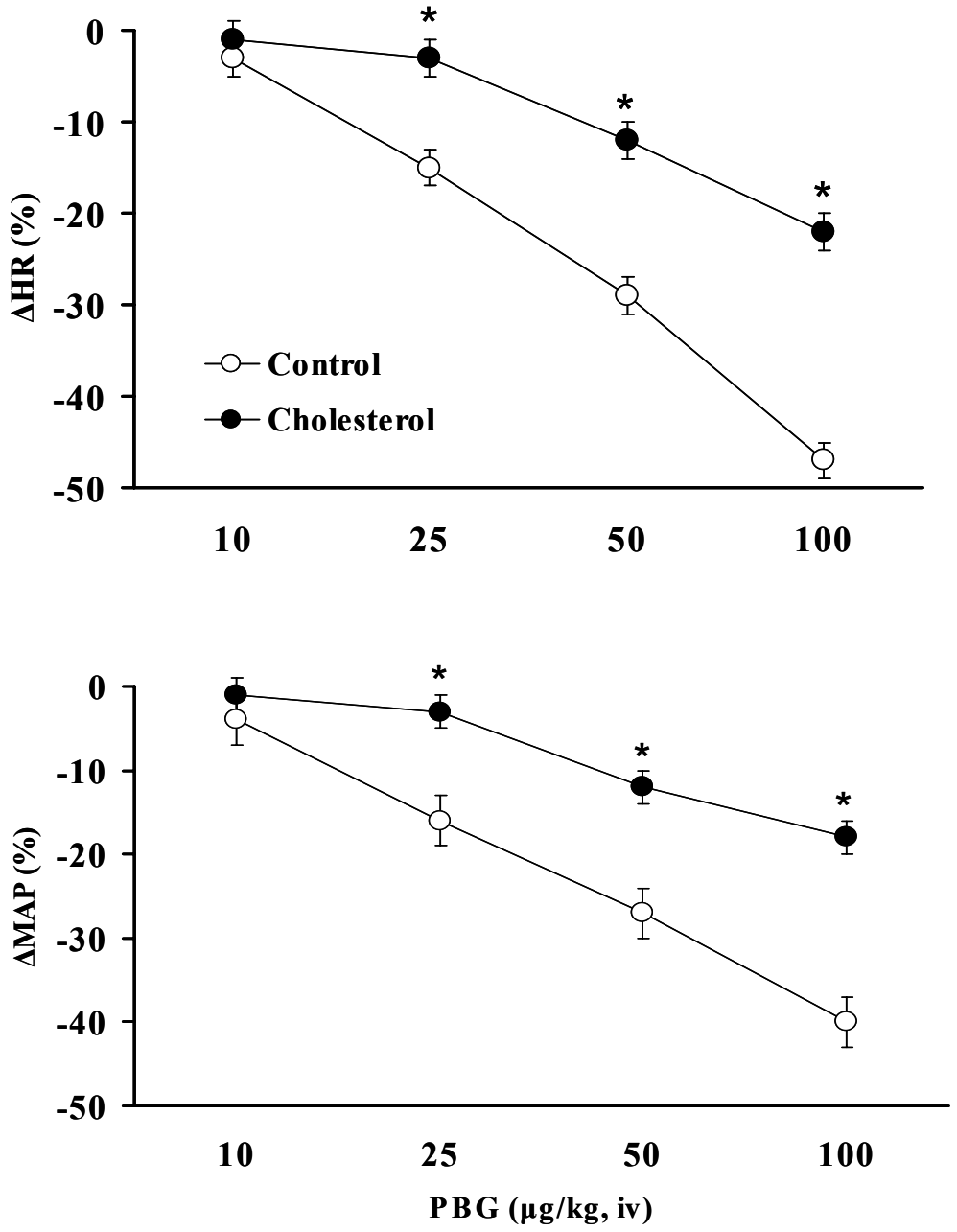


Figure 6.1. A summary of the changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of phenylbiguanide (PBG; 10-100 µg/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *P < 0.05, cholesterol versus control.

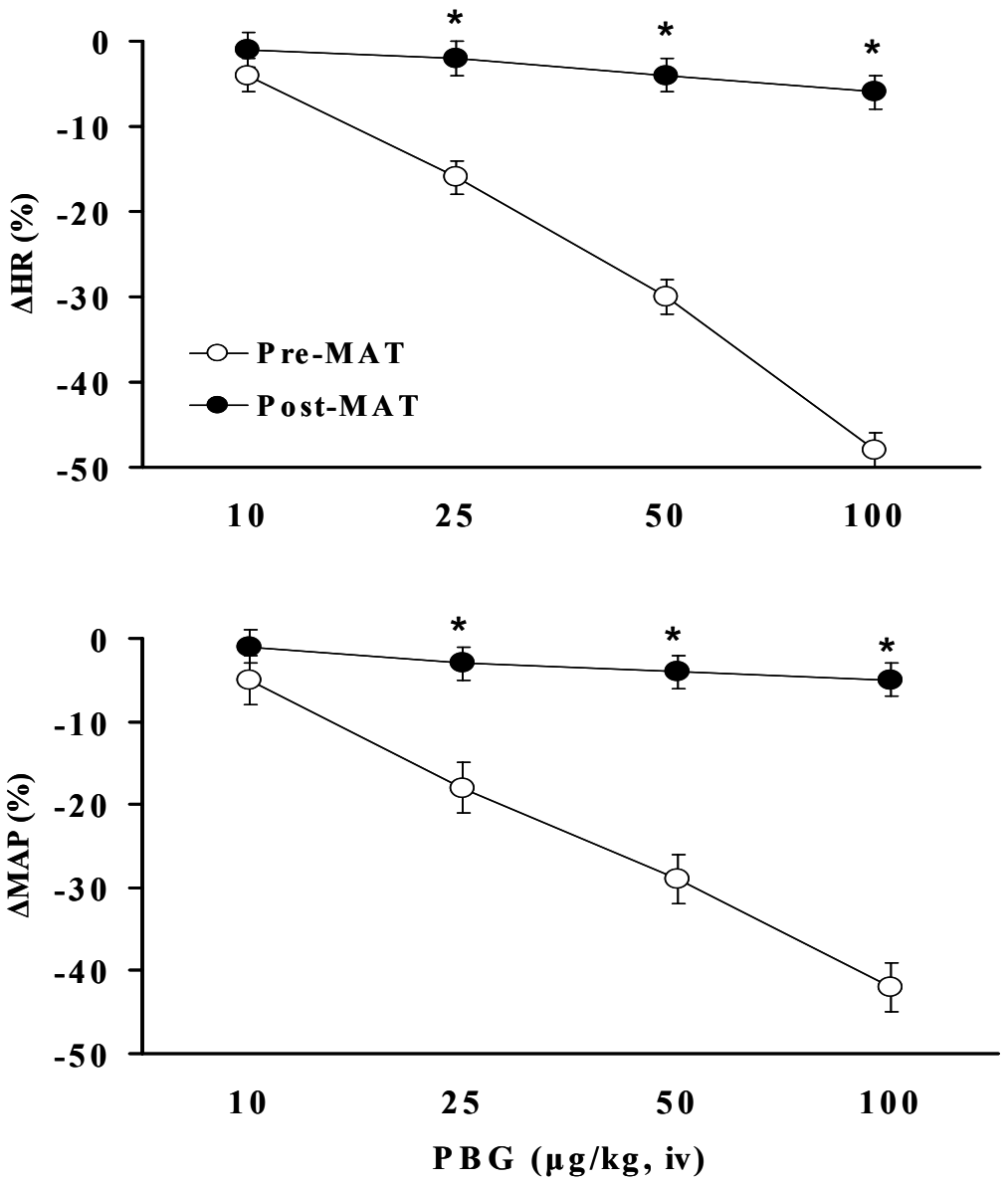


Figure 6.2. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of phenylbiguanide (PBG; 10-100 µg/kg, iv) in conscious control (n=8) guinea pigs, before and after systemic injection of methyl atropine (MAT; 1 mg/kg, iv). The data are shown as mean ± SEM. *$P < 0.05$, cholesterol versus control.

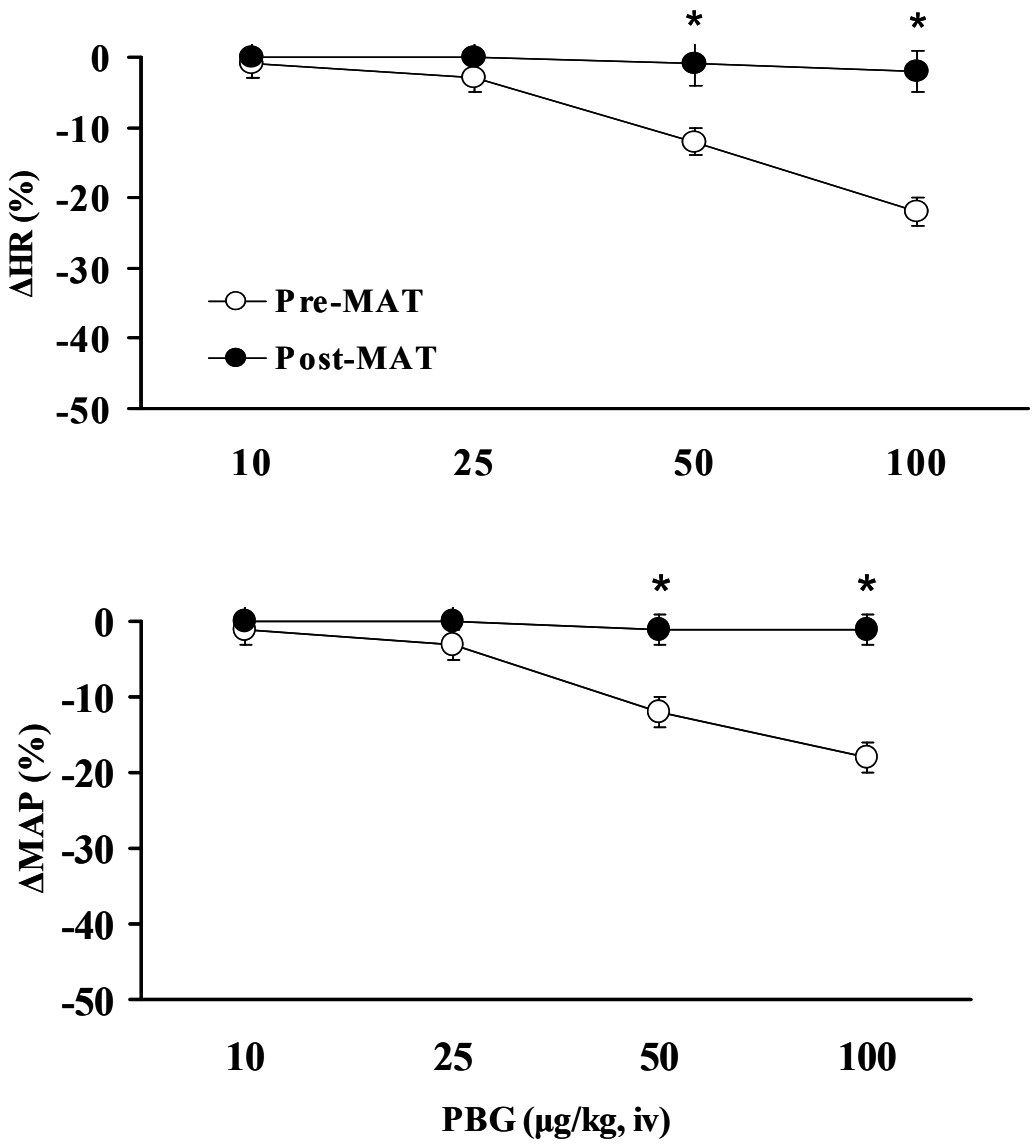


Figure 6.3. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of phenylbiguanide (PBG; 10-100 µg/kg, iv) in conscious cholesterol-fed (n=8) guinea pigs, before and after systemic injection of methyl atropine (MAT; 1 mg/kg, iv). The data are shown as mean ± SEM *$P < 0.05$, cholesterol versus control.

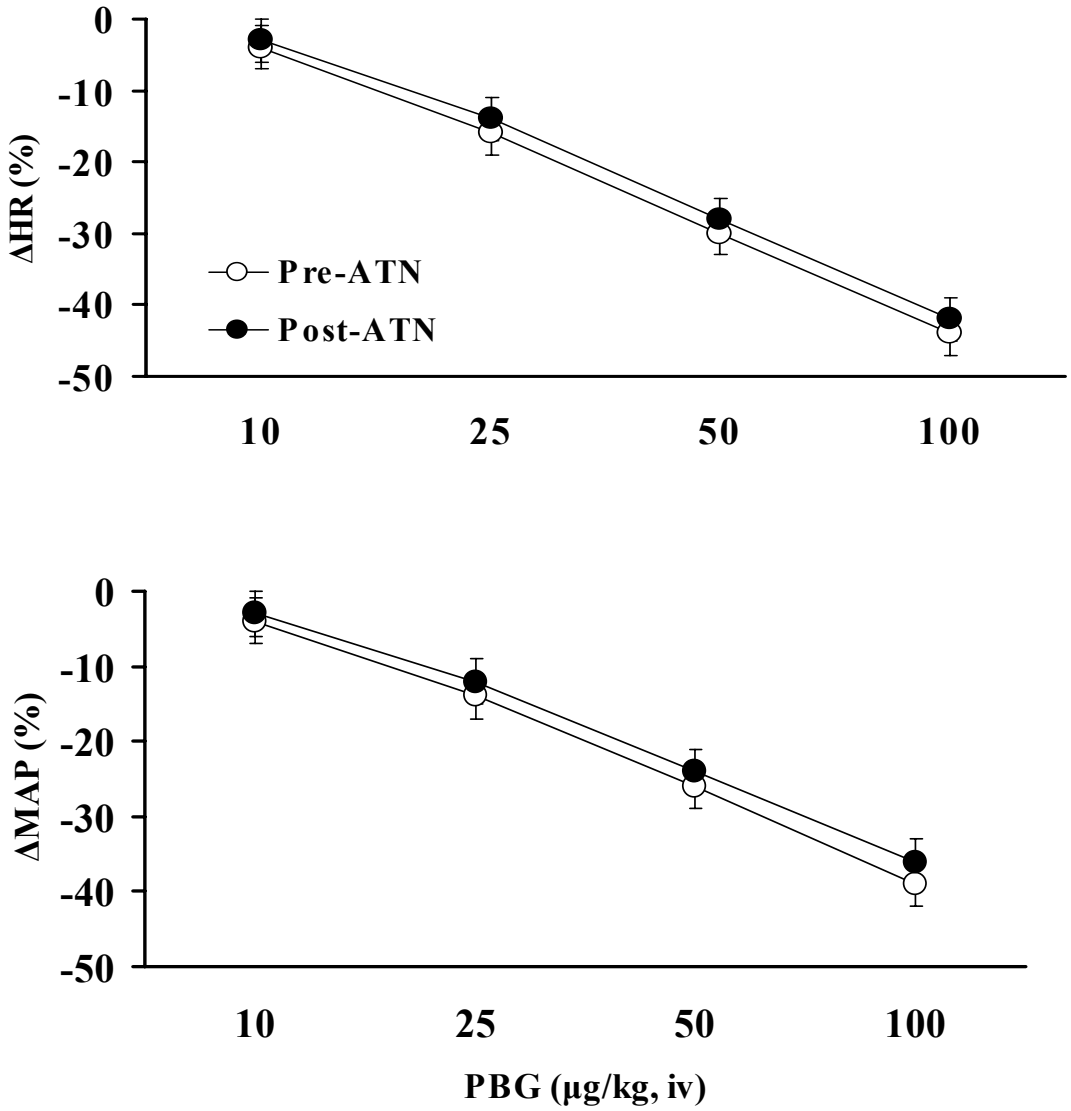


Figure 6.4. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of phenylbiguanide (PBG; 10-100 µg/kg, iv) in conscious control (n=8) guinea pigs, before and after systemic injection of atenolol (ATN; 1 mg/kg, iv). The data are shown as mean ± SEM *$P < 0.05$, cholesterol versus control.

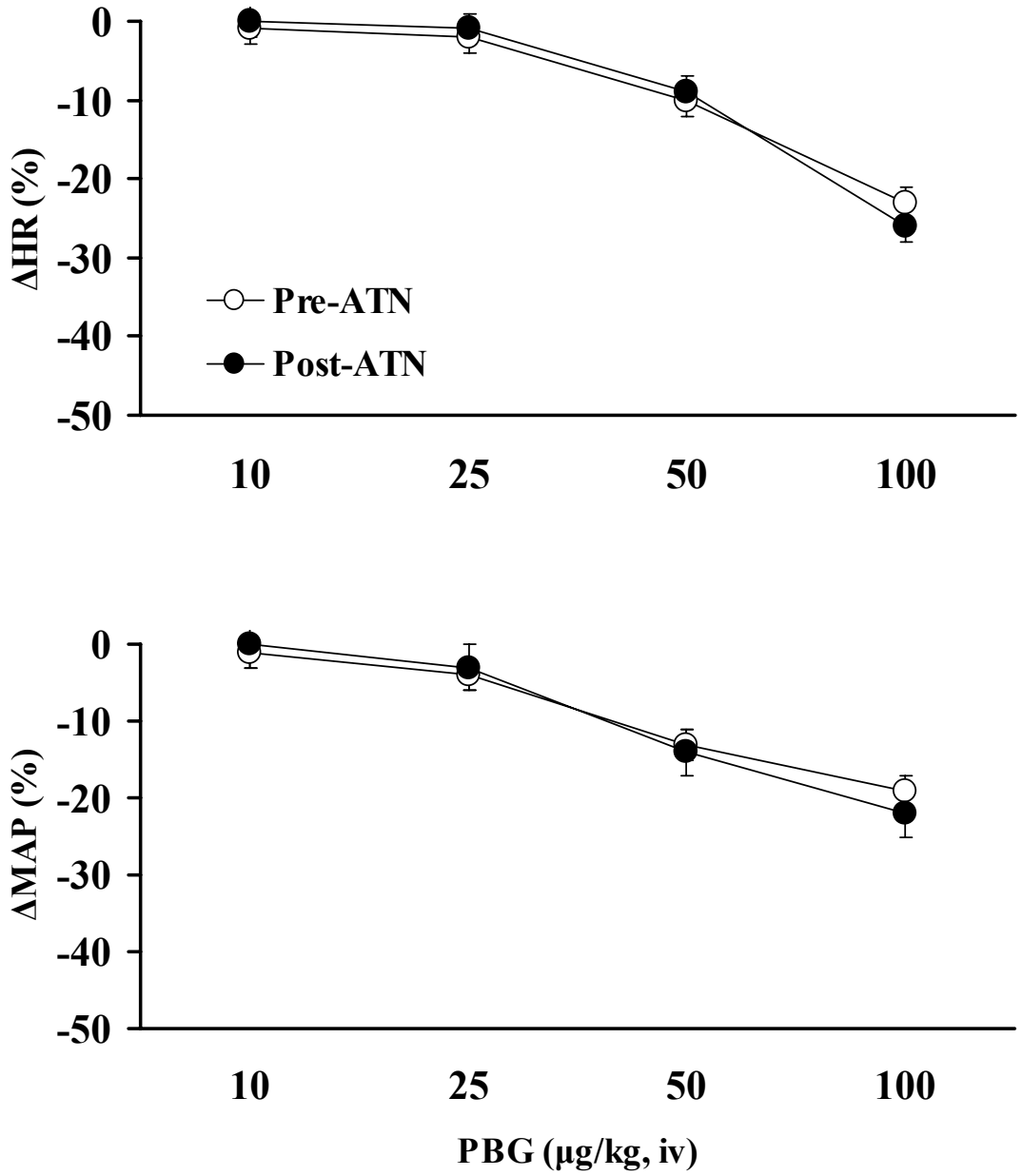


Figure 6.5. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of phenylbiguanide (PBG; 10-100 µg/kg, iv) in conscious cholesterol-fed (n=8) guinea pigs, before and after systemic injection of atenolol (ATN; 1 mg/kg, iv). The data are shown as mean ± SEM **P* < 0.05, cholesterol versus control.

Figure 6.6

Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by 5-hydroxytryptamine (5-HT; 40 μg/kg, iv), L-S-nitrosocysteine (5 μmol/kg, iv), and phenylbiguanide (PBG; 100 μg/kg, iv) in conscious control guinea pigs (n=8). The first bolus injections of 5-HT and L-S-nitrosocysteine were followed by five successive bolus injections of phenylbiguanide, which were followed by the second injections of 5-HT and L-S-nitrosocysteine. The data are shown as mean ± SEM *$P < 0.05$, Injections 2-5 versus Injection 1. $^{\dagger}P < 0.05$, 5-HT responses after the injections of phenylbiguanide versus before. Note, that the L-S-nitrosocysteine responses were similar before and after administration of phenylbiguanide ($P > 0.05$, for both comparisons).

CONTROLS

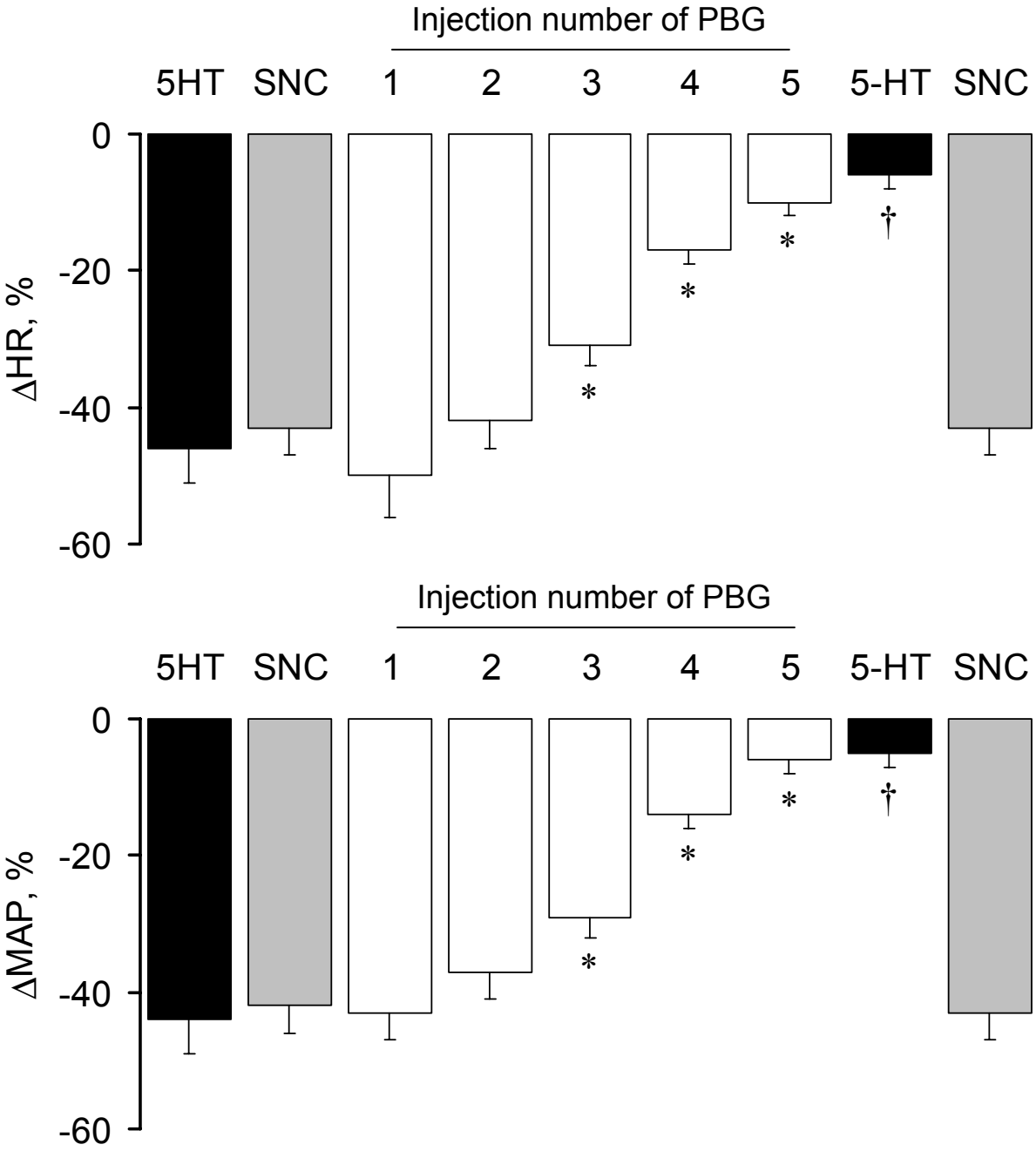
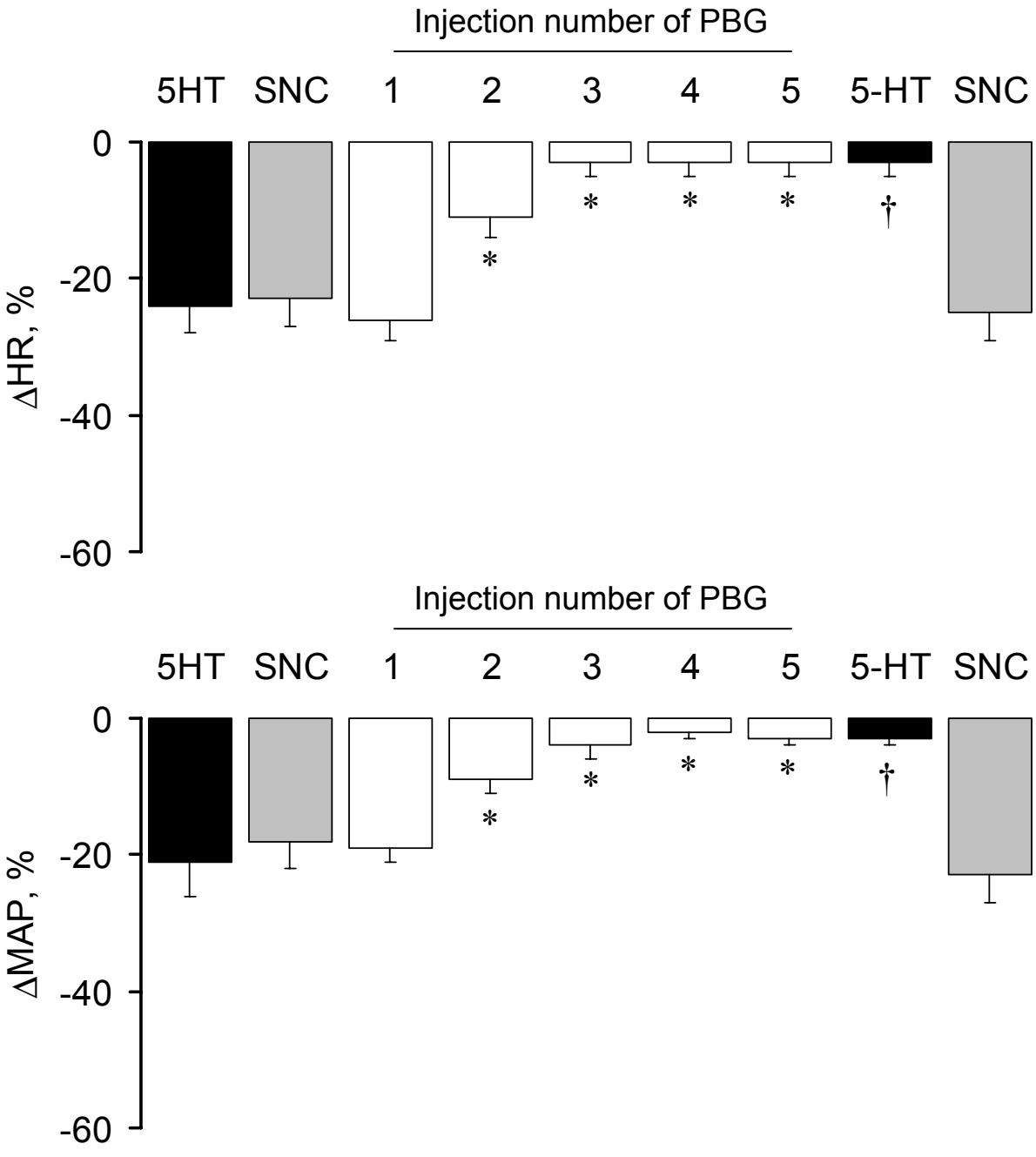

Injection number of PBG
5HT
SNC
1
2
3
4
5
5-HT
SNC
0
-20
-40
-60
ΔHR, %
*
†
Injection number of PBG
5HT
SNC
1
2
3
4
5
5-HT
SNC
0
-20
-40
-60
ΔMAP, %
*
†

Figure 6.7

Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by 5-hydroxytryptamine (5-HT; 40 μg/kg, iv), L-S-nitrosocysteine (5 μmol/kg, iv), and phenylbiguanide (PBG; 100 μg/kg, iv) in conscious cholesterol-fed guinea pigs (n=8). The first bolus injections of 5-HT and L-S-nitrosocysteine were followed by five successive bolus injections of phenylbiguanide, which were followed by the second injections of 5-HT and L-S-nitrosocysteine. The data are shown as mean ± SEM *$P < 0.05$, Injections 2-5 versus Injection 1. $^{\dagger}P < 0.05$, 5-HT responses after the injections of phenylbiguanide versus before. Note, that the L-S-nitrosocysteine responses were similar before and after administration of phenylbiguanide ($P > 0.05$, for both comparisons).

CHOLESTEROL-FED



CHAPTER 7

EFFECTS OF HIGH CHOLESTEROL DIET ON ENDOTHELIUM-DEPENDENT AND ENDOTHELIUM-INDEPENDENT VASODILATION IN CONSCIOUS GUINEA PIGS[1]

[1]Owen JR, Barton MH, Munday JS, Graves JE, Lewis SJ. To be submitted to *British Journal of Pharmacology*.

ABSTRACT

Hypercholesterolemia elicits endothelial dysfunction in humans and experimental animals. The key issue as to whether hypercholesterolemia disturbs the synthesis/release and/or vasodilator potencies of endothelium-derived relaxing factors including nitric oxide and L-S-nitrosocysteine has not been fully established. The objectives of this study were (1) to compare the hypotensive potencies of the endothelium-dependent agonist, acetylcholine, the nitric oxide donor, sodium nitroprusside and L-S-nitrosocysteine, in conscious control and cholesterol-fed (1% cholesterol for 13 weeks) guinea pigs, and (2) to provide evidence as to whether storage/release of putative preformed endothelial pools of L-S-nitrosocysteine are diminished in cholesterol-fed animals.

Bolus injections of acetylcholine (50-500 ng/kg, iv), L-S-nitrosocysteine (25-200 nmol/kg) and sodium nitroprusside (1-8 μg/kg, iv) elicited dose-dependent reductions in mean arterial blood pressure (MAP) in control and cholesterol-fed guinea pigs. However, the hypotensive potencies of acetylcholine and L-S-nitrosocysteine were markedly diminished whereas the hypotensive potency of sodium nitroprusside was augmented in cholesterol-fed guinea pigs.

Bolus injection of nitric oxide synthase inhibitor, N^G-nitro-L-arginine methylester (L-NAME, 50 μmol/kg, iv), elicited a robust hypertension in control guinea pigs and a greater hypertension in cholesterol-fed guinea pigs. The depressor responses elicited by six successive injections of acetylcholine (500 ng/kg, iv) were similar to one another in saline-treated control guinea pigs. The first injection of acetylcholine (500 ng/kg, iv) elicited a substantial depressor response in L-NAME-treated control guinea pigs. However, subsequent injections of acetylcholine elicited progressively and markedly smaller depressor responses. The depressor responses elicited by the

first five injections of acetylcholine (500 ng/kg, iv) were similar to one another in saline-treated cholesterol-fed guinea pigs. However, the sixth injection elicited a smaller depressor response compared to that elicited by the first injection. The first injection of acetylcholine (500 ng/kg, iv) elicited a robust fall in MAP in the L-NAME-treated cholesterol-fed guinea pigs. However, the subsequent injections of acetylcholine elicited progressively and markedly smaller responses.

Our findings demonstrate that the diminished endothelium-dependent vasodilation in cholesterol-fed guinea pigs is associated with the diminished vasodilator potency of L-S-nitrosocysteine but not nitric oxide. The loss of response to acetylcholine in cholesterol-fed guinea pigs may involve the down-regulation of muscarinic receptors on vascular endothelial cells thereby diminishing the activation of nitric oxide synthase and/or release of endothelium-derived L-S-nitrosocysteine. The loss of response to acetylcholine in cholesterol-fed guinea pigs may involve down-regulation of stereoselective L-S-nitrosocysteine recognition sites on vascular smooth muscle. These studies also provide evidence that the putative storage/release of preformed pools of L-S-nitrosocysteine are diminished in cholesterol-fed guinea pigs.

Key words: Endothelium-derived relaxing factors, nitric oxide, S-nitrosothiols.

INTRODUCTION

The catalytically self-contained P_{450} enzyme, nitric oxide synthase (NOS), converts L-arginine to L-citrulline and the free radical nitric oxide (see Ignarro, 1990; Moncada et al., 1991**;** Michel and Feron, 1997; Cooke and Dzau, 1997). NOS activity increases when stimuli promote increases in intracellular calcium concentrations. More specifically, calcium binds to calmodulin and this complex binds to a calcium-calmodulin recognition domain on NOS, which activates the enzyme (see Ignarro, 1990; Moncada et al., 1991). NOS is present in endothelial cells of the vasculature (Moncada et al., 1991**;** Michel and Feron, 1997 Cooke and Dzau, 1997), cardiac muscle and pacemaker cells (Balligand et al., 1994, 1995; Grocott-Mason et al., 1994; Han et al., 1994), and in lumbar autonomic neurons innervating the hindlimb vasculature (Davisson et al., 1994, 1996b,c, 1997a). Endothelium-derived nitric oxide readily diffuses into adjacent vascular smooth muscle where it activates soluble guanylate cyclase to increase production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (Ignarro, 1990; Moncada et al., 1991**;** Michel and Feron, 1997 Cooke and Dzau, 1997). This cGMP activates cGMP-dependent protein kinase, which relaxes vascular smooth muscle by mechanisms such as phosphorylation/inhibition of voltage-sensitive calcium channels (see Travis et al., 2000; Layland et al., 2002). Hence nitric oxide is commonly referred to as an endothelium-derived relaxing factor (EDRF).

There is compelling evidence that the S-nitrosothiol, L-S-nitrosocysteine (NO^+-L-cysteine), is an EDRF (Myers et al., 1990; Bates et al., 1991; Rubanyi et al., 1991, Rosenblum et al., 1992) and an endothelium-derived hyperpolarizing factor (EDHF) (Batenburg et al., 2004a,b). There is also evidence that preformed pools of S-nitrosothiols and/or dinitrosyl iron complexes exist in many tissues including the vasculature (Matsunga and Furchgott, 1991; Chaudry et al., 1993; Venturini

et al., 1993; Kubaszewski et al., 1994; Muller et al., 1996, 2002; Vanin, 1998; Malyshev et al., 1999; Manukina et al., 1999; Pshennikova et al., 2000; Smirin et al., 1999, 2000), brain (Kluge et al., 1997), gastric fundus (Buyukafsar et al., 1999a), corpus cavernosum (Buyukafsar et al., 1999b, 2003) and red blood cells (Jia et al., 1996). Nitric oxide synthase exists in the membranes of cytoplasmic vesicles in endothelial cells and autonomic nerves (Loesch et al., 1993; Loesch and Burnstock, 1994, 1996). The presence of nitric oxide synthase in the membranes of these vesicles raises the possibility that L-S-nitrosocysteine is synthesized and stored in these vesicles. Indeed, there is considerable indirect evidence that vesicular pools of L-S-nitrosocysteine exist in vesicles that are subject to calcium/calmodulin-dependent exocytosis in vascular endothelial cells (Davisson et al., 1996a; Danser et al., 1998, 2000; Kakuyama et al., 1998) and lumbar autonomic neurons (Davisson et al., 1996b,c, 1997a; Colombari et al., 1998; Possas and Lewis, 1997).

The biological actions of L-S-nitrosocysteine only minimally involve its decomposition to nitric oxide (Kowaluk and Fung, 1990; Mathews and Kerr, 1993). S-nitrosothiols regulate the activity of functional proteins such as ion-channels and enzymes via the S-nitrosylation (NO^+ transfer) of key cysteine residues in these proteins (Stamler et al., 1992, 1997; Lipton et al., 1993; Stamler, 1994; Bolotina et al., 1994; Campbell et al., 1996; Lang et al., 2003). L-S-nitrosocysteine and structurally related S-nitrosothiols activate stereoselective recognition sites on vascular smooth muscle (Davisson et al., 1996d, Travis et al., 1996, 1997; Travis and Lewis, 2000; Batenburg et al., 2004a,b; Lewis et al., 2005a,b) and neurons (Lewis et al., 1996; Davisson et al., 1997b; Ohta et al., 1997; Chen et al., 2000; Li et al., 2000; Lipton et al., 2001). Whether these stereoselective recognition sites are novel cell surface receptors or known functional proteins such as calcium-activated potassium channels (Lang et al., 2003), remains to be determined.

Hypercholesterolemia is a major contributor to the development of hemodynamic disorders including endothelial dysfunction in humans and domestic and experimental animals (Saini et al., 2004). Of particular relevance to our studies is the key observation that hypercholesterolemia impairs endothelium-dependent vasodilation in coronary arteries before atherosclerotic lesions are detectable (Yamamoto et al., 1988; Zeiher et al., 1991). This issue has direct relevance to our hypercholesterolemia guinea pig model since these animals display a variety of cardiovascular disorders without visible signs of atherosclerotic lesions (see **Chapter 3**). Taken together, it would seem possible that hypercholesterolemia impairs cell signaling processes before the patho-physiological signs of hypercholesterolemia are evident.

The mechanisms by which hypercholesterolemia disturb endothelium-dependent vasodilation in the absence of atherosclerosis have not been elucidated in humans or experimental animals. In theory, elevations in membrane and/or intracellular cholesterol concentrations may (1) directly or indirectly affect the synthesis and/or storage of EDRFs/EDHFs, (2) accelerate the intracellular and/or extracellular destruction of EDRFs/EDHFs, and/or (3) impair the signaling processes by which EDRFs/EDHFs exert their vasorelaxant effects. The first **specific objective** of this study was to compare the hypotensive potencies of the endothelium-dependent agonist, acetylcholine (see Moncada et al., 1991; Davisson et al., 1996a), the nitric oxide donor, sodium nitroprusside (see Feelisch et al., 1991; Travis et al., 2000), and L-S-nitrosocysteine (see Davisson et al., 1996a,b; Travis et al., 2000), in conscious control and cholesterol-fed (1% cholesterol for 13 weeks) guinea pigs. The second **specific objective** of this study was to provide evidence as to whether the storage and/or release of putative preformed endothelial pools of L-S-nitrosocysteine (see Davisson et al., 1996a) are diminished in cholesterol-fed animals.

MATERIALS AND METHODS

Guinea Pigs and Surgical Procedures

All experimental methodologies were carried out in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. Experimental protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. Male Hartley guinea pigs that were on control or 1% cholesterol diet (Research Diets, Inc) for 12 weeks were anesthetized with acepromazine (120 mg/kg, ip)-ketamine (12 mg/kg, ip) and a catheter (PE-50) was placed in a carotid artery to record arterial blood pressure and heart rate. A catheter (PE-50) was also placed in a jugular vein to administer drugs. The catheters were exteriorized, all wounds sutured closed, and catheters kept patent with daily flushing of heparinized saline. Guinea pigs had 6-7 days to recover from surgery, such that the experiments began after completion of 13 weeks of the dietary regimen.

Protocols

Study 1. The changes in mean arterial blood pressure and heart rate elicited by bolus injections of acetylcholine (50-500 ng/kg, iv), L- and D-S-nitrosocysteine (25-200 nmol/kg, iv) and sodium nitroprusside (1-8 μg/kg, iv), were determined in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. Each injection of these vasodilator agents was given 5 min apart to allow the responses to subside completely before another injection was given.

Study 2. The changes in mean arterial blood pressure and heart rate elicited by six successive bolus injections of acetylcholine (500 ng/kg, iv) were determined before and after administration of saline in control and cholesterol-fed guinea pigs (n=10 per group). The changes in mean

arterial blood pressure and heart rate elicited by six successive bolus injections of acetylcholine (500 ng/kg, iv) were determined before and after injection of L-NAME (50 μmol/kg, iv) in control and cholesterol-fed guinea pigs (n=10 per group). The first injection of acetylcholine after injection of saline or L-NAME was given at 15 min. Bolus injections of L-S-nitrosocysteine and sodium nitroprusside were given before and after the injections of acetylcholine. In control guinea pigs, the doses of L-S-nitrosocysteine and sodium nitroprusside were 100 nmol/kg and 4 μg/kg, respectively. In cholesterol-fed guinea pigs, the doses of L-S-nitrosocysteine and sodium nitroprusside were 200 nmol/kg and 2 μg/kg, respectively. The doses of L-S-nitrosocysteine and sodium nitroprusside were chosen to match the responses of acetylcholine as closely as possible. Each injection of the above vasodilator agents were given 5 min apart to allow the responses to subside completely before another injection was given.

Drugs

All drugs were obtained from Sigma (St. Louis, MO). Ketamine and acepromazine were from Abbott (Chicago, IL). All drugs were dissolved and diluted for injection in sterile saline. L- and D-S-nitrosocysteine were synthesized immediately before use (see Davisson et al., 1996a).

Statistics

The data are shown as mean ± S.E.M. and were analyzed by repeated-measures analysis of variance (Winer, 1971) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means (Wallenstein et al., 1980). A value of $P < 0.05$ was taken to denote statistical significance.

RESULTS

Hypotensive actions of the vasodilators in control and cholesterol-fed guinea pigs

Acetylcholine: Resting mean arterial blood pressures prior to injection of acetylcholine were similar in control and cholesterol-fed conscious guinea pigs (71 ± 3 versus 73 ± 3 mmHg, respectively, $P > 0.05$). Resting heart rates prior to injection of acetylcholine were also similar in control and in cholesterol-fed guinea pigs (267 ± 7 versus 272 ± 8 beats/min, respectively, $P > 0.05$). The cardiovascular responses elicited by systemic injection of acetylcholine in conscious control and cholesterol-fed animals are summarized in **Figure 7.1**. As can be seen, acetylcholine elicited dose-dependent reductions in mean arterial pressure in control guinea pigs which were accompanied by baroreflex-mediated increases in heart rate. The depressor responses elicited by acetylcholine were markedly smaller in cholesterol-fed guinea pigs. The associated baroreflex-mediated increases in heart rate were also smaller.

L- and D-S-nitrosocysteine: Resting mean arterial blood pressure values prior to injection of L- and D-S-nitrosocysteine were similar in control and cholesterol-fed conscious guinea pigs (70 ± 2 versus 72 ± 2 mmHg, respectively, $P > 0.05$). Resting heart rates prior to injection of acetylcholine were also similar in control and cholesterol-fed guinea pigs (269 ± 7 versus 274 ± 8 beats/min, respectively, $P > 0.05$). The cardiovascular responses elicited by systemic injections of L- and D-S-nitrosocysteine in conscious control animals are summarized in **Figure 7.2**. L-S-nitrosocysteine elicited dose-dependent falls in mean arterial pressure which were accompanied by baroreflex-mediated increases in heart rate. As can be seen, the depressor responses elicited by D-S-nitrosocysteine were substantially smaller than those elicited by L-S-nitrosocysteine. The associated baroreflex-mediated increases in heart rate were also smaller. The cardiovascular

responses elicited by L-S-nitrosocysteine in conscious control and cholesterol-fed guinea pigs are summarized in **Figure 7.3**. The dose-dependent falls in mean arterial blood pressure elicited by L-S-nitrosocysteine were substantially smaller in cholesterol-fed guinea pigs. The associated baroreflex-mediated increases in heart rate were also smaller.

Sodium nitroprusside: Resting mean arterial blood pressures prior to injection of sodium nitroprusside were similar in control and cholesterol-fed guinea pigs (74 ± 3 versus 73 ± 3 mmHg, respectively, $P > 0.05$). Resting heart rates prior to injection of sodium nitroprusside were also similar in control and in cholesterol-fed guinea pigs (274 ± 7 versus 272 ± 8 beats/min, respectively, $P > 0.05$). The cardiovascular responses elicited by systemic injection of sodium nitroprusside in conscious control and cholesterol-fed guinea pigs are summarized in **Figure 7.4**. Sodium nitroprusside elicited dose-dependent falls in mean arterial pressure in the control guinea pigs which was accompanied by baroreflex-mediated increases in heart rate. The depressor responses elicited by sodium nitroprusside were markedly greater in cholesterol-fed guinea pigs. However, the associated baroreflex-mediated increases in heart rate in the cholesterol-fed guinea pigs were smaller than in the control animals.

Effects of saline and L-NAME on resting cardiovascular parameters

The effects of an injection of saline or L-NAME (50 μmol/kg, iv) on resting cardiovascular parameters in control and cholesterol-fed guinea pigs are summarized in **Table 1**. The injection of saline did not affect resting mean arterial blood pressure or heart rate in control guinea pigs. The injection of L-NAME elicited a sustained increase in mean arterial blood pressure in control guinea pigs and an even greater sustained increase in mean arterial blood pressure in cholesterol-

fed animals. The increases in mean arterial blood pressures were associated with a sustained bradycardia in control guinea pigs but a sustained tachycardia in the cholesterol-fed guinea pigs.

Repeated administration of acetylcholine in saline-treated control guinea pigs

The falls in mean arterial blood pressure elicited by bolus injections of L-S-nitrosocysteine (100 nmol/kg, iv), sodium nitroprusside (4 μg/kg, iv) and acetylcholine (500 nmol/kg, iv) before and after injection of saline in control guinea pigs are summarized in the **top panel** of **Figure 7.5**. The depressor responses elicited by these vasodilator agents were similar before and after the injection of saline. The depressor responses elicited by six successive injections of acetylcholine (500 nmol/kg, iv) before and after injection of saline in the above conscious control guinea pigs are summarized in the **bottom panel** of **Figure 7.5**. The first L-S-nitrosocysteine and sodium nitroprusside responses and the responses elicited by Injection 1 of acetylcholine are the post-saline responses shown in the **top panel** of **Figure 7.5**. Bolus injections of the above doses of L-S-nitrosocysteine and sodium nitroprusside were also given after the injections of acetylcholine. As can be seen, each injection of acetylcholine elicited similar depressor responses. In addition, the L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine.

Repeated administration of acetylcholine in saline-treated cholesterol-fed guinea pigs

The falls in mean arterial blood pressure elicited by bolus injections of L-S-nitrosocysteine (200 nmol/kg, iv), sodium nitroprusside (2 μg/kg, iv) and acetylcholine (500 nmol/kg, iv) before and after injection of saline in cholesterol-fed guinea pigs are summarized in the **top panel** of **Figure 7.6**. The depressor responses elicited by these vasodilator agents were similar before and after

the injection of saline. The depressor responses elicited by six successive injections of acetylcholine (500 nmol/kg, iv) before and after injection of saline in the above conscious cholesterol-fed guinea pigs are summarized in the **bottom panel** of **Figure 7.6**. The first L-S-nitrosocysteine and sodium nitroprusside responses and the responses elicited by Injection 1 of acetylcholine are the post-saline responses shown in the **top panel** of **Figure 7.6**. Bolus injections of the above doses of L-S-nitrosocysteine and sodium nitroprusside were also given after the injections of acetylcholine. As can be seen, the first five injections of acetylcholine elicited similar depressor responses whereas the sixth injection elicited a depressor response that was less than that elicited by Injection 1. In addition, the L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine.

Repeated administration of acetylcholine in L-NAME-treated control guinea pigs

The falls in mean arterial blood pressure elicited by bolus injections of L-S-nitrosocysteine (100 nmol/kg, iv), sodium nitroprusside (4 μg/kg, iv) and acetylcholine (500 nmol/kg, iv) before and after injection of L-NAME (50 μmol/kg, iv) in control guinea pigs are summarized in the **top panel** of **Figure 7.7**. The depressor responses elicited by L-S-nitrosocysteine and sodium nitroprusside were augmented after administration of L-NAME whereas the depressor response elicited by acetylcholine was of similar magnitude. The depressor responses elicited by six successive injections of acetylcholine (500 nmol/kg, iv) before and after injection of L-NAME in the above conscious control guinea pigs are summarized in the **bottom panel** of **Figure 7.7**. The first L-S-nitrosocysteine and sodium nitroprusside responses and the responses elicited by Injection 1 of acetylcholine are the post-saline responses shown in the **top panel** of **Figure 7.7**. Bolus injections of the above doses of L-S-nitrosocysteine and sodium nitroprusside were also

given after the injections of acetylcholine. As can be seen, the first injection of acetylcholine elicited a robust depressor response. However, subsequent injections elicited progressively smaller responses such that the sixth injection produced a minor response only. The L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine.

Repeated administration of acetylcholine in L-NAME-treated cholesterol-fed guinea pigs

The falls in mean arterial blood pressure elicited by bolus injections of L-S-nitrosocysteine (100 nmol/kg, iv), sodium nitroprusside (4 μg/kg, iv) and acetylcholine (500 nmol/kg, iv) before and after injection of L-NAME (50 μmol/kg, iv) in cholesterol-fed guinea pigs are summarized in the **top panel** of **Figure 7.8**. The depressor responses elicited by sodium nitroprusside were greater after administration of L-NAME whereas the depressor response elicited by L-S-nitrosocysteine and acetylcholine were of similar magnitude. The depressor responses elicited by six successive injections of acetylcholine (500 nmol/kg, iv) before and after injection of L-NAME in the above conscious cholesterol-fed guinea pigs are summarized in the **bottom panel** of **Figure 7.8**. The first L-S-nitrosocysteine and sodium nitroprusside responses and the responses elicited by Injection 1 of acetylcholine are the post-L-NAME responses shown in the **top panel** of **Figure 7.8**. Bolus injections of the above doses of L-S-nitrosocysteine and sodium nitroprusside were also given after the injections of acetylcholine. As can be seen, the first injection of acetylcholine elicited a relatively robust depressor response. Subsequent injections elicited progressively smaller responses such that the sixth injection produced a minor response only. The L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine.

DISCUSSION

Diminished Endothelium-Dependent Vasodilation in Cholesterol-Fed Guinea Pigs

One principal finding of the present study was that the depressor responses elicited by the endothelium-dependent vasodilator, acetylcholine, were substantially diminished in cholesterol-fed guinea pigs. This finding is consistent with substantial evidence that hypercholesterolemia elicits endothelial dysfunction in humans and animals (Saini et al., 2004). Hypercholesterolemia impairs endothelium-dependent vasodilation in coronary arteries before atherosclerotic lesions are detectable (Yamamoto et al., 1988; Zeiher et al., 1991). This is particularly relevant to our studies since the cholesterol-fed guinea pigs do not show obvious signs of atherosclerosis. Accordingly, the effects of high cholesterol on cardiovascular function are likely to involve alterations in cell signaling processes. The diminished responses to acetylcholine could involve (1) down-regulation of muscarinic receptors on vascular endothelial cells and/or reduced intracellular signaling elicited by G protein-coupled muscarinic receptors. This could involve diminished calcium/calmodulin-dependent activation of nitric oxide synthase and/or mobilization of S-nitrosothiol-containing vesicles. Direct evidence that muscarinic receptors on endothelial cells are down-regulated in cholesterol-fed guinea pigs must await appropriate binding studies. However, it should be noted that we provided indirect evidence that cardiac muscarinic receptor function is not compromised in cholesterol-fed guinea pigs (see **Chapter 4**).

Assuming normal muscarinic receptor function, the diminished responses to acetylcholine may involve a (1) a reduction in the amount/activity of nitric oxide synthase in vascular endothelial cells, (2) diminished number of S-nitrosothiol-containing vesicles and/or compromised vesicular exocytotic mechanisms, (3) diminished calcium-dependent activation of nitric oxide synthase

and therefore nitric oxide release, and (4) diminished calcium-dependent release of vesicular stores of S-nitrosothiols. Although the effects of cholesterol on nitric oxide production in the vascular endothelium have yet to be investigated, there is evidence that hypercholesterolemic guinea pigs have reduced endothelial nitric oxide synthase mRNA in cardiac tissue (Schwemmer et al., 2000). This finding raises the possibility that the loss of response to acetylcholine in the cholesterol-fed guinea pigs may be due to diminished nitric oxide synthase function in vascular endothelial cells. The issue as to whether hypercholesterolemia impairs exocytotic mechanisms in endothelial cells (see below) will be a focus of future experiments in our laboratory.

A principal finding of this study was that the hypotensive potency of the nitric oxide donor, sodium nitroprusside, was augmented in cholesterol-fed guinea pigs. This suggests that the cell signaling processes by which nitric oxide relaxes vascular smooth muscle are not compromised in cholesterol-fed guinea pigs. Moreover, these findings suggest that the loss of response to acetylcholine is not due to the diminished biological potency of nitric oxide. We have yet to address the mechanisms by which the hypotensive potency of nitric oxide is augmented in the cholesterol-fed guinea pigs. However, we have established that the vasodilator actions of nitric oxide in rats are mediated almost exclusively via closure of voltage-sensitive calcium channels in vascular smooth muscle (Travis et al., 2000). Moreover, we have obtained recent evidence that the greater vasodilator potency of nitric oxide in spontaneously hypertensive rats is due to the up-regulation of dihydropyridine and non-dihydropyridine classes of voltage-sensitive calcium channels (Lewis et al., 2005c) in vascular smooth muscle in these animals. We are presently performing experiments to investigate the possibility that voltage-sensitive calcium channels are up-regulated in vascular smooth muscle and cardiac cells of cholesterol-fed guinea pigs.

A key finding of this study was that the hypotensive responses elicited by L-S-nitrosocysteine were substantially diminished in the cholesterol-fed animals. This study demonstrated that the hypotensive potency of L-S-nitrosocysteine was greater than that of D-S-nitrosocysteine in the control guinea pigs. This finding is in agreement with evidence that L-S-nitrosocysteine exerts its effects via activation of stereoselective recognition sites (Lewis et al., 1996; 2005a,b; Davisson et al., 1996d, 1997b; Travis et al., 1996, 1997; Ohta et al., 1997; Travis and Lewis, 2000; Batenburg et al., 2004a,b; Chen et al., 2000; Li et al., 2000; Lipton et al., 2001). Whether these recognition sites are novel plasma membrane-bound receptors or known functional proteins such as calcium-activated potassium-channels, which are activated by L- but not D-S-nitrosocysteine (Lang et al., 1993; Batenburg et al., 2004a,b), remains to be determined.

A key finding was that the hypotensive potency of L-S-nitrosocysteine was markedly diminished in cholesterol-fed guinea pigs. This raises the possibility that the reduced hypotensive potency of acetylcholine is due to reduced vasodilator potency of endothelium-derived L-S-nitrosocysteine (see Myers et al., 1990; Rosenblum, 1992). The diminished vasodilator potency of L-S-nitrosocysteine in the cholesterol-fed guinea pigs may be due to the enhanced destruction of L-S-nitrosocysteine, perhaps via enhanced production of reactive oxygen species such as superoxide anion (see Saini et al., 2004). The reduced vasodilator potency of L-S-nitrosocysteine may also be due to down-regulation of stereoselective L-S-nitrosocysteine recognition sites on vascular smooth muscle, and/or the signaling mechanisms activated by these recognition sites (see Travis et al., 2000). The down-regulation of these stereoselective recognition sites in the cholesterol-fed guinea pigs may also be due to increased oxidant stress. More specifically, there is compelling evidence that the oxidation of key cysteine residues in these recognition sites markedly

diminished the activity of these recognition sites (Hoque et al., 1999, 2000; Lewis et al., 2005a,b). Peroxynitrite is a powerful oxidant and nitrating agent that is implicated in the pathophysiology of inflammatory states including hypercholesterolemia (Saini et al., 2004; Lewis et al., 2005b; Graves et al., 2005). We have yet to establish whether the production of peroxynitrite is increased in our hypercholesterolemic guinea pigs. However, we have provided evidence that peroxynitrite down-regulates L-S-nitrosocysteine recognition sites via oxidation of functional cysteine residues and by nitration of key tyrosine residues in these sites (Lewis et al., 2005b; Graves et al., 2005).

Diminished storage/release of putative stores of S-nitrosothiols in cholesterol-fed animals

One primary objective of this study was to provide evidence as to whether the release of putative endothelial stores of L-S-nitrosocysteine (see Davisson et al., 1996a; Danser et al., 1998, 2000) is affected in cholesterol-fed animals. Six successive injections of a higher dose of acetylcholine (i.e., 500 ng/kg, iv) elicited robust and equivalent depressor responses in saline-treated control guinea pigs. The first five of these injections elicited robust and equal depressor responses in saline-treated cholesterol-fed guinea pigs whereas the depressor response elicited by the sixth injection was smaller than the first injection. The loss of response to acetylcholine in the cholesterol-fed guinea pigs may be due to the down-regulation of muscarinic receptors on vascular endothelial cells and/or depletion of putative stores of nitrosyl factors.

An important finding of this study was that the nitric oxide synthase inhibitor, L-NAME, elicited a substantially bigger hypertension in cholesterol-fed than in control guinea pigs. Although there are many possible explanations, it may be that compromised function of nitric oxide synthase

(diminished enzyme expression and/or activity) in cholesterol-fed guinea pigs allows for more substantial effects of the 50 μmol/kg dose of L-NAME to be expressed. The first of six injections of the higher dose of acetylcholine elicited robust depressor response in L-NAME-treated control guinea pigs. Subsequent injections elicited progressively smaller responses that were not due to the diminished vasodilator potency of nitric oxide or L-S-nitrosocysteine. Indeed, the hypotensive potencies of these factors was augmented after injection of L-NAME. An increase in the hypotensive and vasodilator potency of nitric oxide and L-S-nitrosocysteine occurs in L-NAME-treated rats (see Davisson et al., 1996a,b,c; Possas and Lewis, 1997). It would appear that diminished release of endothelium-derived nitrosyl factors up-regulates the signaling mechanisms by which nitric oxide and L-S-nitrosocysteine relaxes vascular smooth muscle (see Davisson et al., 1996a,b,c; Possas and Lewis, 1997). Again the loss of response to acetylcholine may involve down-regulation of muscarinic receptors on vascular endothelial cells. However, previous studies in conscious rats and isolated rat arteries have provided compelling evidence that the loss of response to acetylcholine is due to depletion of vesicular stores of S-nitrosothiols that cannot be replenished in the absence of nitric oxide synthesis rather than the loss of muscarinic receptor function (see Davisson et al., 1996a; Danser et al., 1998, 2000).

The finding that the loss of response to acetylcholine in L-NAME-treated cholesterol-fed guinea pigs occurred much more rapidly than in control guinea pigs also supports the contention that vesicular storage of S-nitrosothiols may be diminished in these animals. We are performing ultra-structural studies to determine whether the number of vesicles *per se* are diminished in the endothelial cells of cholesterol-fed guinea pigs and/or whether the actual synthesis and storage of S-nitrosothiols such as L-S-nitrosocysteine are diminished in these vesicles. It should be noted

that whereas the vasodilator actions of sodium nitroprusside were augmented after administration of L-NAME in the cholesterol-fed guinea pigs, the vasodilator actions of L-S-nitrosocysteine were not. This finding tentatively suggests that the up-regulation of stereoselective recognition sites uner conditions of diminished exposure to endothelium-derived nitrosyl factors does not occur in hypercholesterolemic guinea pigs.

SUMMARY

To our knowledge, this is the first study to address endothelium-dependent vasodilation (i.e., that elicited by acetylcholine) in a normotensive model of cholesterol-induced cardiac concentric remodeling. Our data suggests that reduced endothelium-dependent vasodilation in cholesterol-fed guinea pigs is involved the reduced potency of endothelium-derived L-S-nitrosocysteine. Moreover, our studies suggest that vesicular storage of S-nitrosothiols may be diminished in the endothelial cells of resistance arteries of cholesterol-fed guinea pigs. These possibilities will be addressed in future functional and ultra-structural *in vitro* studies on arteries taken from control and cholesterol-fed guinea pigs. These studies will establish whether (1) endothelial cells in resistance arteries of guinea pigs contain cytoplasmic vesicles subject to exocytosis such as those that exist in endothelial cells of rats (see Bruns et al., 1968a,b; Bungaard et al., 1979; Mazzone and Kornblau, 1980, Wagner and Casley-Smith, 1981; Loesch et al., 1993, 1994; Lane et al., 1995; Moldovan et al., 1995; Loesch and Burnstock, 1996), (2) whether endothelial cells contain fusion proteins that support vesicular exocytosis as in endothelial cells of rats (see Schnitzer et al., 1995; Sudhof 1995), and (3) whether vesicles in endothelial cells of guinea pigs contain nitric oxide synthase as do rat endothelial vesicles (Loesch et al., 1993, 1994, 1996). Mice lacking type I (neuronal) and type III (endothelial) nitric oxide synthase develop concentric remodeling of the

heart, which leads to increased mortality, myocyte hypertrophy, and an age-associated increase in ventricular stiffness (Barouch et al., 2003). Schwemmer et al. (2000) determined that cardiac tissue of hypercholesterolemic guinea pigs has reduced endothelial nitric oxide synthase mRNA. Our data supports the contention that hypercholesterolemia disturbs nitric oxide synthase-dependent signaling processes (and in particular those involving S-nitrosocysteine) in vascular endothelial cells of resistance arteries as well as cardiomyocytes.

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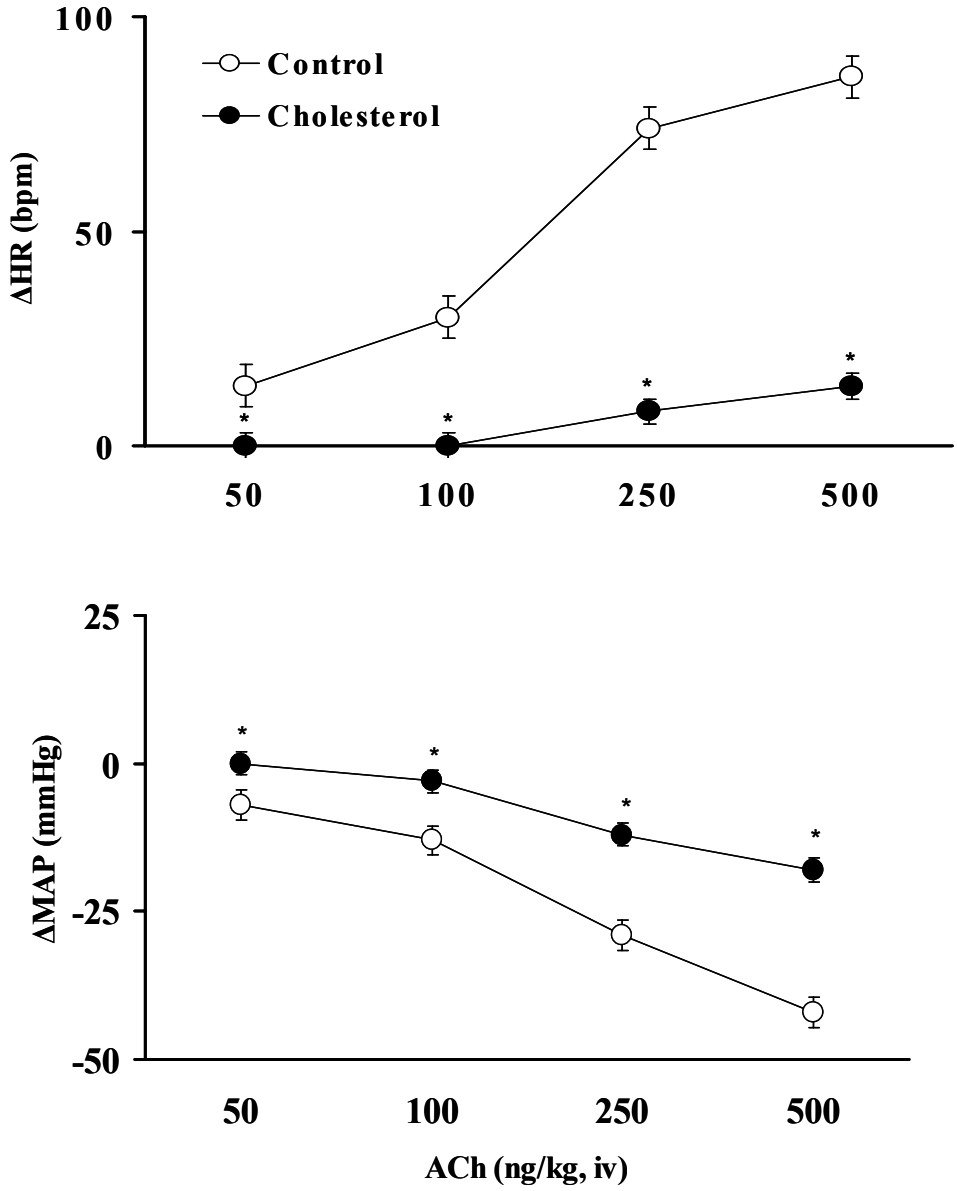

Figure 7.1. A summary of the changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of acetylcholine (ACh; 50-500 ng/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol-fed versus control.

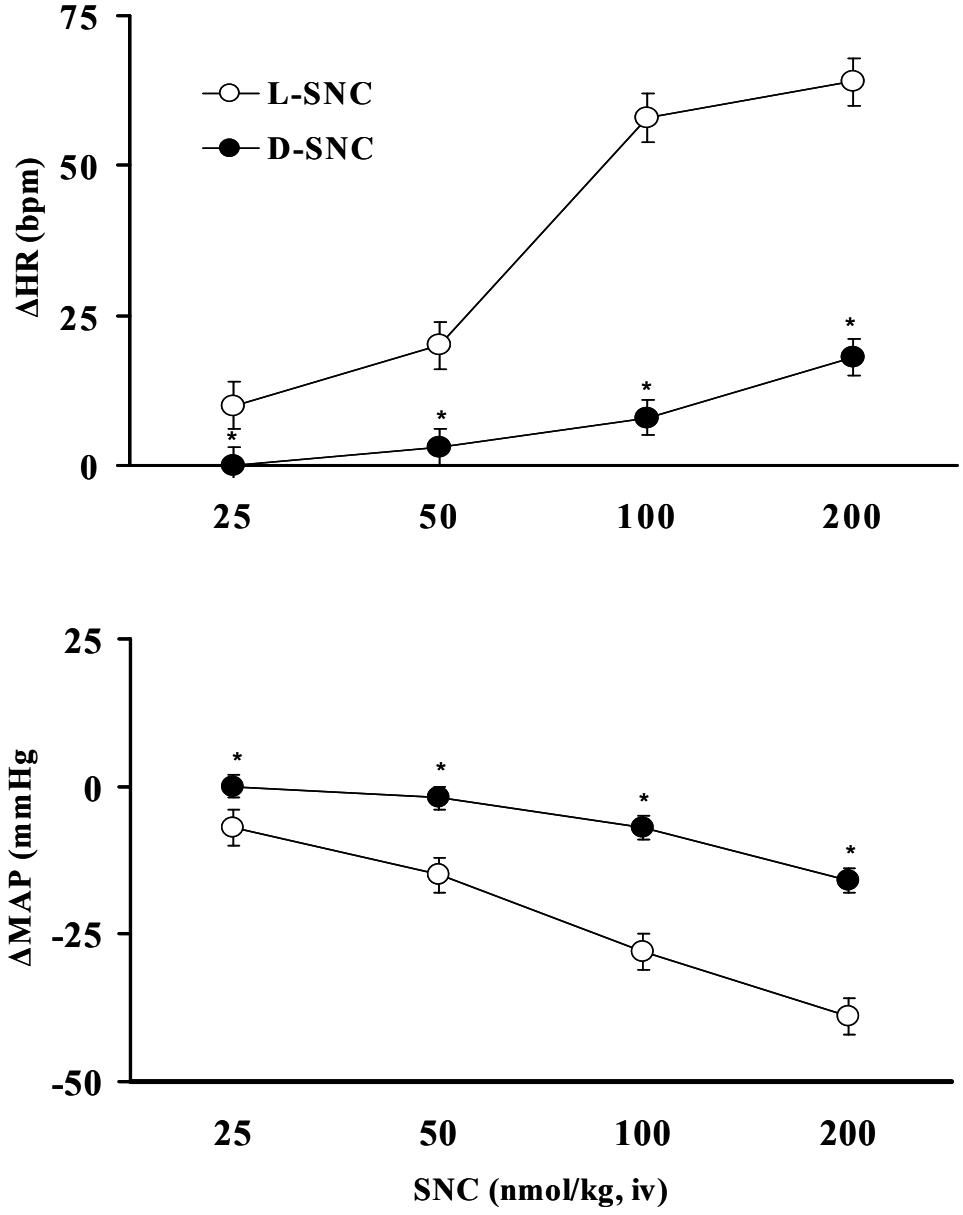

Figure 7.2. Changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of L-S-nitrosocysteine or D-S-nitrosocysteine (L-SNC and D-SNC; 25-200 nmol/kg, iv) in conscious control (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, D-SNC versus L-SNC.

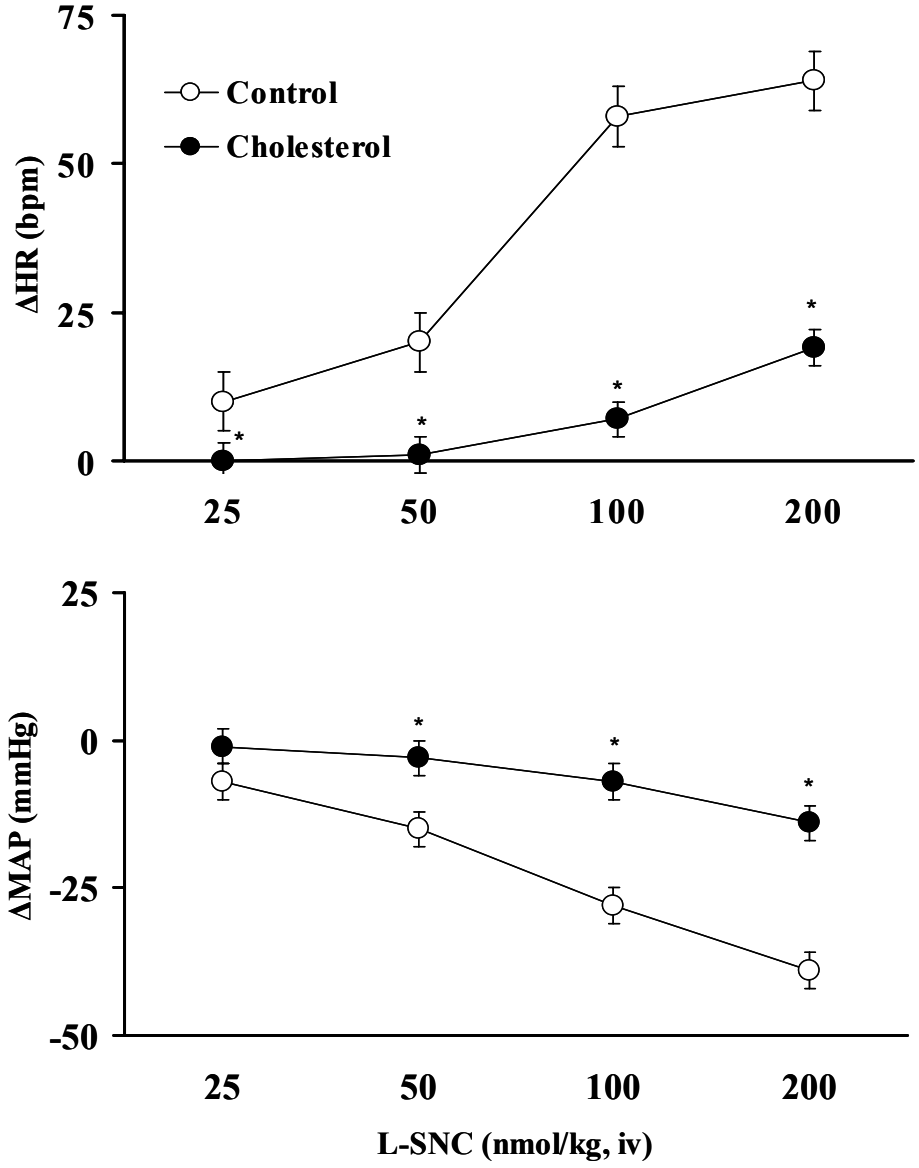

Figure 7.3. A summary of the changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of L-S-nitrosocysteine (SNC; 25-200 nmol/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. **P* < 0.05, cholesterol-fed versus control.

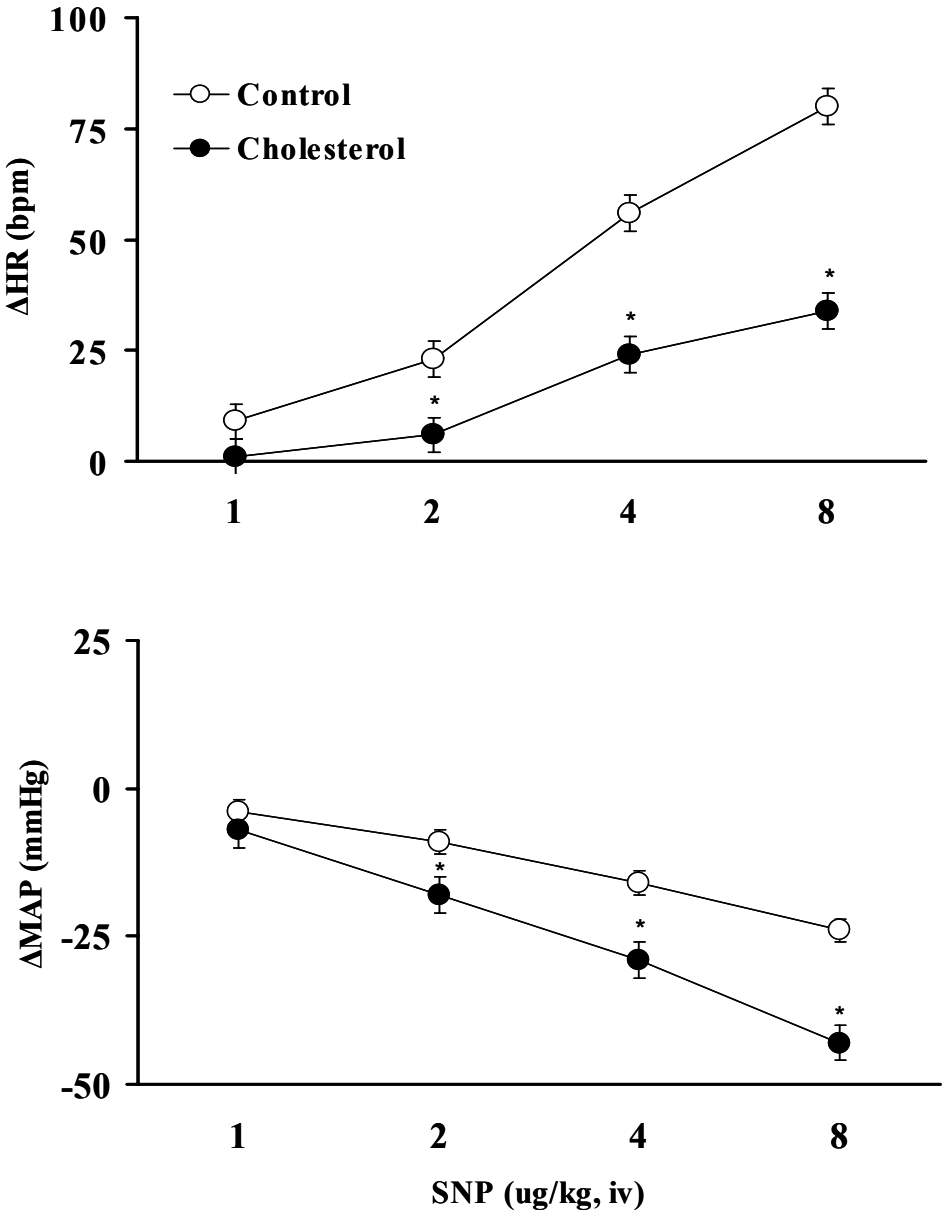

Figure 7.4. A summary of the changes in heart rate (HR) and mean arterial pressure (MAP) elicited by systemic injections of the nitric oxide donor, sodium nitroprusside (SNP; 1-8 μg/kg, iv) in conscious control (n=8) and cholesterol-fed (n=8) guinea pigs. The data are shown as mean ± SEM. *$P < 0.05$, cholesterol-fed versus control.

Figure 7.5

Top Panel. Reductions in mean arterial blood pressure (MAP) elicited by bolus injections of L-S-nitrosocysteine (SNC; 100 nmol/kg, iv), sodium nitroprusside (SNP; 4 μg/kg, iv) and acetylcholine (ACh; 500 nmol/kg, iv) before and after a bolus injection of saline in conscious control guinea pigs (n=10). **Note** that there were no differences in the responses before and after injection of saline ($P > 0.05$ for all comparisons).

Bottom Panel. Reductions in mean arterial blood pressure (MAP) elicited by six successive injections of acetylcholine (ACh; 500 nmol/kg, iv) before and after injection of saline in the above conscious control guinea pigs (n=10). Bolus injections of L-S-nitrosocysteine (L-SNC; 100 nmol/kg, iv) and sodium nitroprusside (SNP, 4 μg/kg, iv) were given before and after the injections of acetylcholine. **Note** that each injection of acetylcholine elicited similar responses and that the L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine ($P > 0.05$ for all comparisons).

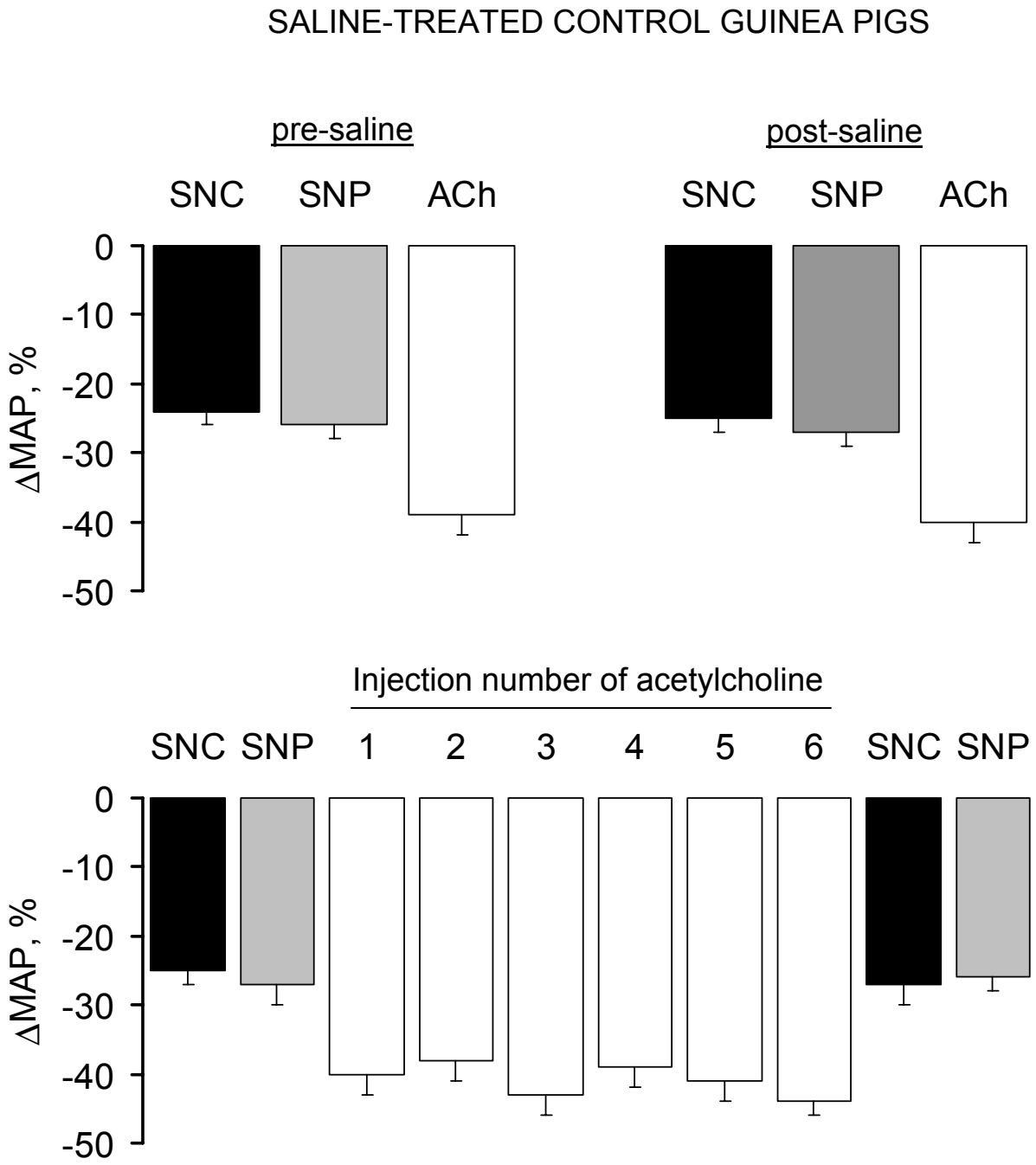
SALINE-TREATED CONTROL GUINEA PIGS
pre-saline
post-saline
SNC
SNP
ACh
SNC
SNP
ACh
0
-10
-20
-30
-40
-50
ΔMAP, %
Injection number of acetylcholine
SNC
SNP
1
2
3
4
5
6
SNC
SNP
0
-10
-20
-30
-40
-50
ΔMAP, %

Figure 7.6

Top Panel. Reductions in mean arterial blood pressure (MAP) elicited by bolus injections of L-S-nitrosocysteine (SNC; 200 nmol/kg, iv), sodium nitroprusside (SNP; 2 μg/kg, iv) and acetylcholine (ACh; 500 nmol/kg, iv) before and after a bolus injection of saline in conscious cholesterol-fed guinea pigs (n=10). **Note** that there we no differences in the responses before and after injection of saline ($P > 0.05$ for all comparisons).

Bottom Panel. Reductions in mean arterial blood pressure (MAP) elicited by six successive injections of acetylcholine (ACh; 500 nmol/kg, iv) before and after injection of saline in the above conscious cholesterol-fed guinea pigs (n=10). Bolus injections of L-S-nitrosocysteine (L-SNC; 200 nmol/kg, iv) and sodium nitroprusside (SNP, 2 μg/kg, iv) were given before and after the injections of acetylcholine. *$P < 0.05$, Injections 2-6 of acetylcholine versus Injection 1. **Note** that the L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine ($P > 0.05$ for all comparisons).

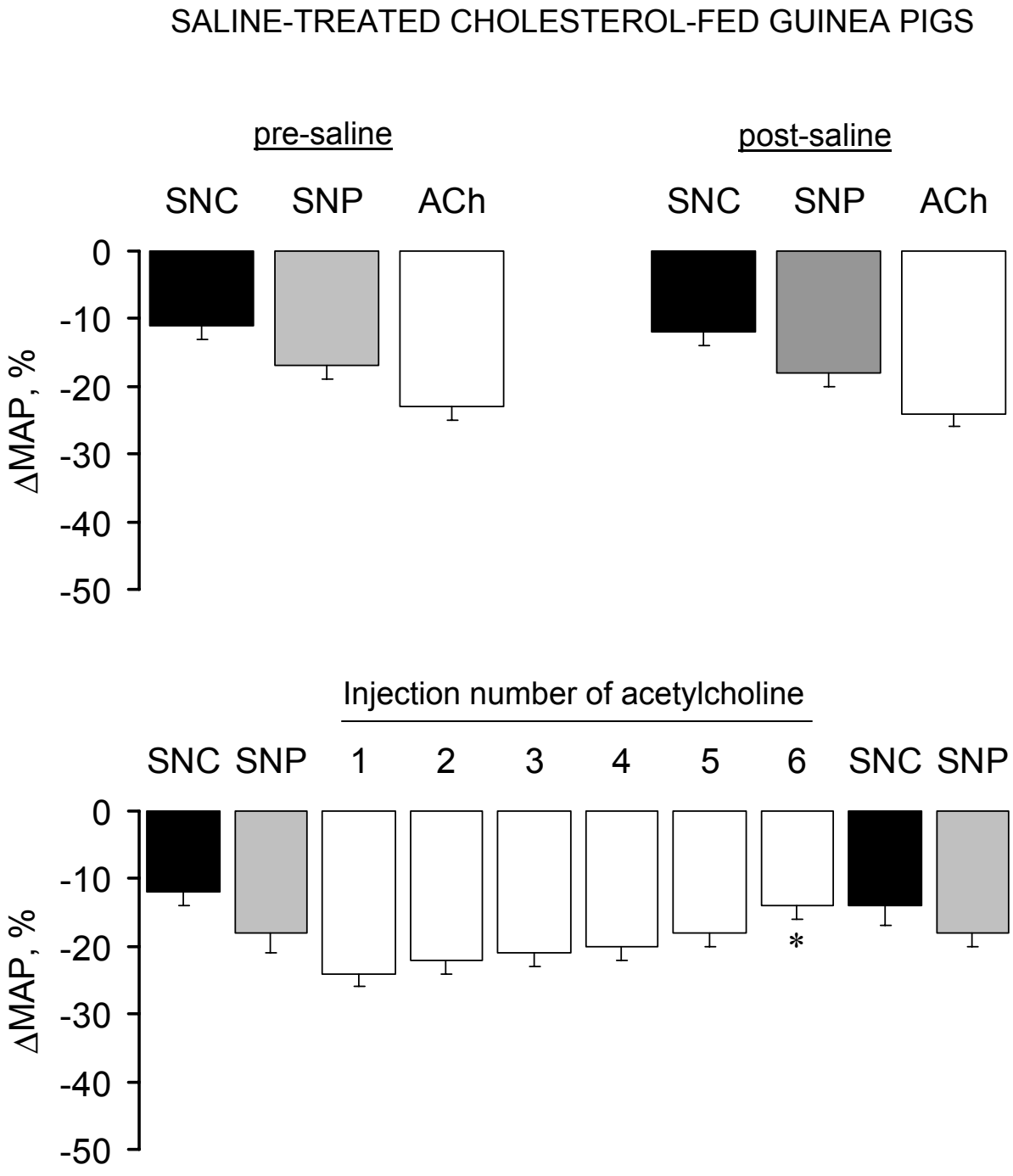
SALINE-TREATED CHOLESTEROL-FED GUINEA PIGS
pre-saline
post-saline
SNC
SNP
ACh
SNC
SNP
ACh
0
-10
-20
-30
-40
-50
ΔMAP, %
Injection number of acetylcholine
SNC
SNP
1
2
3
4
5
6
SNC
SNP
0
-10
-20
-30
-40
-50
ΔMAP, %
*

Figure 7.7

Top Panel. Reductions in mean arterial blood pressure (MAP) elicited by bolus injections of L-S-nitrosocysteine (SNC; 100 nmol/kg, iv), sodium nitroprusside (SNP; 4 μg/kg, iv) and acetylcholine (ACh; 500 nmol/kg, iv) before and after a bolus injection of L-NAME (50 μmol/kg, iv) in conscious control guinea pigs (n=10). *$P < 0.05$, post-L-NAME versus pre.

Bottom Panel. Reductions in mean arterial blood pressure (MAP) elicited by six successive injections of acetylcholine (ACh; 500 nmol/kg, iv) before and after injection of saline in the above conscious control guinea pigs (n=10). Bolus injections of L-S-nitrosocysteine (L-SNC; 100 nmol/kg, iv) and sodium nitroprusside (SNP, 4 μg/kg, iv) were given before and after the injections of acetylcholine. *$P < 0.05$, Injections 2-6 of acetylcholine versus Injection 1. **Note** that the L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine ($P > 0.05$ for all comparisons).

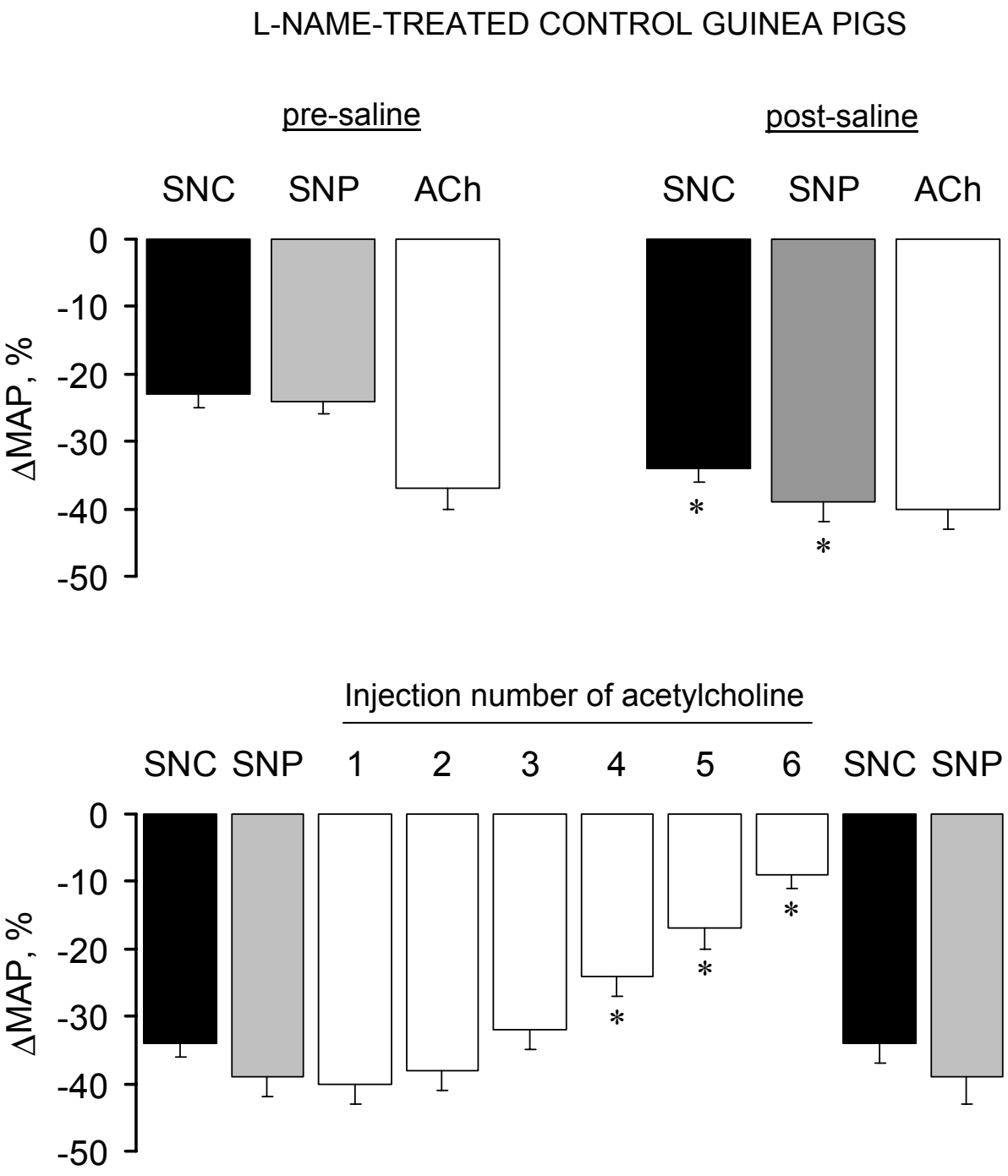
L-NAME-TREATED CONTROL GUINEA PIGS
pre-saline
post-saline
SNC
SNP
ACh
SNC
SNP
ACh
0
-10
-20
-30
-40
-50
ΔMAP, %
*
*
Injection number of acetylcholine
SNC
SNP
1
2
3
4
5
6
SNC
SNP
0
-10
-20
-30
-40
-50
ΔMAP, %
*
*
*

Figure 7.8

Top Panel. Reductions in mean arterial blood pressure (MAP) elicited by bolus injections of L-S-nitrosocysteine (SNC; 200 nmol/kg, iv), sodium nitroprusside (SNP; 2 μg/kg, iv) and acetylcholine (ACh; 500 nmol/kg, iv) before and after an injection of L-NAME (50 μmol/kg, iv) in conscious cholesterol-fed guinea pigs (n=10). *$P < 0.05$, post-L-NAME versus pre.

Bottom Panel. Reductions in mean arterial blood pressure (MAP) elicited by six successive injections of acetylcholine (ACh; 500 nmol/kg, iv) before and after injection of L-NAME in the above conscious cholesterol-fed guinea pigs (n=10). Bolus injections of L-S-nitrosocysteine (L-SNC; 200 nmol/kg, iv) and sodium nitroprusside (SNP, 2 μg/kg, iv) were given before and after the injections of acetylcholine. *$P < 0.05$, Injections 2-6 of acetylcholine versus Injection 1. **Note** that the L-S-nitrosocysteine- and sodium nitroprusside-induced responses were similar before and after the injections of acetylcholine ($P > 0.05$ for all comparisons).

L-NAME-TREATED CHOLESTEROL-FED GUINEA PIGS

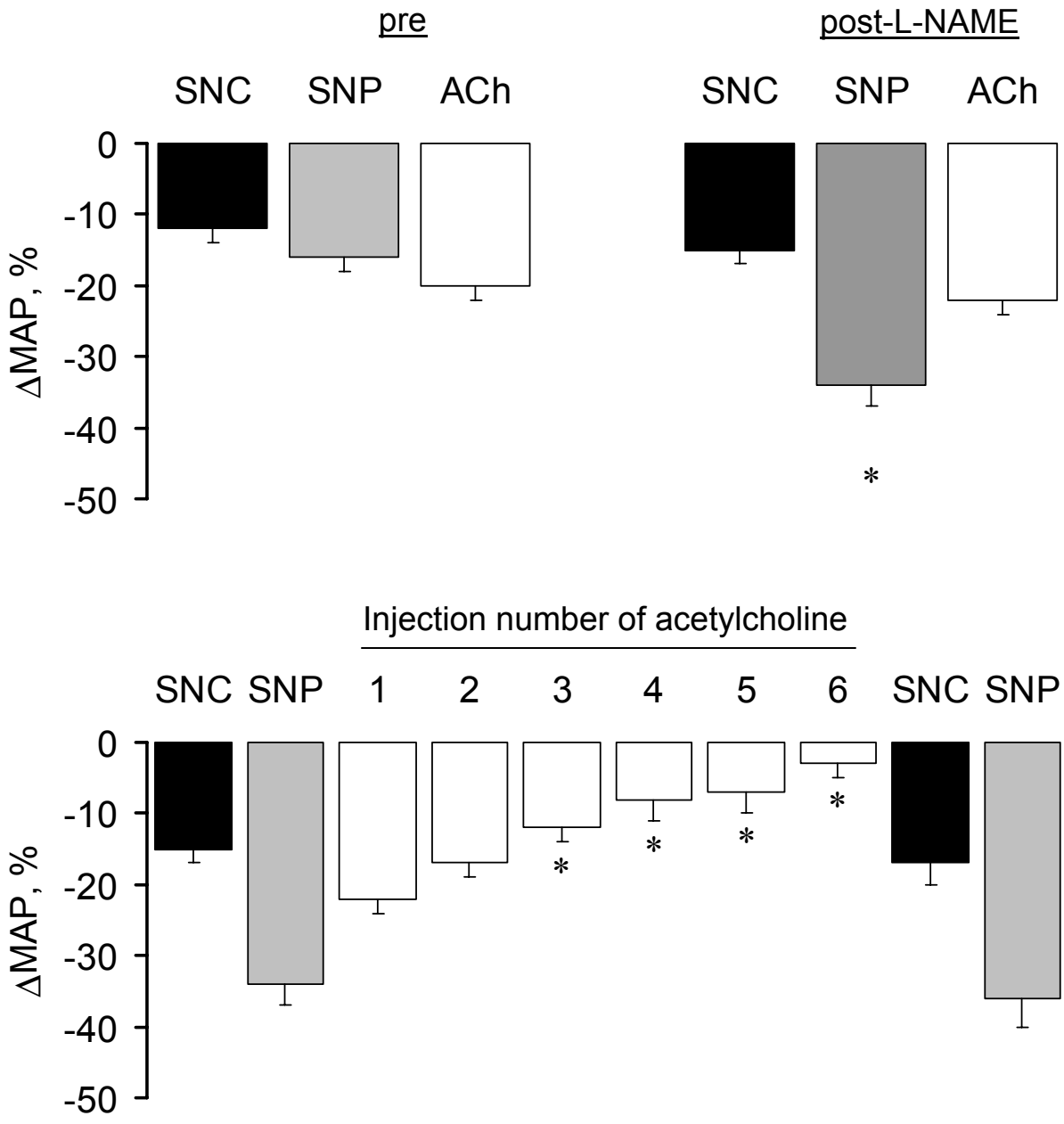

Table 7.1

Resting hemodynamic parameters before and after injection of saline or L-NAME

Parameter	Treatment	Group	Pre	Post	Actual Change
MAP, mmHg	Saline	Control	75 ± 4	76 ± 4	+1 ± 2
		Cholesterol-fed	74 ± 3	74 ± 3	0 ± 2
	L-NAME	Control	73 ± 4	92 ± 4	+19 ± 2*
		Cholesterol-fed	76 ± 3	119 ± 4	+43 ± 4*,†
HR, beats/min	Saline	Control	274 ± 8	273 ± 7	-1 ± 4
		Cholesterol-fed	268 ± 9	266 ± 9	-2 ± 2
	L-NAME	Control	270 ± 7	253 ± 6	-17 ± 3*
		Cholesterol-fed	271 ± 8	279 ± 8	+8 ± 3*,†

The data are presented as mean ± SEM. MAP = mean arterial blood pressure. HR = heart rate. L-NAME = N^G-nitro-L-arginine methylester (50 μmol/kg, iv). There were 10 animals in each group. *$P < 0.05$, significant change from pre values. †$P < 0.05$, cholesterol-fed versus control.

CHAPTER 8

ENDOGENOUS NITROSYL FACTORS MAY INHIBIT THE DESENSITIZATION OF 5-HT_3 RECEPTORS ON VAGAL CARDIOPULMONARY AFFERENTS[1]

[1]Owen JR, Bates JN, Lewis SJ. 2005. *Brain Res*earch. 1059: 167-172.
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ABSTRACT

The pronounced tachyphylaxis to the Bezold-Jarisch reflex responses elicited by systemic injections of the 5-HT_3 receptor agonists such as phenylbiguanide may involve desensitization and/or reduced rate of resensitization of 5-HT_3 receptors on vagal cardiopulmonary afferents. The presence of nitric oxide synthase in vagal afferents suggests that endogenous nitrosyl factors regulate the status of 5-HT_3 receptors in these afferents. The aim of this study was to determine whether inhibition of nitric oxide synthase alters the development of tachyphylaxis to the Bezold-Jarisch reflex responses elicited by phenylbiguanide in conscious rats. The first injection of phenylbiguanide (100 μg/kg, i.v.) elicited robust reductions in heart rate, diastolic arterial blood pressure and cardiac output in saline-treated rats. Subsequent injections elicited progressively smaller responses such that the sixth injections elicited minor responses only. The first injection of phenylbiguanide (100 μg/kg, i.v.) in rats treated with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methylester (L-NAME; 25 μmol/kg, i.v.) elicited similar reductions in heart rate, diastolic arterial blood pressure and cardiac output as in saline-treated rats. However, the rate of development of tachyphylaxis to the Bezold-Jarish reflex responses elicited by phenylbiguanide was markedly faster in the L-NAME-treated rats. The Bezold-Jarisch reflex responses elicited by 5-HT (40 μg/kg, i.v.) were markedly attenuated after the development of tachyphylaxis to phenylbiguanide in saline- and in L-NAME-treated rats whereas the Bezold-Jarisch reflex responses elicited by the S-nitrosothiol, L-S-nitrosocysteine (5 μmol/kg, i.v.), were not attenuated in either group. Taken together, these findings suggests that tachyphylaxis to phenylbiguanide was not due to the loss of central or efferent processing of the Bezold-Jarisch reflex. Collectively, these findings suggest that nitric oxide synthase exists in

vagal cardiopulmonary afferents mediating the Bezold-Jarisch reflex, and that nitrosyl factors influence 5-HT_3 receptor function.

Keywords: Bezold-Jarisch reflex; serotonin, 5-HT_3 receptors, nitric oxide, tachyphylaxis, rat

INTRODUCTION

Systemic injections of 5-hydroxytryptamine (5-HT) elicit the Bezold-Jarisch reflex (Thoren, 1979; Fozard, 1984; Richardon and Engel, 1986; Aviado and Guevara-Aviado, 2001) by activating 5-HT_3 ion-channel receptors (Andrade and Chaput, 1991; Derkach et al., 1989; Maricq et al., 1991; Zifa and Fillion, 1992) on vagal cardiopulmonary afferents (Thoren, 1979; Aviado and Guevara-Aviado, 2001). The Bezold-Jarisch reflex responses include pronounced drops in mean arterial blood pressure, due primarily to vagal efferent-mediated reductions in heart rate and cardiac output (see Whalen et al., 2000a,b). The Bezold-Jarisch reflex responses elicited by the 5-HT_3 receptor agonists, phenylbiguanide and 2-methyl-5-HT (Fozard, 1990; Richardson and Engel, 1986; Zifa and Fillion, 1992), are subject to the development of tachyphylaxis in rats (Pires et al., 1990; Whalen et al., 2000a,b). This tachyphylaxis appears to be due to the desensitization and/or reduced rate of resensitization of 5-HT_3 receptors on vagal afferents rather than a loss of central or efferent processing of the Bezold-Jarisch reflex (Whalen et al., 2000a,b).

Vagal afferent neurons contain nitric oxide synthase (Koike et al., 1998; Lin et al., 1998), which is Ca^{2+}-calmodulin-dependent enzyme (Ignarro, 1990). To our knowledge there is no definitive evidence that nitric oxide synthase exists in cardiopulmonary vagal afferents mediating the Bezold-Jarisch reflex. However, activation of these afferents by natural stimuli such as elevated cardiopulmonary blood pressures or circulating 5-HT acting via 5-HT_3 receptors would elicit increases in intracellular Ca^{2+} levels via depolarization-induced activation of voltage-gated Ca^{2+}-channels (see Abdel-Latif, 1986; Andrade and Chaput, 1991; Zifa and Fillion, 1992). As such, these increases in intracellular Ca^{2+} levels in the cardiopulmonary afferent terminals would have

the propensity to increase nitric oxide synthase activity and the generation of nitrosyl factors, which in turn may regulate the status of 5-HT_3 receptors.

The hypothesis of this study is that nitric oxide and/or nitrosyl factors such as S-nitrosothiols (Brody et al., 1990; Ignarro, 1990; Myers et al., 1990; Stamler et al., 1992; Stamler et al., 1997; Davisson et al., 1996a,b,c,d, 1997a,b) generated by nitric oxide synthase play a role in preventing the desensitization of 5-HT_3 receptors in vagal cardiopulmonary afferents. The aim of this study was to determine whether inhibition of nitric oxide synthase in vagal cardiopulmonary afferents affects the rate of development of tachyphylaxis to the reflex responses elicited by systemic injections of phenylbiguanide and 2-methyl-5-HT in conscious rats.

MATERIALS AND METHODS

Rats and Surgical procedures

All studies were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. The protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250-300 g were anesthetized with a mixture of ketamine (12 mg/kg, i.p.) and acepromazine (120 mg/kg, i.p.). A catheter was placed into a femoral vein to inject drugs. A catheter was also placed into a femoral artery to measure MAP and pulsatile (PP) and diastolic arterial blood pressure and to determine heart rate. A thoracotomy was performed and a pulsed Doppler flow probe was placed around the ascending aorta to measure cardiac output and to determine total peripheral resistance (see Whalen et al., 2000a). The catheters and Doppler leads were exteriorized at the back of the neck, all wounds sutured closed, and the rats

were returned to their individual cages. The rats were given 5 days to recover before use.

Protocols

On the day of the experiments, the arterial catheter was connected to a Beckman Dynagraph-coupled pressure transducer to measure PP, MAP and diastolic arterial blood pressure. Heart rate was determined from PP by a cardiotachometer. The Doppler flow probe wires were connected to a Doppler Flowmeter (Davisson et al., 1996a,b,c,d, 1997a,b; Whalen et al., 2000a). The Beckman Dynagraph did not accurately determine the changes in MAP due to the rapid and transient nature of the BJR responses. Accordingly, the BJR changes in diastolic arterial blood pressure were reported. The hypertension elicited by the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), was much more gradual and longer in duration. These responses were accurately monitored by the Beckman Dynagraph and so changes in MAP were reported.

In study 1, separate groups of rats received a bolus intravenous injection of saline (n = 8 rats), or L-NAME (25 μmol/kg, n = 8) or the inactive enantiomer, D-NAME (25 μmol/kg, n = 5). The hemodynamic effects of L-NAME reached their maximum after 10-15 min and were sustained at these levels for the remainder of the experiments. After 15-20 min, the saline-, L-NAME- or D-NAME-treated rats received a bolus intravenous injection of a dose of L-S-nitrosocysteine (5 μmol/kg), which activates the Bezold-Jarisch reflex (Whalen et al., 2000a,b). After 10 min, all rats received a bolus intravenous dose of 5-HT (40 μg/kg), which activates the Bezold-Jarisch reflex (Whalen et al., 2000a,b). After 10 min, all rats received six consecutive intravenous injections of phenylbiguanide (100 μg/kg), given 5-7.5 min apart. After 10 min, all rats received a bolus injection L-S-nitrosocysteine (5 μmol/kg) and after 10 min a bolus injection of 5-HT (40 μg/kg). The time period between all of the above injections was sufficient to allow the responses

elicited by each injection to subside completely before another injection was given. In study 2, the protocols were identical to those in Study 1 except that the rats received six consecutive injections of 2-methyl-5-HT (100 μg/kg) rather than phenylbiguanide.

A flow chart showing the times of injection of the test agents in saline-treated, L-NAME-treated and D-NAME-treated rat is shown in **Table 8.1**. These rats received an injection of one of the above agents at Time 0 min. After 20 min, the rats received an injection of a dose of L-S-nitrosocysteine. After 10 min (Time 30 min), the rats received an injection of 5-HT. After 10 min (Time 40 min), the rats received the first of six injections of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2). Injections 2-6 of phenylbiguanide were given at 5 min intervals (Times 45, 50, 55, 60 and 65 min). After 10 min (Time 75 min), the rats received an injection of L-S-nitrosocysteine (5 μmol/kg). After 10 min, (Time 85 min), the rats received an injection of 5-HT.

Drugs

All drugs were from Sigma (St. Louis, MO, USA) except for phenylbiguanide, which was obtained from Research Biochemicals (Natick, MA, USA). L-S-nitrosocysteine was synthesized immediately before use as described previously (Davisson et al., 1996a,b,c,d, 1997a,b).

Statistics

The data are presented as mean ± S.E.M. The data were analyzed by repeated measures analysis of variance (ANOVA) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVAs (Davisson et al., 1996a,b,c,d, 1997a,b).

RESULTS

Hemodynamic actions of L-NAME

The hemodynamic responses elicited by saline or L-NAME in Study 1, are summarized in **Table 8.2**. L-NAME elicited a sustained increase in MAP due to an increase in total peripheral resistance. The effects of L-NAME were accompanied by decreases in heart rate and cardiac output. The maximal effects of L-NAME occurred within 10 min. The values listed in the Plateau column are the average of the values recorded 20-90 min after injection of L-NAME. This Plateau period was the time the test agents were given. The Plateau period values wre generally lower than the maximal responses although all values were higher than pre-injection values ($P < 0.05$, for all comparisons). The injection of saline (or D-NAME, data not shown) did not affect resting hemodynamic parameters ($P > 0.05$, for all responses). The effects of saline and L- and D-NAME in Study 2 were very similar to those observed in Study 1.

Bezold-Jarisch reflex responses elicited by phenylbiguanide and 2-methyl-5-HT

The Bezold-Jarisch reflex responses elicited by the six injections of phenylbiguanide in saline or L-NAME-treated rats are summarized in **Table 8.3**. The first injection of phenylbiguanide elicited marked falls in heart rate, diastolic arterial blood pressure and cardiac output. These responses were similar in saline- and L-NAME-treated rats. The Bezold-Jarisch reflex responses elicited by subsequent injections of phenylbiguanide were subject to pronounced tachyphylaxis in both groups. However, tachyphylaxis to phenylbiguanide developed more rapidly in L-NAME than in saline-treated rats. Tachyphylaxis was evident for the second injection of phenylbiguanide in L-NAME-treated rats but not in the saline-treated rats. The maximal loss of responses to phenylbiguanide was similar in both groups ($P > 0.05$, for all comparisons). The

rate of development of tachyphylaxis to phenylbiguanide in D-NAME-treated rats was similar to that in the saline-treated rats ($P > 0.05$, for all comparisons) since (1) tachyphylaxis to phenylbiguanide was not observed until the third dose was given, and (2) the maximal loss of responses to phenylbiguanide was similar to that in saline-treated rats. For example, the percent falls in heart rate elicited by injections 1 to 6 of phenylbiguanide in D-NAME-treated were; -62 ± 7, -63 ± 6, -52 ± 4, -39 ± 5, -22 ± 4 and -11 ± 3, respectively. The responses elicited by injections 3 (-16 ± 4% of injection 1), 4 (-36 ± 5%), 5 (-65 ± 7%) and 6 (-82 ± 7%) were significantly smaller than injection 1 ($P < 0.05$, for all comparisons).

As expected (Whalen et al., 2000a), the Bezold-Jarisch reflex responses elicited by the 100 μmol/kg dose of 2-methyl-5-HT in saline and in L-NAME-treated rats were similar to those elicited by the 100 μmol/kg dose of phenylbiguanide. Moreover, the rate of development of tachyphylaxis to 2-methyl-5-HT was similar to that of phenylbiguanide in saline- and in L- or D-NAME-treated rats ($P > 0.05$, for all comparisons, data not shown) with tachyphylaxis occurring much faster in L-NAME-treated rats. For example, the percent reductions in heart rate elicited by injections 1 to 6 of 2-methyl-5-HT in saline-treated rats were; injection 1, -61 ± 7 ($P < 0.05$); injection 2, -60 ± 6 (-2 ± 4% of response elicited by injection 1, $P > 0.05$); injection 3, -52 ± 4 bpm (-14 ± 4%, $P < 0.05$); injection 4, -39 ± 5 (-37 ± 5%, $P < 0.05$); injection 5, -22 ± 4 (-64 ± 7%, $P < 0.05$); and injection 6, -11 ± 3 (-83 ± 8%, $P < 0.05$), respectively. The percent reductions in heart rate elicited by injections 1 to 6 of 2-methyl-5-HT in L-NAME-treated rats were; injection 1, -64 ± 8 bpm ($P < 0.05$); injection 2, -43 ± 4 (-33 ± 4% of response elicited by injection 1, $P < 0.05$); injection 3, -28 ± 3 bpm (-56 ± 8%, $P < 0.05$); injection 4, -19 ± 4 (-70 ±

6%, $P < 0.05$); injection 5, -10 ± 3 (-83 ± 6%, $P < 0.05$); and injection 6, -9 ± 3 (-86 ± 7%, $P < 0.05$), respectively.

Bezold-Jarisch reflex responses elicited by 5-HT and L-S-nitrosocysteine

The Bezold-Jarisch reflex responses elicited by 5-HT or L-S-nitrosocysteine before and after injections of phenylbiguanide in saline- or L-NAME-treated rats are summarized in **Table 8.4**. The selected doses of 5-HT and L-S-nitrosocysteine elicited robust and equivalent Bezold-Jarisch reflex responses prior to injection of any treatment. The Bezold-Jarisch reflex-induced responses elicited by 5-HT were markedly reduced after development of tachyphylaxis to phenylbiguanide in saline- and in L-NAME-treated rats. The loss of response to 5-HT was similar in each group ($P > 0.05$, for all comparisons). The Bezold-Jarisch reflex-induced responses elicited by L-S-nitrosocysteine in saline-treated rats were similar before and after injections of phenylbiguanide. The Bezold-Jarisch reflex responses elicited by the first injection of L-S-nitrosocysteine in L-NAME-treated rats (i.e., prior to injections of phenylbiguanide) were larger than in saline-treated rats. However, the L-S-nitrosocysteine-induced responses in L-NAME-treated rats were similar before and after the injections of phenylbiguanide. Virtually identical results were found in the 2-methyl-5-HT study (data not shown).

DISCUSSION

Effects of L-NAME on Bezold-Jarisch reflex responses

This study confirms that the systemic injection of a 25 μmol/kg dose of L-NAME elicits a sustained hypertension in conscious rats via an increase in total peripheral resistance (Davisson et al., 1996a,b). The increase in MAP was associated with (presumably) baroreflex-mediated

reductions on cardiac output and heart rate. The injections of D-NAME did not affect resting hemodynamic parameters or the rate of development of tachyphylaxis to the BJR responses elicited by the 5-HT_3 receptors agonists. Chronic administration of L-NAME enhances the BJR responses to 5-HT in conscious rats (Araujo et al., 1995, 1998), although this is due in large part to an increase in responsiveness of cardiac pacemaker cells to vagal cholinergic stimulation (Araujo et al., 1998). In contrast, the chronic administration of L-NAME reduces Bezold-Jarisch reflex-mediated responses induced by phenylbiguanide in urethane-anesthetized mice (Peotta et al., 2001) by as yet undertermined mechanisms.

To our knowledge, the effects of acute administration on NO synthase inhibitors on 5-HT-mediated activation of the Bezold-Jarisch reflex have not been investigated. The present results demonstrate that an acute injection of a 25 μmol/kg dose of L-NAME does not affect the BJR responses elicited by selective 5-HT_3 receptors. We selected the 25 μmol/kg dose of L-NAME for the present study on the basis that (i) this dose does not impair the central processing of vagal (i.e., superior laryngeal) afferent input in anesthetized rats whereas higher doses do have significant effects in the brain pathways processing vagal afferent input (Possas and Lewis, 1997), and (ii) this dose of L-NAME markedly affects endothelium-dependent vasodilation and lumbar sympathetic vasodilation in paradigms used to demonstrate the possible existence of preformed stores of nitrosyl factors (Davisson et al., 1996a,b,c;1997a,b). The present finding that the first injections of phenylbiguanide and 2-methyl-5-HT elicited BJR responses in L-NAME-treated rats of equal magnitude to those in saline-treated rats suggests that L-NAME did not affect 5-HT_3 receptors status or the central and efferent processing of vagal cardiopulmonary afferent input.

Effects of L-NAME on tachyphylaxis to phenylbiguanide and 2-methyl-5-HT

This study confirms that the Bezold-Jarisch reflex responses elicited by the selective 5-HT_3 receptor agonists, phenylbiguanide and 2-methyl-5-HT, are subject to pronounced tachyphylaxis in conscious rats (Whalen et al., 2000a,b). One principal finding of this study was that the rate of development of tachyphylaxis to phenylbiguanide and 2-methyl-5-HT was faster in L-NAME-treated than in saline-treated rats. Specifically, tachyphylaxis in L-NAME-treated rats was evident for the second injection of phenylbiguanide or 2-methyl-5-HT. It therefore appears that the rate of desensitization of 5-HT_3 receptors occurs faster or that the rate of resensitization of these receptors is slower, after inhibition of NO synthesis. As expected (see Whalen et al., 2000a), the Bezold-Jarisch reflex responses elicited by 5-HT were markedly reduced after development of tachyphylaxis to phenylbiguanide in saline- and in L-NAME-treated rats. This provides further evidence that the loss of response to the synthetic 5-HT_3 receptor agonist induces tachyphylaxis to the endogenous ligand for 5-HT_3Rs. The finding that the BJR responses elicited by L-S-nitrosocysteine were not diminished after development of tachyphylaxis to phenylbiguanide or 2-methyl-5-HT suggests that (i) the loss of response to the 5-HT_3R agonists may be due to the down-regulation of 5-HT_3 receptors rather than a loss of central and efferent processing of the BJR, and (ii) L-S-nitrosocysteine activates cardiopulmonary afferents by mechanisms other than activation of 5-HT_3 receptors.

The other principal finding of this study was that the Bezold-Jarisch reflex responses elicited by the 5 μmol/kg dose (and lower doses, data not shown) of L-S-nitrosocysteine were augmented in L-NAME-treated rats. The biological actions of L-S-nitrosocysteine involve (i) its decomposition to NO (Ignarro, 1990), (ii) nitrosation of functional proteins such as ion-channels

(Stamler et al., 1992, 1997) and (iii) the activation of stereoselective S-nitrosothiol recognition sites (Lewis et al., 1996; Davisson et al., 1996d, 1997b). We have obtained preliminary evidence that D-S-nitrosocysteine and large doses of NO-donors such as sodium nitroprusside do not elicit the BJR in conscious rats and that the BJR responses elicited by L-S-nitrosocysteine are virtually absent in adult rats that were treated with capsaicin as neonates (Brody et al., 1990). These findings contrast with evidence that the Bezold-Jarisch reflex responses elicited by 5-HT are not diminished in anesthetized adult rats that were treated neonatally with capsaicin (see Meller et al., 1991). Taken together, these findings suggest that (i) 5-HT elicits the Bezold-Jarisch reflex via activation of sub-populations of vagal cardiopulmonary afferents in addition to small diameter unmyelinated C-fiber afferents, and (ii) L-S-nitrosocysteine elicits the Bezold-Jarisch reflex via activation of stereoselective recognition sites on vagal small diameter cardiopulmonary afferents, and (iii) these stereoselective recognition sites (or their signaling cascades) are up-regulated in cardiopulmonary afferents after inhibition of nitric oxide synthesis in these afferents. However, other mechanisms such as enhanced vagal efferent-mediated signaling processes cannot be discounted.

PERSPECTIVES

Although nitric oxide synthase is present in most vagal afferents (Ruggiero et al., 1996; Koike et al., 1998; Lin et al., 1998), there is to our knowledge no definitive evidence that this enzyme exists in small- or large-diameter unmyelinated vagal cardiopulmonary afferents mediating the Bezold-Jarisch reflex. The present findings suggest that this is indeed the case and that endogenous nitrosyl factors play a functional role in regulating 5-HT_3 receptors in these afferents. Specifically, it appears that nitrosyl factors in vagal afferent terminals regulate the

desensitization and/or resensitization of 5-HT_3 receptors. Co-activation of G protein-coupled 5-HT_2 receptors (Probst et al., 1992) allows the full expression of 5-HT_3 receptor-mediated activation of the Bezold-Jarisch reflex (Meller et al., 1991a,b). As such, elevations in intracellular Ca^{2+} elicited by activation of 5-HT_2 receptors may regulate the desensitization and or resensitization of 5-HT_3 receptors on cardiopulmonary vagal afferents via an increase in NOS activity and generation of nitrosyl factors. Finally, the present findings complement evidence that endothelium-derived nitrosyl factors regulate the desensitization and/or resensitization of G_s protein-coupled receptors in vascular smooth muscle (Travis et al., 1997; Whalen et al., 1999a,b; 2000c).

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Table 8.1

Typical time-line for injections of test agents

Time (min)	Injection regime
0	Injection of saline, L-NAME or D-NAME (Study 1 and 2)
20	Injection of L-S-nitrosocysteine (Study 1 and 2)
30	Injection of 5-HT (Study 1 and 2)
40	Injection 1 of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2)
45	Injection 2 of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2)
50	Injection 3 of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2)
55	Injection 4 of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2)
60	Injection 5 of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2)
65	Injection 6 of phenylbiguanide (Study 1) or 2-methyl-5-HT (Study 2)
75	Injection of L-S-nitrosocysteine (Study 1 and 2)
85	Injection of 5-HT (Study 1 and 2)

L-NAME = N^G-nitro-L-arginine methylester. D-NAME = N^G-nitro-L-arginine methylester. 5-HT = 5-hydroxytryptamine.

Table 8.2

Hemodynamic responses elicited by saline and N^G-nitro-L-arginine methyl ester

		Pre	Maximum		Plateau	
Study	Parameter	Value	Value	%Change	Value	%Change
Saline	HR, bpm	349 ± 13	345 ± 13	0 ± 3	351 ± 13	+1 ± 4
	MAP, mmHg	106 ± 3	106 ± 3	0 ± 2	108 ± 4	0 ± 2
	CO, kHz	2.48 ± 0.27	2.53 ± 0.24	+2 ± 4	2.46 ± 0.31	-1 ± 5
	TPR, mmHg/kHz	42 ± 6	42 ± 7	0 ± 3	44 ± 5	+4 ± 4
L-NAME	HR, bpm	356 ± 11	325 ± 8	-9 ± 2*	337 ± 11	-5 ± 1*,†
	MAP, mmHg	108 ± 3	141 ± 3	+31 ± 3*	132 ± 3	+22 ± 3*,†
	CO, kHz	2.56 ± 0.35	1.89 ± 0.24	-26 ± 5*	2.08 ± 0.22	-17 ± 6*,†
	TPR, mmHg/kHz	43 ± 4	77 ± 5	+78 ± 9*	64 ± 6	+51 ± 6*,†

The data are presented as mean ± S.E.M. HR = heart rate. MAP = mean arterial blood pressure. CO = cardiac output. TPR = total peripheral resistance. The dose of L-NAME was 25 μmol/kg, i.v. There were eight rats in each group. *$P < 0.05$, Maximum or Plateau versus Pre. †$P < 0.05$, Plateau versus Maximum.

Table 8.3

Hemodynamic responses elicited by systemic injections of phenylbiguanide in saline- or L-NAME-treated rats

		Injection number of phenylbiguanide					
Parameter	Pretreatment	1	2	3	4	5	6
ΔHR, %	Saline	-63 ± 6	-60 ± 5	-51 ± 5*	-38 ± 4*	-25 ± 3*	-10 ± 2*
	L-NAME	-59 ± 5	-43 ± 4*,†	-31 ± 5*,†	-17 ± 4*,†	-8 ± 3*,†	-6 ± 2*
ΔBP_D, %	Saline	-64 ± 5	-61 ± 5	-53 ± 6	-43 ± 4*	-28 ± 3*	-11 ± 2*
	L-NAME	-62 ± 6	-45 ± 5*,†	-34 ± 4*,†	-23 ± 3*,†	-16 ± 3*,†	-10 ± 3*
ΔCO, %	Saline	-62 ± 6	-59 ± 5	-48 ± 4*	-39 ± 3*	-26 ± 3*	-10 ± 3*
	L-NAME	-61 ± 5	-42 ± 5*,†	-31 ± 4*,†	-21 ± 4*,†	-14 ± 3*,†	-9 ± 3*
ΔTPR, %	Saline	-5 ± 6	-4 ± 4	-7 ± 5	-6 ± 5	-5 ± 3	-4 ± 4
	L-NAME	-3 ± 5	-6 ± 4	-6 ± 4	-5 ± 6	-7 ± 5	-3 ± 4

Data are presented as mean ± S.E.M. HR = heart rate. BP_D = diastolic arterial blood pressure. CO = cardiac output. TPR = total peripheral resistance. The dose of phenylbiguanide was 100 μg/kg i.v. There were 8 rats in each group. L-NAME = N^G-nitro-L-arginine methylester (25 μmol/kg i.v.). Note that each response was significant ($P < 0.05$, for all responses). *$P < 0.05$, 2nd-6th injections versus 1st injection. †$P < 0.05$, L-NAME versus saline.

Table 8.4

Bezold-Jarisch reflex responses elicited by L-S-nitrosocysteine or 5-HT before and after administration of phenylbiguanide in saline- or L-NAME-treated rats

		Treatment Groups			
		Saline		L-NAME	
Compound	Parameter	Pre	Post-PBG	Pre	Post-PBG
5-HT	ΔHR, %	-56 ± 5	-6 ± 2*	-50 ± 6	- 5 ± 2*
	ΔBP_D, %	-48 ± 4	-7 ± 2*	-43 ± 4	-6 ± 2*
	ΔCO, %	-49 ± 6	-9 ± 3*	-44 ± 5	- 7 ± 3*
	ΔTPR, %	+5 ± 6	+7 ± 5	+8 ± 6	+9 ± 6
L-SNC	ΔHR, %	-52 ± 5	-53 ± 6	-69 ± 5†	-66 ± 6
	ΔBP_D, %	-44 ± 5	-43 ± 6	-64 ± 5†	-63 ± 7
	ΔCO, %	-45 ± 6	-44 ± 8	-63 ± 5†	-65 ± 8
	ΔTPR, %	+4 ± 4	+6 ± 5	+7 ± 5	+6 ± 5

Data are presented as mean ± S.E.M. HR = heart rate. ΔBP_D = diastolic arterial blood pressure. CO = cardiac output. TPR = total peripheral resistance. Rats received injections of 5-HT (40 μg/kg, i.v.) and L-S-nitrosocysteine (L-SNC, 5 μmol/kg, i.v.) before and after six injections of phenylbiguanide (PBG, 100 μg/kg, i.v.) in saline-treated or in N^G-nitro-L-arginine (L-NAME, 25 μmol/kg i.v.)-treated rats. There were 8 rats in each group. Changes in HR, MAP and CO elicited by the first injections of 5-HT and L-SNC were significant ($P < 0.05$) whereas the changes in TPR were not ($P > 0.05$). *$P < 0.05$, Post-phenylbiguanide versus Pre. †$P < 0.05$, L-NAME versus Saline.

CHAPTER 9

SUMMARY AND CONCLUSIONS

BACKGROUND

Elevated plasma cholesterol level is a major risk factor for cardiovascular disease. Concentric remodeling of the left ventricle occurs in a substantial sub-population of normotensive hypercholesterolemic patients. This left ventricular geometric pattern is characterized by an increased relative wall thickness occurring concurrently with normal left ventricular mass. The exact mechanisms leading to the progression of cardiac concentric remodeling have yet to be elucidated. We used the high cholesterol-fed guinea pig as a normotensive small animal model of human hypercholesterolemia in order to examine the mechanisms by which high cholesterol elicits changes in cardiac morphology and function. We also used this high cholesterol-fed guinea pig model to examine the mechanisms responsible for the effects of hypercholesterolemia on autonomic and cardiovascular function.

OBJECTIVES

The objectives of this dissertation project were to (1) use echocardiographic and morphological techniques to characterize the changes in cardiac geometry in cholesterol-fed guinea pigs, (2) use cardiovascular and pharmacological methods to determine the effects of cholesterol-induced cardiac concentric remodeling on baroreceptor reflex function in conscious guinea pigs, (3) to use a variety of cardiovascular techniques to elucidate the mechanisms involved in the regulation of cardiovascular autonomic function in cholesterol-fed conscious and anesthetized guinea pigs displaying cardiac concentric remodeling, 4) examine the effects of cholesterol-induced cardiac concentric remodeling on Bezold-Jarisch Reflex function as elicited by activation of 5-HT_3 ion channel receptors on vagal cardiopulmonary afferents in conscious guinea pigs, and 5) evaluate the effects of hypercholesterolemia on endothelium-dependent vasodilator mechanisms.

FINDINGS

Concentric Cardiac Remodeling

We found that guinea pigs that consumed a 1% cholesterol diet for 13 weeks displayed left ventricular cardiac concentric remodeling without any signs of hypertrophy. Importantly, these animals were normotensive indicating that the geometric changes of the left ventricle were not driven by changes in mean arterial blood pressure. Echocardiographic measurements showed that relative left ventricular wall thickness was substantially increased in the cholesterol-fed guinea pigs whereas left ventricular mass remained unchanged. Cardiac contractility, as measured by fractional shortening, was similar in control and cholesterol-fed guinea pigs. Taken together, it was evident that the cholesterol-fed guinea pigs exhibited classic cardiac concentric remodeling. In support of this evidence, morphologic analyses of the hearts of hypercholesterolemic guinea pigs determined that these hearts had smaller left ventricular chambers, left ventricular wall volumes and total left ventricular volumes. Minimum left ventricular wall thickness, maximum left ventricular wall thickness, and mean left ventricular wall thickness was similar in both normocholesterolemic and hypercholesterolemic guinea pigs.

Our results provide functional and morphological evidence that guinea pigs fed 1% cholesterol for 13 weeks developed concentric remodeling of the left ventricle. This remodeling is likely to result from the disruption of normal physiological processes that modulate cardiac morphology, including apoptotic removal of and replication of myocardial tissue, along with fluctuations in the genetic expression of key signaling elements such as nitric oxide synthase. Our laboratory is currently performing experiments with Dr. David Hurley (Large Animal Medicine, University of Georgia) to address the temporal sequence of events that result in concentric remodeling.

Baroreceptor Heart Rate Reflex

To determine if cholesterol-induced left ventricular remodeling alters cardiovascular homeostatic mechanisms, baroreceptor heart rate reflex activity was measured in conscious control and hypercholesterolemic guinea pigs. Baroreflex function was markedly impaired in cholesterol-fed guinea pigs. More specifically, the gain (sensitivity) and range (difference between upper and lower heart rate plateaus) were markedly diminished in the cholesterol-fed animals. The high cholesterol-induced alterations in baroreceptor reflex range and sensitivity was attributable to possible abnormalities in the baroafferents themselves and to the central processing of the input from the baroafferent fibers. Whether the central abnormalities are focused within the nucleus of the tractus solitarius or other key brain regions such as the rostroventral lateral medulla will be the focus of future studies.

As mentioned, studies evaluating the mechanisms involved in baroreceptor heart rate reflex dysfunction in hypercholesterolemic guinea pigs provided evidence that central processing of baroafferent input was disturbed in these animals. Further studies provided clear evidence that the function of cardiac β-adrenoceptors on cardiac pacemaker and ventricular muscle cells was markedly impaired in the cholesterol-fed guinea pigs and that intrinsic heart rate was elevated in these animals. In contrast, evidence was obtained that cardiovagal drive was not impaired in the cholesterol-fed guinea pigs. Our findings demonstrated that autonomic function is compromised in cholesterol-fed guinea pigs. This may be may be due to the ability of cholesterol to perturb the integrity of lipid rafts/caveolae microdomains, which play a major role in facilitating signal transduction pathways involved in β-adrenoceptor signaling processes, central processing of the baroreceptor reflex, and regulation of intrinsic heart rate by the pacemaker cells of the heart.

Bezold-Jarisch Reflex

Bezold-Jarisch reflex function was analyzed via administration of systemic injections of the selective 5-HT_3 receptor agonist, phenlybiguanide, in conscious control and cholesterol-fed guinea pigs. Phenylbiguanide exerted pronounced depressor and bradycardic responses due almost entirely to activation of cardiovagal drive. The key finding was that the Bezold-Jarisch reflex responses elicited by phenylbiguanide were markedly reduced in cholesterol-fed guinea pigs. A series of successive systemic injections of phenylbiguanide resulted in the development of tachyphylaxis to the 5-HT_3 receptor agonist in control and cholesterol-fed guinea pigs. The rate of development of tachyphylaxis was substantially faster in the cholesterol-fed animals. The Bezold-Jarisch reflex responses elicited by 5-HT were substantially diminished whereas those elicited by L-S-nitrosocysteine were not affected upon injection into guinea pigs subjected to the development of tachyphylaxis to phenylbiguanide. These data suggest that guinea pigs with cholesterol-induced cardiac concentric remodeling have markedly impaired Bezold-Jarisch reflex function via a disruption of 5-HT_3 receptor function and that hypercholesterolemia affects the rate of desensitization/resensitization of 5-HT_3 receptors.

Endothelium-dependent Vasodilation

Our studies provided evidence that the vasodilator potencies of the endothelium-dependent agonist, acetylcholine, and the endothelium-derived S-nitrosothiol, L-S-nitrosocysteine, were substantially diminished in the normotensive, hypercholesterolemic guinea pigs with cardiac concentric remodeling. In contrast, the vasodilator potency of the nitric oxide donor, sodium nitroprusside, was not diminished in the cholesterol-fed guinea pigs. Our studies also provided evidence for a loss of and/or mobilization of preformed vesicular pools of L-S-nitrosocysteine in

cholesterol-fed guinea pigs. This loss of response to acetylcholine may be due to the down-regulation of muscarinic receptors on vascular endothelial cells. However, our findings raise the possibility that the loss of response to acetylcholine in cholesterol-fed guinea pigs may involve diminished biological potency of L-S-nitrosocysteine due to down-regulation of stereoselective L-S-nitrosocysteine recognition sites on vascular smooth muscle.

SUMMARY

Our findings suggest that a high (1%) cholesterol diet for 13 weeks causes cardiac concentric remodeling of the left ventricle in guinea pigs and that this geometric change in heart shape has profound effects on cardiovascular homeostatic mechanisms. Cellular membranes maintain an intricate balance between cholesterol and sphingolipids/phospholipids that provide functional support for signal transduction pathways co-localized in lipid rafts/caveolae microdomains and are involved in the pathogenesis of cardiac morphology, cardiac homeostatic mechanisms and endothelium-derived relaxation. This project provided substantial experimental evidence to support the idea that our novel animal model of human hypercholesterolemia may contribute to the development of pharmacological therapies used to treat and prevent cholesterol-induced cardiovascular disease states. Our future studies will address the temporal sequence of events that allow hypercholesterolemia to induce cardiac concentric remodeling.