DISSERTATION

FATTY ACIDS AND ENDOTHELIAL DYSFUNCTION IN OBESITY: ROLE OF β-ADRENERGIC STIMULATED LIPOLYSIS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION

FATTY ACIDS AND ENDOTHELIAL DYSFUNCTION IN OBESITY: ROLE OF β-ADRENERGIC STIMULATED LIPOLYSIS

Obesity is a risk factor for vascular disease, and endothelial dysfunction is common to both. The mechanism linking obesity and endothelial dysfunction is unclear. Serum free fatty acids (FFAs) are elevated in obesity, and impair endothelium-dependent vasodilation by inducing inflammation and oxidative stress. Elevated FFAs in obesity are partly due to enhanced triglyceride lipolysis, which is increased by sympathetic stimulation of the β-adrenoreceptor. There is evidence of augmented sympathetic activity in obesity.

It was hypothesized that β-antagonism would reduce serum FFAs and thereby improve endothelium-dependent vasodilation in fat-fed rats. Further, that β-antagonist-mediated reduction in FFAs would be associated with decreased serum markers of inflammation and oxidation.

Male Sprague-Dawley rats (n = 8) were fed a high-fat diet for 16 weeks. During the final 4 weeks, rats received β_1 and β_3-antagonists (atenolol and SR59230A) by subcutaneous slow-release pellets. Endothelium-dependent vasodilation was assessed in vivo by laser Doppler flowmetry of the femoral artery to measure the flow-time integral (i.e. area under the flow curve) in response to 0.25, 0.75, and 2.5 μg/kg intra-arterial acetylcholine. Markers of

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inflammation [C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)] and oxidative stress [thiobarbituric acid reactive substances (TBARS), arterial nitrotyrosine residues] were also measured.

Chronic $\beta_1\beta_3$-antagonism elevated serum FFAs ($p < 0.01$) and increased body weight ($p < 0.001$) in normal and fat-fed rats compared to placebo-pelleted animals. High-fat feeding increased serum triglyceride and leptin concentrations, and this was not increased further by β-antagonist treatment. Antagonist treatment did not change the flow-time integral ($p = 0.17$). $\beta_1\beta_3$-antagonism was associated with decreased serum CRP, IL-6 and TNF-α, and increased TBARS. Nitrotyrosine residues were primarily localized to the endothelium, followed by the subendothelium and vascular media. Slot blot analysis of proteins revealed increased nitrosylated protein in both normal and fat-fed animals that received β-antagonist treatment.

Increased serum FFAs after chronic $\beta_1\beta_3$-antagonism was possibly due to attenuation of brown adipose fatty acid oxidation which would prevent uptake of circulating FFAs. Additionally, lipolytic pathways that are independent of β-adrenergic control exist, and may have compensated for attenuated β-mediated lipolysis. Despite elevated FFAs in $\beta_1\beta_3$-treated rats and known pathologic effects of FFAs on the vascular endothelium, $\beta_1\beta_3$-antagonist treatment preserved endothelium-dependent vasodilation. This suggests that beneficial effects of β-antagonists on the vasculature are not mediated by FFA-lowering properties. Markers of systemic inflammation were reduced in $\beta_1\beta_3$-antagonist-treated rats

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despite increased FFAs. Because FFAs are known to be pro-inflammatory, β-antagonism may preserve endothelium-dependent vasodilation by attenuating FFA-mediated inflammation. In contrast, TBARS and nitrotyrosine were increased with β-antagonism, possibly due to unmitigated FFA-mediated oxidative stress.

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CHAPTER I

INTRODUCTION

The dramatic increase in prevalence of overweight and obese individuals in the United States is commonly described as an epidemic.[1] Individuals who are overweight or obese have increased risk of hypertension, dyslipidemia, insulin resistance, and coronary artery disease.[1,2] Endothelial cell dysfunction has been documented in obese individuals as well as individuals with the above-associated pathologies, and may represent the common link between obesity and its sequelae.

Obesity is associated with elevated serum free fatty acids (FFAs). FFAs promote acute and chronic endothelial dysfunction when administered intravenously or by dietary alterations.[3-6] This effect may be due, at least in part, to induction of inflammation and increased oxidative stress.[7-10]

Pro-inflammatory effects of FFAs have been attributed to protein emulsification by detergent activity[10] and stimulation of inflammatory cytokines.[7] Increased serum cytokines, including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) have been associated with overweight

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and obese individuals,[11-13] and weight loss lowers concentrations.[14-18] Small elevations in CRP have been correlated with increased risk for cardiovascular events in healthy individuals and in those with existing atherosclerosis.[15,19] Elevated serum CRP, IL-6, and TNF-α were shown to be independent predictors of vascular events in the elderly.[20] In addition to serving as markers of vascular pathology, the inflammatory cytokines induce endothelial dysfunction.[21-23] Thus, the relationship between obesity and vascular disease may partly be explained by FFA activation of pro-inflammatory pathways.

Fatty acids are thought to increase oxidative stress by both engaging oxidative mechanisms[8] and depleting endogenous antioxidants.[24] A critical pathologic sequelae is depletion of nitric oxide (NO), a factor believed to confer vascular protection by maintaining normal endothelial function. Reactive oxygen species (ROS) quench NO[7,8,25] and they may in turn interfere with regulation of vascular tone and maintenance of endothelial integrity by this effect. Antioxidants have been shown to attenuate endothelial dysfunction attributable to high-fat feeding in humans and rodents,[26,27] suggesting that oxidative stress is a mechanism by which FFAs, elevated with high-fat intake, impair normal endothelial function.

If FFAs are responsible for obesity-related endothelial dysfunction, then the mechanism of FFA elevations in obese individuals becomes important. Obesity-induced elevations in FFAs can be partly attributed to enhanced lipolysis of both dietary triglyceride (TG)-rich particles and of adipocyte TG stores.[28]

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Stimulation of the β-adrenergic receptors of the sympathetic nervous system (SNS) exerts pro-lipolytic effects, and there is aberrant β-mediated lipolysis in the obese state. Obese, normotensive individuals have enhanced sympathetic activation,[29] and weight loss results in decreased sympathetic nerve activity.[30] Thus, the amplified sympathetic tone associated with obesity may contribute to enhanced lipolysis and elevated FFAs.

A perpetuating cycle may ensue. Postprandial FFAs enhance SNS activity in adults with Type II diabetes.[31] Additionally, the obesity-induced reduction in NO may also augment β-mediated lipolysis.[32] A positive feedback loop involving elevated FFA, NO depletion, and adrenergic stimulation may thus develop. By reducing serum FFAs and attenuating β-stimulated lipolysis, it may be possible to restore NO and preserve endothelial function in obese individuals.

β-antagonist drugs exert protective effects in patients with coronary artery disease, myocardial infarction, hypertension, and congestive heart failure. These agents are associated with reduced mortality and morbidity.[33-36] In addition to favorable effects on the myocardium,[35,37-39] β-antagonists have beneficial effects on the vasculature.[40-44] The mechanisms by which β-antagonists exert these favorable effects remain unclear. Some β-antagonists may exert antiatherosclerotic activity by enhancing NO activity as evidenced by improved endothelium-dependent relaxation.[43,45-49] β-antagonism is associated with reduced CRP concentrations in patients with angina and coronary artery disease,[50] and thus may improve endothelial function by directly reducing

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inflammation and by attenuating the injurious effects of inflammatory cytokines. This study will investigate the idea that β-antagonists may attenuate NO depletion, decrease inflammation, and reduce oxidative stress by reducing serum FFAs through inhibition of β-mediated lipolysis.

SUMMARY

The prevalence of obesity in the United States has doubled in the past 20 years. Obesity is an independent risk factor for the development of vascular complications. Multiple interdependent processes contribute, including hypertension, diabetes, and dyslipidemia. A pathologic mechanism common to each as well as to obesity, is endothelial dysfunction. The mechanism linking obesity and endothelial dysfunction is unclear.

Obesity is accompanied by elevated serum FFA concentrations, which cause acute and chronic endothelial dysfunction. FFAs impair endothelium-dependent vasodilation and promote atherogenesis by exerting proinflammatory effects and enhancing generation of ROS.

Obesity-induced elevations in FFAs are partly attributed to enhanced TG lipolysis. Lipolytic liberation of FFAs is increased by β-adrenoreceptor stimulation of the SNS. There is evidence of enhanced sympathetic activity in obesity.

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HYPOTHESIS

The proposed study will test the hypotheses that under conditions of obesity and elevated serum FFAs, β-antagonism will reduce serum FFAs, and thereby attenuate endothelial dysfunction. The mechanism of FFA-mediated endothelial dysfunction will be by inflammation or oxidation, or both (Figure 1.1). β-antagonism will protect against endothelial dysfunction by blocking the increase in FFAs or by decreasing inflammatory and oxidative mechanisms, or both.

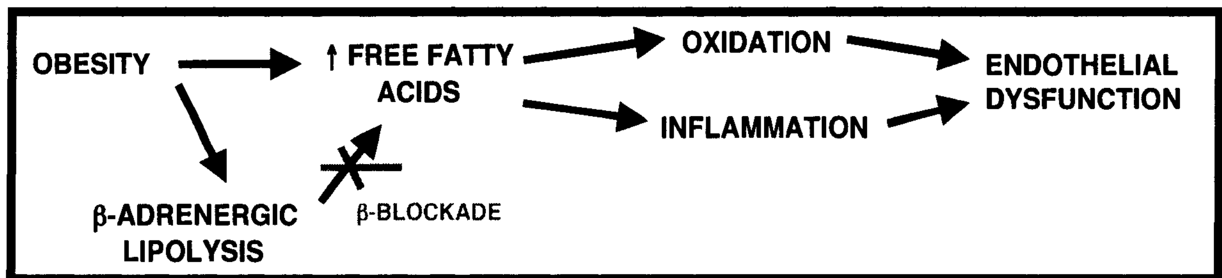

Figure 1.1 Diagrammatic representation of hypothesis.

The SPECIFIC AIMS of this study are to:

1. **MEASURE FFAS AND IN VIVO ENDOTHELIAL FUNCTION IN NORMAL AND FAT-FED RATS WITH AND WITHOUT β-ANTAGONISM.**
2. **MEASURE MARKERS OF INFLAMMATION AND OXIDATIVE STRESS IN NORMAL AND FAT-FED RATS WITH AND WITHOUT β-ANTAGONISM.**

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CHAPTER II

LITERATURE REVIEW

THE PROBLEM OF OBESITY

The prevalence of obesity, defined as a body mass index (BMI) ≥ 30, has doubled in the United States over the past 20 years. The Center for Disease Control and Prevention reported a 15% prevalence in U.S. adults in 1976-1980, which rose to 30% in 1999-2000.[51] Concomitantly, the prevalence of overweight (BMI ≥ 25) and extremely obese (BMI ≥ 40) individuals has increased to 34% and 5%, respectively.[51] Treatment of obesity-related cardiovascular disease added an estimated $22.17 billion to treatment of non-obesity related cardiovascular disease in 1996.[52] Obesity increases medical service costs by 37%, and medication costs by 77%, which exceeds that of smoking or alcoholism.[53]

More critical than the increased cost of care, obese individuals experience greater morbidity and mortality than individuals of normal weight. Obesity is an independent risk factor for coronary atherosclerosis,[1,54,55] and is strongly correlated with prevalence of cardiovascular disease.[52] Obese individuals with

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preexisting cardiovascular disease experience a 24% increase in relative risk of death from that disease.[56]

ADIPOCYTE PHYSIOLOGY AND CONTROL OF LIPOLYSIS

The lipolytic pathway

Far from being viewed as passive lipid depots, adipocytes are currently appreciated as multifaceted cells under intricate regulatory control, and the source of secretogues of systemic consequence. The role of adipocytes most relevant to this study is that of a reservoir for FFAs that are normally released with cold or fasting. Quantitatively, FFAs are the principle moiety secreted from adipocytes.[57] In response to stimulation of β-adrenergic receptors by catecholamines, stimulatory G-proteins mediate activation of adenylyl cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine 3',5'-monophosphate (cAMP).[58] Subsequently, cAMP activates protein kinase A (PKA), which phosphorylates hormone-sensitive lipase (HSL) and perilipin. Phosphorylation of perilipin physically alters the lipid droplet it encases, increasing permeability and allowing increased exposure of the TG substrate to the HSL. Active HSL hydrolyzes TGs to glycerol and FFA, which are released into the blood. Stimulation of the adipocyte adrenergic α_2-receptor acts via inhibitory G-proteins to attenuate adenylyl cyclase and inhibit lipolysis[58,59] (Figure 2.1). The distribution and density of these opposing receptors, along with the physiologic milieu, determine which influence prevails at any given adipose site.

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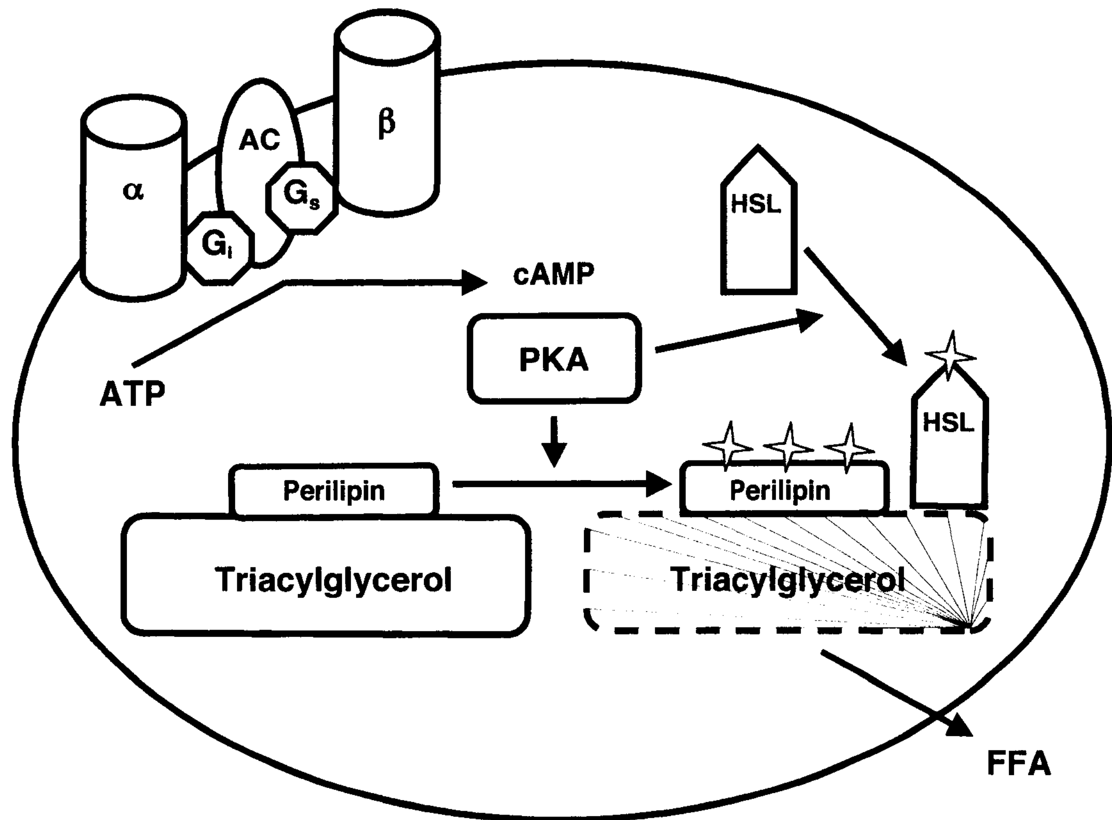

Figure 2.1 Sympathetic control of lipolysis within the adipocyte. Effectors bind to β-adrenergic receptors (β) and activate stimulatory G proteins (G_s). Subsequent stimulation of adenylyl cyclase (AC) converts adenosine triphosphate (ATP) to cyclic adenosine 3',5'-monophosphate (cAMP), which activates protein kinase A (PKA). PKA phosphorylates hormone-sensitive lipase (HSL) and perilipin. Upon phosphorylation, HSL moves from the cytosol to the lipid droplet surface. Perilipin, located on the surface of these droplets, becomes less uniform, presumably facilitating HSL-mediated lipolysis and subsequent release of FFAs into the plasma. Stimulation of α-adrenergic receptors (α) inhibits AC via inhibitory G proteins (G_i). Stars represent phosphorylation. Adapted from: Londos C et al. On the control of lipolysis in adipocytes. *Annals of the New York Academy of Sciences.* November 18 1999;892:155-168.

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Adrenergic modulation of the adenylyl cyclase-PKA cascade is not the sole means by which lipolysis is controlled. The adenylyl cyclase-PKA pathway is recruited by other stimulatory (adrenocorticotropic hormone, glucagons, thyroid stimulating hormone, parathyroid hormone) and inhibitory (adenosine, prostaglandin, nicotinic acid) factors to alter lipolytic generation of FFAs.[59] Additionally, genetically altered mice devoid of adipose β-receptors demonstrated enhanced lipolysis in response to fasting when compared to wild type mice,[60] suggesting that alternative pathways of intracellular SNS control of lipolysis exist. Moreover, there is evidence of a β-mediated, PKA-independent lipolytic cascade characterized by activation of the mitogen-activated protein (MAP) kinase and extracellular signal-regulated kinase (ERK) pathway.[61] Blocking this pathway eliminates 30% of catecholamine-mediated lipolysis,[62] and may preferentially be activated at high catecholamine doses after the PKC pathway is fully stimulated.[63]

Conditions that characterize the obese state influence the SNS and lipolysis. Just as sympathetic tone exerts effects on serum FFA concentrations, circulating FFAs may augment sympathetic activity.[64,65] In support of this idea is the observation that postprandial FFAs enhance sympathetic activity in adults with Type II diabetes.[31] Additionally, obesity-induced depletion of NO may augment β-mediated lipolysis.[32] Thus, a cycle of elevated FFA, NO depletion, and increased sympathetic tone exists in the obese individual.

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Peripheral and central nervous system control of lipolysis

Three β-receptor subtypes (β_1, β_2, β_3) are expressed in adipocyte membranes, and contribute to sympathetic control of lipolysis. Due to interspecies variability in density and sensitivity, the contribution of a specific β-receptor subtype cannot be generalized. Recruitment of β-receptors and associated lipolysis occurs with high concentrations of endogenous catecholamines.[66] Binding and functional studies using isolated rodent white adipocytes have shown β_1- and β_3-receptors to be the primary subtypes by which lipolysis is mediated, and support a minor role of the β_2-subtype.[67,68] Indeed, stimulation of the β_3-receptor fully activates lipolysis in rodents but has minimal effect in humans.[69] In contrast, human adipose primarily expresses β_1- and β_2-receptor subtypes.[63] Also in humans, antilipolytic α_2-receptors predominate, and in contrast to β-receptors, are preferentially stimulated at lower catecholamine concentrations. There is little α_2-expression in rodent adipocytes;[70] thus, potentiation of β-mediated lipolysis with α_2-receptor inhibition is negligible in rodents, but does occur in humans.[69]

In addition to the action of circulating catecholamines on adipocyte β-receptors, there is evidence of central nervous system (CNS) mediation of lipolysis via neural pathways linking nuclei within the brainstem and spinal cord to adipocytes stores. Retrograde neural tracers have identified sympathetic[71] and parasympathetic[72] innervation of white adipose with distinct CNS origins. Functional studies have demonstrated increased lipolysis with electrical

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stimulation of sympathetic nerve endings, and attenuation with denervation.[73] With parasympathetic denervation, glucose and FFA uptake into adipocytes was inhibited, and HSL activity increased.[72] Evidence of afferent pathways also exists, with obliteration of capsaicin-sensitive sensory nerves leading to attenuation of brown and white adipose mass.[74]

The sympathetic nervous system in obesity

The state of the SNS in obesity is controversial, and many conflicting studies exist. Obese, normotensive individuals have enhanced sympathetic activation,[29] and weight loss results in decreased nerve activity.[30] Specifically, obesity is associated with enhanced SNS activity in skeletal and cardiac muscle[30,31,75,76] and increased adipose β_2-receptor density in humans.[77] In contrast, there is evidence that adipocyte β-receptor responsiveness may be impaired,[77-80] and plasma noradrenaline concentrations decreased,[81] in obese individuals. One study, however, showed that lipolytic response did not vary between lean and obese individuals when expressed as a function of fat-free mass, suggesting the decrease in lipolysis may simply reflect the reduced metabolic requirements of obese individuals.[82] Others have suggested that regional differences exist. In subcutaneous adipocytes, obesity alters β-receptor function to favor decreased lipolysis. In contrast, obesity increases β-adrenergic function and downstream signaling in visceral adipocytes, augmenting the lipolytic response.[83] Obese men, who have increased risk of visceral adiposity

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and cardiovascular complications than obese women,[84,85] also have significantly greater β_3- and cAMP-mediated lipolysis.[86] Others have shown that individuals with visceral obesity have higher lipolytic rates in both visceral and subcutaneous adipocytes.[87]

Other determinants of serum FFAs

Serum FFA concentrations in obesity are not solely modulated by lipolytic generation within white adipose tissue. Chronic administration of a β_3-adrenergic agonist (CL-316243) to obese rats resulted in paradoxically reduced serum FFAs, and this was attributed to increased thermogenesis in brown adipose tissue.[88] Because FFAs are the primary substrate of brown adipose thermogenesis, it was postulated that FFA consumption prevailed over β-stimulated FFA generation, resulting in a net decrease in serum FFA concentration.[88] The same β_3-adrenergic agonist, when given as a single dose to wild-type mice, induced a 2-fold rise in serum FFAs.[89] This effect was absent in genetically altered mice lacking white and brown adipose tissue. These findings suggest that chronicity of β-adrenergic modulation and relative mass of adipose (i.e. normal vs. obese) may determine net effects on serum FFAs.

In addition to the action of HSL in adipocytes, circulating FFAs are determined by the activity of lipoprotein lipase (LPL) located on the capillary endothelium. This enzyme facilitates the breakdown of circulating TGs, contained in chylomicrons and lipoproteins, into FFA and glycerol. Liberated FFAs are

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either taken up into adipocytes and reesterified as stored TGs, or remain in the circulation bound to albumin.[58] While some report that stimulation of β-receptors suppresses LPL gene expression and enzyme activity,[90,91] other investigators have observed an increase in LPL gene expression and concentration with β-adrenergic stimulation.[92,93] More recent investigation shows that long-term β-stimulation with adrenaline may displace active LPL from the adipose capillary endothelium to the plasma.[92]

Adipocyte-derived leptin and control of metabolism

In addition to FFAs, adipocytes release other secretogues that act upon CNS targets and on local sympathetic nerves. Leptin, a product of the ob gene, is the principle hormone relevant to both adipose metabolism and the sympathetic nervous system. Leptin is secreted by white adipocytes and crosses the blood-brain barrier to act on the hypothalamus,[94,95] thereby contributing to energy homeostasis. Mice with a mutation in the ob gene are characterized by obesity, diabetes, inactivity, and reduced metabolic rate.[96] Daily treatment with leptin increases lean body mass, metabolism, and physical activity, and decreases food intake and body weight. Additionally, serum insulin and glucose levels were reduced.[96] More specifically, in rats, leptin infused centrally increases muscle glucose utilization, and promotes use of metabolic substrates with energy dissipation via increased uncoupling protein expression in adipose and muscle.[97] In murine skeletal muscle segments, leptin increases fatty acid oxidation and

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decreases their assimilation into TGs.[98] Cultured rat adipocytes exposed to leptin display decreased lipogenesis and increased efflux of FFAs.[99] The authors propose that, *in vivo*, leptin increases both the formation and breakdown of adipocyte TGs, and that this modification of FFA flux increases delivery of FFAs to non-adipose tissues capable of enhanced FFA oxidation.[99] The role of leptin as a catabolic hormone appears to be in direct contrast to the observation that serum leptin concentrations are proportional to adipose mass, and that obese humans have higher serum leptin concentrations.[100] This suggests that obesity is characterized by leptin resistance,[100] a phenomenon that is widely accepted and the subject of much investigation.[101]

Leptin and the sympathetic nervous system

The close association between leptin and the SNS is illustrated by a number of studies. Injection of rat adipose with leptin increases nerve signals recorded from an afferent nerve originating in the adipose.[102] Additionally, leptin causes increased sympathetic nerve activity, and decreased parasympathetic activity, in efferent nerves innervating epididymal adipose.[103] In humans, plasma leptin concentrations are correlated with renal catecholamine spillover.[104]

Just as leptin modulates the SNS, sympathetic tone, in turn, mediates leptin activity. The effect of the SNS on leptin activity is apparent under conditions of cold or starvation, which augment sympathetic tone and lead to inhibition of catabolism. This may be partly explained by observing stimulation of

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the sympathetic β-receptors in rat white adipose and associated inhibition of leptin secretion.[105] Increased adipocyte leptin levels in overfed rats are decreased by treating the animals with a β_3-agonist.[106] Others have shown that reduced ob gene expression occurs concomitantly with β_3-mediated reduction in plasma leptin.[107,108] Inhibition of leptin by β_3-agonists is believed to be dependent upon activation of cAMP-dependent protein kinases.[109,110] Functional studies reveal that the correlation between resting metabolic rate and plasma leptin that exists in normal humans is absent in those individuals with damage to the SNS from spinal cord injury.[111] Recently, however, it was shown that intact efferent sympathetic innervation of white fat in rodents is not required for low-dose leptin-induced reduction of adipose mass,[112] suggesting that alternative mechanisms of leptin modulation exist.

Visceral versus subcutaneous adipose: implications for vascular disease

Obesity is particularly pathogenic when the excess adipose is contained within visceral (or omental) storage sites. In fact, the amount of visceral, relative to subcutaneous, adipose is more relevant in predicting the presence of cardiovascular risk factors than overall body weight.[1,113-115] It is now known that visceral and subcutaneous adipose are biologically distinct, and that these differences account for the increased pathogenicity of visceral stores.[116] Using retrograde neural markers, it has been shown that sympathetic efferents to subcutaneous adipose have a central neural origin that is distinct from those of

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visceral depots.[72] Gene microarray analysis shows that the two depots have markedly different gene expression.[117] Multiple investigators have demonstrated a higher sensitivity to the lipolytic catecholamines in visceral adipose,[83,118,119] partly due to increased β-receptor density.[120] Additionally, the visceral adipose is more resistant to the antilipolytic effects of insulin.[121,122] The net effect of increased visceral lipolysis is increased FFAs directed to the liver via the portal circulation.[123] This increased flux of FFAs provides substrate by which the liver generates very low density lipoproteins (VLDL), and ultimately, more pathogenic lipid moieties.[124-126] The contribution of visceral fat to elevated serum FFAs has been further characterized by surgical removal of visceral adipose in obese rats, which results in normalization of serum FFA concentrations.[127]

Summary

It is evident that the relationship between the autonomic nervous system and adipocytes is complex, and requires appropriate efferent and afferent signaling between the two systems for functional regulation of systemic energy homeostasis to occur. Figure 2.2 is a simplified, diagrammatic representation of the regulatory loop that exists between adipocyte stores and the autonomic nervous system. Absent from this diagram is the additional influence of humoral modulation of the SNS exerted by endogenous catecholamines.

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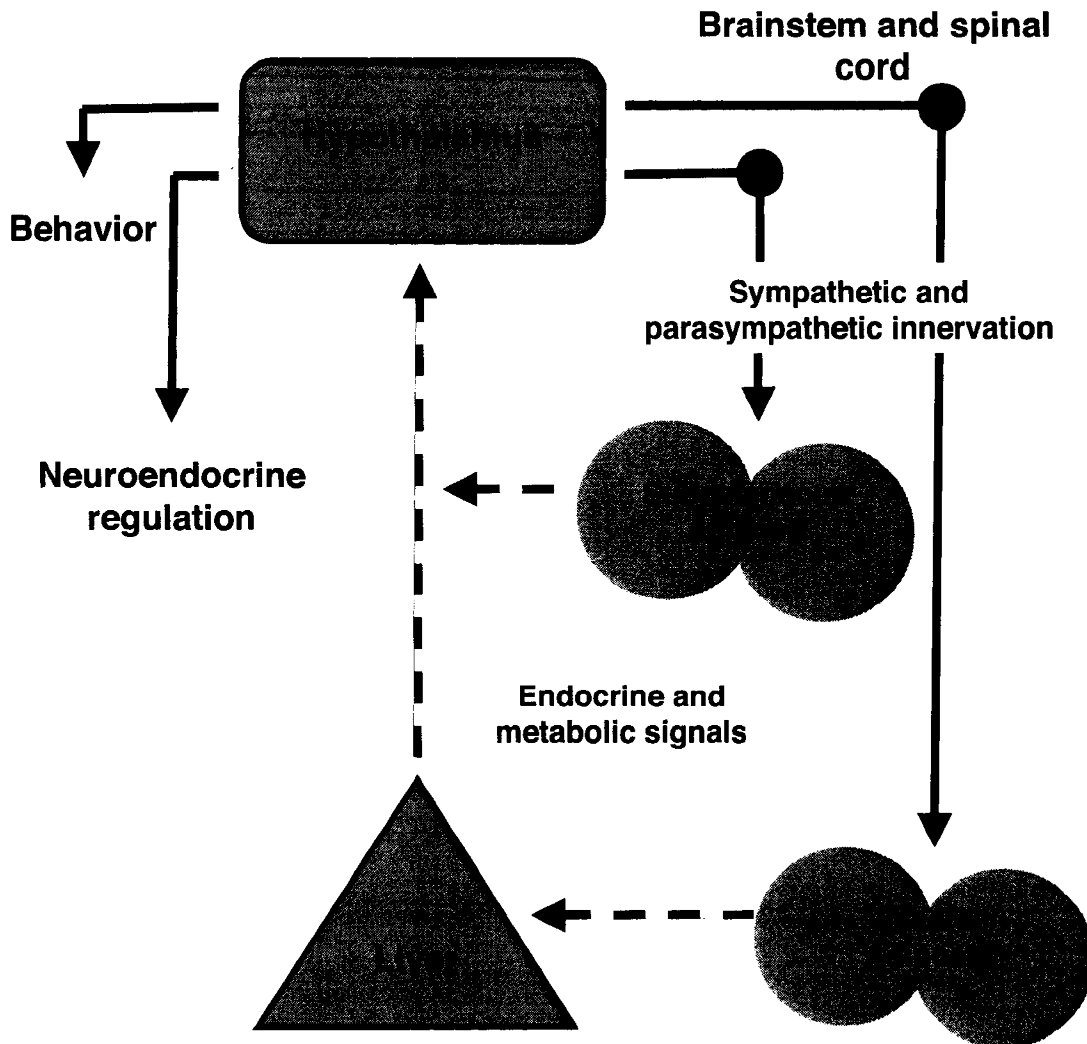

Figure 2.2 Diagrammatic representation of neural, metabolic, and endocrine signals conveyed between adipose stores and the autonomic nervous system. Metabolic and endocrine efferent signals originating in the adipose exert effects in the CNS via the hypothalamus. Autonomic pathways join the hypothalamus to brainstem nuclei, which provide sympathetic and parasympathetic innervation to adipocyte stores, modulating metabolic processes. As indicated schematically, the subcutaneous and visceral adipose sites are innervated by neurons that originate from distinct and separate regions within the brain stem and spinal cord. Adapted from: Fliers E et al. White adipose tissue: Getting nervous. *Journal of Neuroendocrinology.* November 2003;15(11):1005-1010.

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THE ENDOTHELIUM

The principle focus of these studies is the effect of elevated serum FFAs on the vascular endothelium, and the efficacy of β-antagonists in attenuating FFA-mediated endothelial dilator dysfunction. As noted, obese individuals have higher morbidity and mortality attributable to vascular disease. It is proposed that elevated FFAs in obesity contribute to this by promoting endothelial dysfunction. To better appreciate this phenomenon, it is helpful to review relevant biology of the vascular endothelium.

Functions of the normal endothelium

The luminal surface of arteries is lined with a monolayer of endothelial cells, which overlay a medial layer of smooth muscle cells and an external layer of connective tissue.[58] In health, endothelial cells release mediators that facilitate the appropriate regulation of multiple processes, including vascular permeability, inflammation and cell adhesion, coagulation, maintenance of intercellular matrix, lipid metabolism, and vascular reactivity.[128-130] Specifically, endothelial cells provide a barrier between the blood and interstitium, with passage of nutrients, ions, water, and cells through selective transporters, caveolae-facilitated transcytosis, and intercellular tight junctions.[128] The endothelium is also the source of cytokines which modulate host inflammatory and immune responses.[128] Cellular adhesion molecules [i.e. intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM), P-selectin, E-selectin] facilitate

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leukocyte rolling and subsequent adherence to the endothelium.[128,131] Transmigration of leukocytes from the blood into the interstitium requires the expression of platelet/endothelial cell adhesion molecule-1 (PECAM-1) and integrins in the endothelial intercellular junction.[130,131]

Relative to coagulation and hemostasis, endothelial cells express surface receptors that bind blood-borne coagulation proteins. Protein-receptor interactions activate endothelial cells and induce expression of factors mediating coagulation, inflammation, and vascular reactivity.[128] Endothelial cells synthesize procoagulant factors such as vonWillebrand factor and thrombospondin, which promote platelet adherence and aggregation.[130] Endothelium-derived fibronectin stabilizes fibrin monomers and promotes clot formation.[130] Basal generation of tissue factor pathway inhibitor, as well as binding of surface thrombomodulin with thrombin and activation of protein C, are two principle means by which endothelial cells inhibit inappropriate activation of procoagulant pathways.[128,130]

Vascular endothelial growth factor (VEGF) is synthesized by endothelial cells and modulates growth of new blood vessels (angiogenesis).[128] The endothelium also modulates the dynamic response to changes in shear stress through acute and chronic changes in intracellular signaling, gene expression, and factor regulation to alter cytoskeletal structure and endothelial cell alignment.[132]

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The role of nitric oxide in preservation of endothelial function

Products generated by endothelial cells exert effects locally and at distant sites throughout the body.[128] A principle product synthesized by endothelial cells is NO, which plays a critical role in functions aimed at preserving healthy endothelium.[131] In addition to contributing to endothelium-dependent vasodilation (see below), NO optimizes endothelial function by other mechanisms. NO inhibits platelet[133] and leukocyte[134-136] adhesion to the endothelium, thereby attenuating inappropriate pro-coagulant and pro-inflammatory processes. Cultured endothelial cells exposed to TNF-α display increased expression of adhesion molecules and inflammatory cytokines, and pretreatment with an NO donor reduces this effect.[137,138] Treatment with an NO donor also attenuated endothelial cytotoxicity and neutrophil adhesion attributable to the ROS superoxide.[139] Mechanical injury to arterial segments from obese rats caused more profound expression of adhesion molecules, macrophage infiltration, and intimal thickening than in control animals, and exogenous NO attenuated the magnified response to injury.[140] Inhibition of vascular smooth muscle proliferation by NO reduces the severity of pathologic intimal thickening, a condition seen with atherosclerosis and mechanical damage to the endothelium.[141] Further, NO appears to attenuate the rise in tissue factor, a procoagulant glycoprotein, that occurs in the presence of cytokines and endotoxin.[142] It is evident that diminished NO production or enhanced NO depletion may contribute to profound disruption in normal

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endothelial function. With so many critical functions, it is easily appreciated how dysfunction of the endothelium can lead to critical pathologic sequelae.

ENDOTHELIUM-DEPENDENT VASODILATION

Overview

As noted, an important function of the endothelium is maintenance of appropriate dilatory and constrictor responses to circulating and local stimuli. Vascular reactivity may be determined by eliciting vasodilation using agonists [i.e. acetylcholine (Ach), bradykinin] or mechanical stimuli (i.e. shear stress),[58,130] the latter being the predominant physiologic stimulus to the endothelium.[143] In response, vascular endothelial cells generate the vasodilators NO, prostacyclin (PGI_2), and endothelium-dependent hyperpolarizing factor (EDHF), resulting in relaxation of underlying vascular smooth muscle cells. NO and prostacyclin act primarily via cyclic guanosine monophosphate (cGMP) and cAMP pathways, respectively, which decrease smooth muscle cell cytosolic calcium concentrations, resulting in vessel relaxation. EDHF, a process or factor yet to be identified, produces vasodilation by hyperpolarizing vascular smooth muscle cells. It is believed that NO serves as the primary mediator of relaxation in conduit arteries, while EDHF is the principle dilator in resistance arteries.[144-146] Relative EDHF:NO activity is not wholly correlated with vessel diameter, however, so additional unidentified factors also direct the heterogenous distribution of these vasodilators[145] (Figure 2.3).

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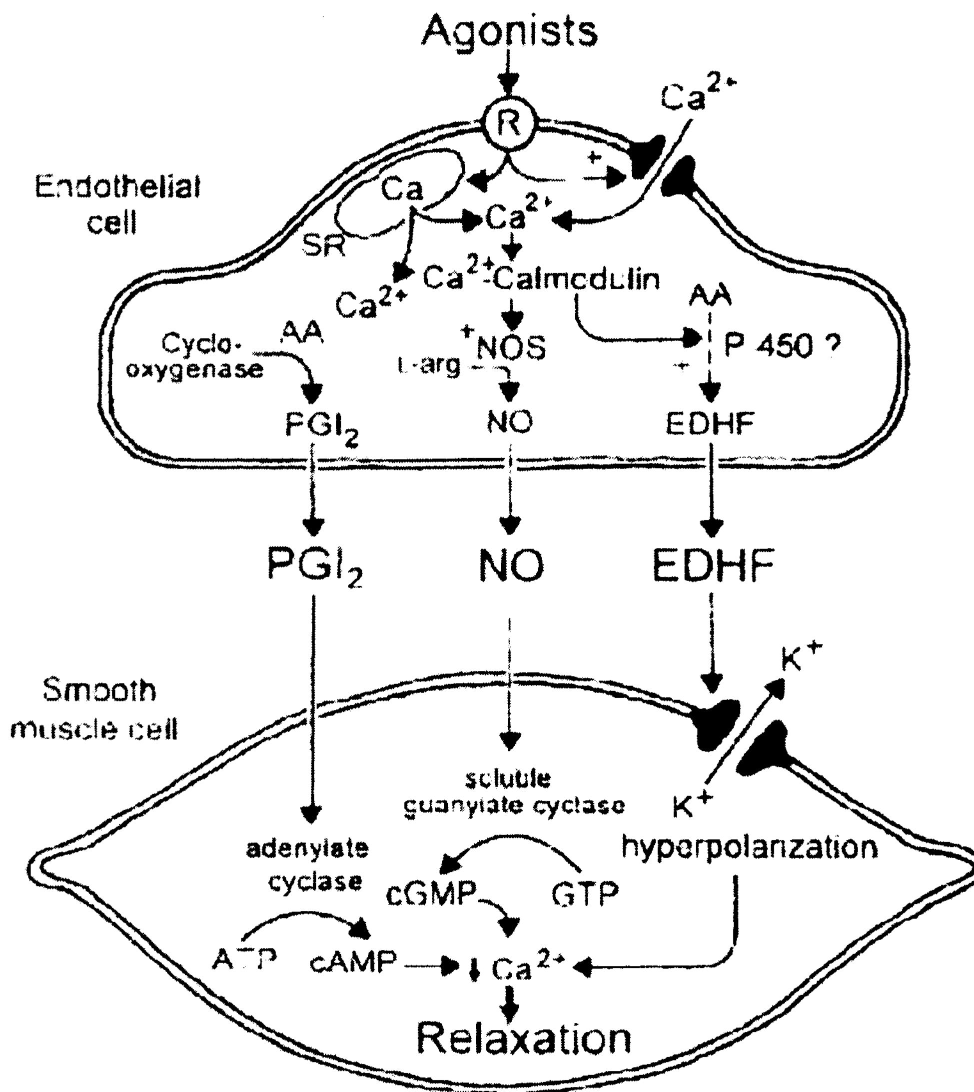

Figure 2.3 Schematic representation of endothelium-derived vasodilators (PGI_2, NO, EDHF) and the pathways involved in relaxation of the underlying smooth muscle. (L-arg, L-arginine; SR, sarcoplasmic reticulum; R, receptor; NOS, nitric oxide synthase; AA, arachadonic acid). From: Mombouli J, Vanhouette PM. Endothelial dysfunction: From physiology to therapy. *J Mol Cell Cardiol.* January 1999;31(1):61-74.

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The extent of interdependence of the systems responsible for endothelium-dependent vasodilation is not known. Mâny animal models using artery ring myography or perfused vessel systems have been studied. Some authors suggest that EDHF serves as a "back-up" in states of reduced NO activity, such as hypercholesterolemia and hypertension.[143,147,148] Others maintain that insufficient cross talk occurs for such a mechanism to be present.[149] The profile of relative contributions of various vasodilatory pathways in health versus disease remains to be defined.

Nitric oxide as a contributor to endothelium-dependent vasodilation

The conversion of L-Arginine to NO and citruline is facilitated by the enzyme nitric oxide synthase (NOS).[58] The endothelial isoform, NOSIII, is anchored to the plasma membrane and constitutively produces NO.[150] The stimulus for NO production by NOSIII may occur as shear stress, a frictional force applied to the vessel wall by the flow of blood,[151] or as receptor activation by agonists such as Ach, bradykinin, and ATP.[150]

Shear stress, the most physiologically relevant activator of NOSIII, regulates vessel diameter and capacitance in response to the change in flow that occurs with each myocardial contraction.[150,151] Shear stress produces an acute, transient increase in NOSIII mRNA transcription and enzyme activity, as well as a prolonged stabilization of NOSIII mRNA.[152,153] These two pathways are distinct, though activation of the tyrosine kinase c-Src is common to both.[153]

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Acute application of shear stress enhances NOSIII enzyme activity in the following manner. Inactive NOSIII is localized to calveolae, invaginations in the endothelial cell membrane that are capable of transmission of luminal changes in mechanical forces.[152] Shear stress activates calveolar NOSIII, and also causes dissociation of NOSIII from the calveolar protein calveolin with simultaneous binding to calcium-calmodulin complexes.[152] Association with calmodulin further enhances NOSIII activity by inducing allosteric changes favoring electron flux and NO formation[151,154] (see below). The subsequent acute rise in NO is followed by a prolonged, modest increase in NO in the face of sustained shear stress.[151] This latter phase is independent of increased intracellular calcium, and believed to be modulated by phosphorylation of NOSIII by tyrosine kinases.[155,156] In addition to the low levels of NO that are continuously produced by normal shear stress via NOSIII phosphorylation, humoral agonists such as Ach can induce acute vasodilation by increased NO activity. These pathways are believed to be principally modulated by increased intracellular calcium and promotion of NOSIII-calmodulin complexes, and preexisting NOS phosphorylation from chronic shear may lower the amount of calcium required for NOSIII activation and facilitate agonist-mediated dilation.[150]

Two identical monomers make up NOSIII. Each monomer has a carboxy-terminal reductase domain which binds dihydronicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide and flavin mononucleotide, and an amino-terminal oxidase domain which contains binding sites for heme and

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tetrahydrobiopterin.[150,151,157] The conversion of L-arginine and O_2 to NO and citrulline requires that electrons derived by NADPH flow from the reductase domain of one NOSIII monomer to the heme iron of the oxygenase domain located on the other monomer.[151] Subsequent formation of reduced oxygen favors oxidation of L-arginine to NO and citrulline.[158] Tetrahydrobiopterin serves to structurally stabilize the enzyme so that the flow of electrons and subsequent NO production is favored.[159] In the absence of tetrahydrobiopterin, NOSIII is uncoupled and superoxide is preferentially generated.[159,160] Superoxide quenches NO, resulting in NO depletion and endothelial dysfunction in many disease states.[160-163] Once formed in the endothelial cell, NO activates guanylate cyclase in the underlying smooth muscle. The resultant increase in cGMP and reduced intracellular calcium causes vasodilation.[58,131]

Endothelium-dependent hyperpolarizing factor as a contributor to endothelium-dependent vasodilation

Unlike NO- and PGI_2–mediated vasodilation, EDHF-mediated vasodilation is strongly dependent on hyperpolarization of the vascular smooth muscle underlying the endothelium from which the EDHF originates.[144,164] Three major hypotheses of EDHF-mediated hyperpolarization and vasodilation exist. It is probable that portions of each of these mechanisms are contributory, and may act synergistically.

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One mechanistic theory is that Ach increases intracellular calcium concentrations in endothelial cells. Increased intracellular calcium results in opening of endothelial calcium-activated potassium channels (K_{Ca}), which increases potassium concentrations in the extracellular space between the endothelial and smooth muscle cells. Increased extracellular potassium stimulates Na^+/K^+-ATPase and inwardly rectifying potassium channels (K_{ir}) on the smooth muscle cell membrane, resulting in net outward movement of positive charge and hyperpolarization of the smooth muscle cell.[165] While potassium may contribute to EDHF-mediated vasodilation in specific arterial beds, it is unlikely to be a universal EDHF.[166] An alternative hypothesis is that smooth muscle cell hyperpolarization results from transmission of endothelial hyperpolarization or EDHF via myoendothelial gap junctions,[167] rather than from increased extracellular potassium concentrations. Lastly, studies have demonstrated attenuation of EDHF activity by inhibitors of cytochrome P450, suggesting that EDHF may be a product of arachidonic acid metabolism.[167] It has been suggested that increased intracellular calcium within the endothelial cell results in enhanced cytochrome P450 epoxygenase activity in selected vessels.[167] It is likely that the role of P450 metabolites in EDHF-mediated vasodilation is dependent on the vessel and preparation under study, and is more permissive than causal to EDHF activity.

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Insulin-mediated vasodilation

Relevant to vascular reactivity under conditions of metabolic aberrancy is the role of insulin as a vasodilator. Under normal conditions, insulin causes local dilation and increased blood flow. Enhanced flow causes increased glucose delivery to skeletal muscle, thereby facilitating efficient extraction of glucose from the blood to the muscle.[168,169] A blunted vasodilatory response has been observed in skeletal muscle of obese, insulin-resistant humans,[170] and in mesenteric arteries of insulin-resistant rats.[171] This vascular insulin resistance may further contribute to impaired glucose clearance.[168]

The mechanism of insulin-mediated vasodilation has been attributed to prostacylin- and ATP-dependent potassium channels,[171] calcium-activated potassium channels,[172] and increased NO.[173] Others have reported increased skeletal muscle SNS activity with hyperinsulinemia in humans,[174] indicating that insulin-mediated vasoreactivity may be mediated by the autonomic nervous system. In rat mesenteric arteries, vascular resistance to insulin was attenuated by application of an endothelin receptor antagonist, suggesting that increased endothelin activity contributes to impaired insulin-mediated dilation.[171]

Relative to the contribution of NO to insulin-dependent dilation, in coronary arteries of obese Zucker rats, insulin-dependent vasodilation was impaired due to ROS depletion of NO.[175] The normal dilatory response to insulin was restored with inhibition of vascular NADPH oxidase, suggesting that this enzyme mediates the increase in ROS.[175] Elevated endogenous and exogenous serum FFAs impair

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insulin-mediated increases in leg blood flow and glucose disposal in humans,[176,177] and this was due to impaired NO production.[176] Acute hyperinsulinemia attenuates flow-mediated brachial artery dilation independent of insulin sensitivity and serum cholesterol levels in humans, providing evidence that, in high concentrations, insulin may directly contribute to endothelial dilatory dysfunction.[178]

ENDOTHELIAL DYSFUNCTION IN OBESITY

Free fatty acids and endothelial dysfunction in obesity

Elevated serum FFAs, as in obesity, promote acute and chronic endothelial vasodilator dysfunction when introduced intravenously or by dietary alterations, respectively.[3-6] FFAs exert this effect by induction of insulin resistance, enhanced generation of ROS, and proinflammatory effects.[7-10,179-182] The first two mechanisms serve to deplete NO through generation of ROS, while the latter reduces NO by direct injury to endothelial cells. These processes will be described in detail in the following paragraphs.

Free fatty acids and inflammation

Elevated inflammatory cytokines, including CRP, IL-6 and TNF-α, have been associated with overweight and obese individuals,[11-13] and weight loss lowers serum concentrations.[14-18] FFAs induce protein emulsification by detergent activity,[10] and are highly associated with increased IL-6, TNF-α, and CRP in

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normal and obese adults .[7,183] Small elevations in CRP have been correlated with increased risk for cardiovascular events in healthy individuals and in those with existing atherosclerosis.[15,19]

The cytokines are not simply markers of inflammatory processes. The relationship between obesity, inflammation, and vascular disease may partly be attributed to impaired endothelial function that occurs as a result of elevated cytokine concentrations.[21-23] Elevated serum CRP, IL-6, and TNF-α were shown to be independent predictors of vascular events in the elderly.[20] Specifically, CRP induces expression of monocyte chemoattractant peptide (MCP-1), partly via stimulation of NF-κB and IL-6.[184] Additionally, CRP increases expression of adhesion molecules (i.e. ICAM-1, VCAM-1) in human endothelial cells.[185] CRP also induces expression of NOS II (inducible NOS, or iNOS) RNA, which is postulated to favor peroxynitrite formation and increased oxidative stress.[184] In endothelial cells, diminished eNOS mRNA stability leading to impaired NO bioactivity has also been attributed to CRP.[186,187] Moreover, CRP-mediated inhibition of NO has been demonstrated in vascular smooth muscle cells.[23] The inflammatory cytokine IL-6 also induces direct injury to the endothelium, causing increased permeability in cultured monolayers, thereby attenuating endothelial barrier function.[22] TNF-α contributes to endothelial dysfunction by attenuating insulin-mediated eNOS expression in diabetic individuals.[188] These studies suggest that FFAs exert proinflammatory effects through multiple mechanisms,

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including NF-κB activation (see above), induction of direct cytotoxicity, and generation of inflammatory cytokines.

Free fatty acids and oxidative stress

There is a positive association between waist-to-hip ratio and markers of systemic oxidative stress,[189] and dietary restriction leads to reduced oxidative damage to lipids and proteins in obese individuals.[190] Serum FFAs, elevated in individuals with increased waist-to-hip ratio, increase oxidative stress.[24,191] As noted, FFAs induce endothelial dysfunction. Vitamin C, an antioxidant, reverses endothelial vasodilator dysfunction seen with oral or intravenous fatty acid administration.[26,192,193] Additionally, antioxidants attenuate endothelial dysfunction attributable to high-fat feeding in both humans and rodents.[26,27] These findings suggest that obesity-associated endothelial dilator dysfunction is partly attributable to the pro-oxidant nature of circulating FFAs.

FFAs increase oxidative stress through generation of ROS and depletion of endogenous antioxidants.[24] Specifically, FFAs promote uncoupling of oxidative phosphorylation in the mitochondria of several cell types, which favors mitochondrial ROS generation.[194-196] FFAs also promote the formation of ROS by vascular endothelial and smooth muscle NADPH oxidase.[8] Further, exposure of vascular endothelial cells to exogenous unsaturated fatty acids increased the proportion of carbon-carbon double bonds in cell membranes through replacement of endogenous fatty acids. This increase in double bonds

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augmented the susceptibility of the cell membrane to oxidative damage by ROS.[197] Tipping the balance further in favor of oxidative stress is the FFA-mediated depletion of glutathione, an endogenous antioxidant, in vascular endothelial cells.[198]

In addition to the direct damage to proteins, lipids, and DNA imposed by excess ROS, accumulated superoxide reacts with NO to form peroxynitrite, thereby depleting NO and reducing its bioactivity.[7,8,25] Rats with dietary obesity and hypertension exhibit decreased NO bioavailability attributable to increased oxidative stress.[199] In hypercholesterolemic rabbits, endothelial dysfunction was attributable to reduced NO, and improved vasodilation was associated with reduced oxidative stress.[200] Investigation of EDHF impairment with oxidative stress includes examination of the vasodilatory profile in a rat model of Type I diabetes. Rats with streptozotocin-induced (Type I) diabetes have impaired EDHF-mediated vasodilation[201] which is improved with antioxidant treatment.[202,203] In contrast, renal artery rings from hypercholesterolemic rabbits had mildly impaired vasodilation in response to Ach, but increased vasodilatory activity attributable to EDHF.[147] Others have demonstrated focal EDHF inhibition with ascorbate.[204] The degree to which EDHF activity is attenuated or enhanced by increased oxidative stress, as well as compensatory capabilities of the EDHF/NO system, remain unclear, and is likely specific to the disease state, regional EDHF:NO ratio, and experimental model.

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Both ROS and FFAs activate the transcription factor nuclear factor (NF)-κB, promoting the expression of a number of genes for chemokines, adhesion molecules, and immune receptors.[24,205] Inappropriate regulation of NF-κB is associated with two disease states characterized by endothelial dysfunction, diabetes and atherosclerosis.[24] NF-κB activation may therefore be an additional mechanism by which FFAs contribute to ROS-mediated endothelial dysfunction.

Finally, FFAs contribute to oxidant-mediated endothelial dysfunction by promoting insulin resistance and hyperglycemia. Obesity, dyslipidemia, and insulin resistance are principle abnormalities in the clinical condition termed the metabolic syndrome.[206] Acute elevation in exogenous FFAs induces insulin resistance in both normal and diabetic individuals.[180,207,208] Additionally, reducing serum FFAs with an antilipolytic agent improves insulin sensitivity in lean and obese humans, and in obese rats.[209,210] The mechanism of FFA-induced insulin resistance remains unclear, but it is likely to be multifactorial as distinct defects emerge with varied durations of FFA elevation.[211] At the cellular level, FFA infusion causes inhibition of glucose transport in skeletal muscle, possibly by attenuation of insulin receptor subtrate-1-associated PI3-kinase activity and associated Glut 4 transporter inhibition.[180] Others have demonstrated FFA-induced increases in diacylglycerol and protein kinase-C in muscle, and propose that this may lead to insulin resistance through insulin receptor phosphorylation, induction of oxidative stress, and/or activation of NFκB.[212] Inhibition of glucose transport leads to decreased muscle glycogen synthesis and glucose

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oxidation.[182] Subsequent hyperglycemia contributes to oxidative stress and endothelial dysfunction,[213-215] and therefore is an indirect mechanism by which elevated FFAs contribute to vascular complications in obesity.

Triglycerides and endothelial dysfunction in obesity

The contribution of serum TGs to endothelial dysfunction remains unclear. Investigators have observed impaired Ach-mediated relaxation in arterial rings from dietary obese rats that is significantly correlated with serum TGs, but not FFAs.[216] Postprandial hypertriglyceridemia is associated with impaired endothelium-dependent vasodilation and attributable to increased oxidative stress.[217-219] In contrast, humans with chronic hypertriglyceridemia have impaired endothelium-dependent vasodilation that is correlated with insulin resistance, including elevated FFAs, but not with serum TGs.[220] A partial explanation for these divergent data is the observation that hypertriglyceridemic patients with impaired LPL activity do not demonstrate attenuated endothelial dilator function.[221] This suggests that it is not the TGs, but the products of TG hydrolysis (i.e. FFAs), that contribute to the endothelial dysfunction in these patients. Additionally, the majority of investigators who attribute endothelial dysfunction to serum TGs do not include serum FFA measurements in the study design.

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Leptin and endothelial dysfunction in obesity

Modulation of metabolism by leptin, and the interaction of leptin with the SNS, have been previously discussed. As noted, serum leptin concentrations are proportional to adipose mass, being higher in obese individuals.[100] In addition to modulating energy homeostasis, leptin exerts effects on the vasculature. Rats exposed to 7 days of leptin infusion exhibited increased MAP and tachycardia, responses that were absent in animals administered concomitant β-antagonists.[222] Rats administered intravenous leptin produced dose-dependent increases in sympathetic nerve activity to brown adipose, kidneys, and the hind limb.[223] Though this group of rats did not exhibit elevated MAP or HR, others have demonstrated increased MAP and HR with infused leptin.[224] In addition to systemic hemodynamic effects modulated through the SNS, leptin exerts direct vasodilatory effects through both NO- and EDHF-mediated mechanisms.[225,226] The net result of these contrasting influences is believed to lie in favor of sympathetically-mediated vasoconstriction, which overwhelms vasodilation attributable direct leptin stimulation.[227] In contrast, concentrations of leptin present in obese individuals were shown to inhibit Ach-mediated vasodilation in canine coronary arteries despite enhanced NO production.[228] The adverse effect on endothelium-dependent vasodilation may in part be attributed to accumulation of ROS involved in leptin signalling,[229] as well as leptin-induced antioxidant depletion.[230]

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EFFECTS OF β-ADRENERGIC ANTAGONISM

Protective effects of β-antagonists in cardiovascular disease

β-antagonists exert protective effects in patients with coronary artery disease, myocardial infarction, hypertension, and congestive heart failure, and are associated with reduced mortality and morbidity.[33-36] In addition to favorable effects on the myocardium,[35,37-39] β-antagonists have beneficial effects in the vasculature. β-antagonists are effective in decreasing blood pressure in hypertension.[42,43] Third-generation β-antagonists reduce endothelin-1 secretion and cell proliferation in culture, which may contribute to antiproliferative effects in vivo.[40] Additional antiatherogenic effects include slowed progression of vessel intima-media thickness[44] and enhanced NO activity as evidenced by improved endothelium-dependent relaxation.[43,45-49] Exposure of cultured adipocytes to the β-agonist isoprenaline increased the release of IL-6,[231] and β-antagonism reduces CRP concentrations in patients with angina and coronary artery disease.[50] Thus, β-antagonism may also improve endothelial function by attenuating inflammation and reducing the detrimental effects of CRP on cellular adhesion and NO availability. The mechanisms by which β-antagonists exert these protective effects remain poorly defined. While β_2-mediated vasodilation requires NO,[232] modulation of vascular β-receptors do not appear to be a mechanism by which some β-antagonists activate eNOS and improve endothelial function.[233] This suggests that alternative mechanisms of β-antagonist mediated

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vascular protection likely exist, and outweigh the possible decrement in β-agonist induced increases in NO.

Effects of β-antagonists on cardiovascular parameters

Hemodynamic variables such as heart rate, cardiac contractility, and vascular tone are also mediated by the β-adrenergic nervous system.[58] Stimulation of myocardial β_1- and β_2-receptors increases contractility and heart rate,[58] and this is the basis for the use of β-antagonists as antihypertensive agents. Stimulation of β_2-receptors results in dilation of systemic veins and the arterioles supplying the cardiac and skeletal muscle, lungs, abdominal viscera, and kidneys.[58] In humans, β_3-receptor mRNA is primarily expressed in abdominal adipocyte depots, with less expression in subcutaneous stores.[234] No β_3-receptor mRNA was detected in human skeletal muscle, heart, liver, lung, kidney, or thyroid.[234] Species variation in receptor distribution exists. In dogs, β_3-agonists increase myocardial contractility, whereas this effect is less pronounced in rats and absent in nonhuman primates.[235] In rats, β_3-antagonism increases blood flow to brown fat stores.[236]

Effects of β-antagonists on glucose and lipid metabolism

Because of the heterogeneity of lipophillicity, sympathomimetic activity, receptor selectivity, and agonism within the class of β-antagonists, effects on the lipid profile are specific to the β-antagonist being examined. Cardioselective β-

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antagonists that possess partial agonist activity have favorable effects on lipid profiles in dyslipidemic individuals.[237] Nonselective β-antagonists have adverse effects on blood lipids, causing increased plasma TG and decreased high-density lipoprotein (HDL) levels. One study demonstrated no change in FFAs with β-antagonism.[238] Others have shown a reduction in serum FFAs during exercise with administration of β_1-selective antagonists and lipid-lowering compounds.[239] Of related interest is the effect of β-antagonists on body weight. Long-term use of β-antagonists for treatment of hypertension in humans results in an average weight gain of 1.2 kg,[240,241] and this has been attributed to a decrease in resting metabolic rate.[242]

There is controversy regarding the effects of β-antagonists on insulin sensitivity. This is important when one recalls that increased serum glucose concentrations, which can result from progressive insulin resistance, attenuate endothelium-dependent vasodilation. Atenolol, a selective β_1-antagonist, is the agent most commonly associated with impaired insulin sensitivity,[243,244] despite efficacy in reducing blood pressure.[245,246] Newer β-antagonists with vasodilatory properties attributed to partial β_2-agonism do not worsen insulin resistance,[247-249] suggesting that alterations in peripheral blood flow may contribute to the metabolic aberrancies seen with pure β_1-antagonists.[247]

Changes in heart rate, cardiac contractility, and vascular tone, then, are also important mediators of lipid metabolism, as it is the rate of blood flow that determines the concentration of substrates and regulatory hormones to which the

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adipocytes are exposed.[250] In addition to attenuation of NO-mediated endothelial dilator function, insulin resistance in obese individuals impairs the normal increase in adipose blood flow that occurs after meal ingestion.[251] This is accompanied by reduced plasma norepinephrine, suggesting that the attenuation in post-meal blood flow may be mediated by blunted insulin-mediated sympathetic stimulation of flow.[251] Normal increases in adipose blood flow prevent accumulation of FFAs derived from lipase activity and preserve efflux of FFAs from adipocytes.[252] It has been proposed that the accumulation of plasma glucose and TGs that occurs with impaired flow-mediated extraction may contribute to vascular disease, and that β-adrenergic antagonists may contribute to this phenomenon.[253]

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CHAPTER III

RESEARCH DESIGN AND METHODS

AIM 1: MEASURE FFAS AND IN VIVO ENDOTHELIAL FUNCTION IN NORMAL AND FAT-FED RATS WITH AND WITHOUT β-ANTAGONISM.

Rationale/strategy

Endothelial dysfunction is present in obese individuals and likely plays an important role in obesity-associated vascular events. Serum FFAs are increased in the obese state and are known to induce endothelial dysfunction. Obesity-induced elevations in FFAs can in part be attributed to enhanced TG lipolysis, which in turn is the result of regional sympathetic overactivity known to occur in obese individuals. β-antagonism may attenuate sympathetic lipolysis, decrease FFAs, and thereby improve endothelium-dependent vasodilation.

In preliminary studies, the **acute** effects of selective β-antagonists (i.e. β_1, β_2, β_3) on serum FFAs and endothelial function were studied in **normal** rats with isoproterenol (ISO)-induced lipolysis.[69] The effects of **long-term** β-antagonism on serum FFAs and endothelial function were studied in **fat-fed** (dietary obese) rats.

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Design: Effects of acute β-antagonism in normal rats

Fifty-six pairs of normal (i.e. non-fat-fed) male Sprague-Dawley rats were randomly assigned to 1 of 7 groups: 0.9% saline (NS) + NS; NS + ISO; dimethyl sulfoxide (DMSO) + ISO; β_1 + ISO; β_2 + ISO; β_3 + ISO; β_1+ β_3 + ISO (β_x = antagonist receptor subtype). For each treatment, one group of rats was used for serum and tissue sampling, and a separate group used for measurement of endothelium-dependent vasodilation. Rats were fasted overnight and body weights were measured. General anesthesia was induced with ketamine 80 mg/kg and xylazine 12 mg/kg injected in the intraperitoneal (IP) space. A 1:1 mixture of ketamine 100 mg/ml and xylazine 20 mg/ml was used for subsequent anesthetic dosing, the need for which was determined by assessment of anesthetic plane conducted at least every 3 minutes. Regional delivery of 95% oxygen/5% CO_2 was achieved by maintenance of a mask just cranial to the nares. This respiratory protocol was established to avoid artifact induced by mechanical ventilation. Additionally, arterial blood gas measurement revealed adequate oxygenation and only mild and intermittent respiratory acidosis at the conclusion of experiments. It was anticipated that the stress of handling during anesthetic induction might increase endogenous catecholamines[254,255] and activate lipolytic pathways; thus, a 30-minute equilibration time was integrated between anesthetic induction and experimental initiation.

The β-antagonists used in this study include: β_1=atenolol 6 mg/kg (24 mg/ml DMSO); β_2=butoxamine 2.5 mg/kg (12.5 mg/ml water); β_3=SR 59230A 1

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mg/kg (4 mg/ml DMSO). Route of administration was IP injection. Sixty minutes after β-antagonist treatment, 0.05 mg/kg ISO or equal volumes of NS were injected subcutaneously (SC). Blood samples were obtained 15 minutes after ISO or NS administration based on previous work showing a 94% increase in FFAs in rats with a similar ISO dose and sampling regimen[256] (Figure 3.1).

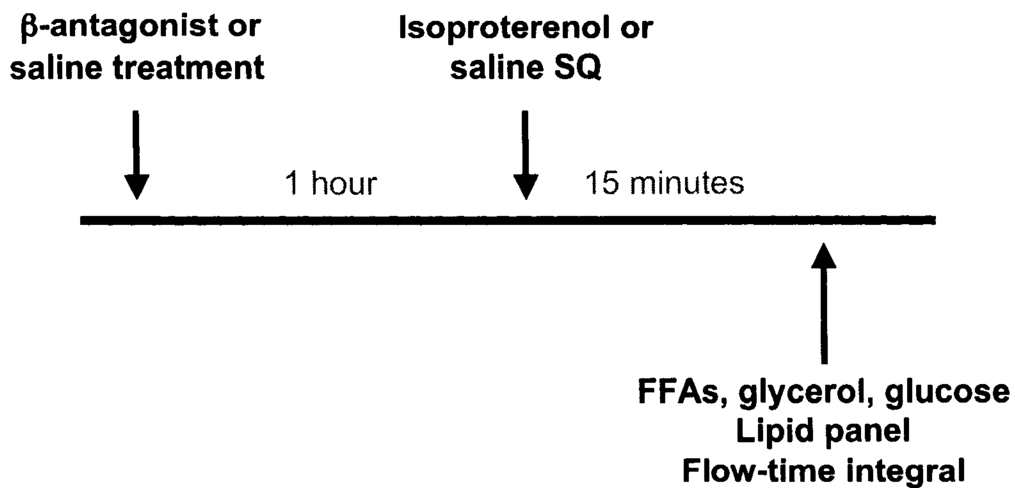

Figure 3.1 Experimental design of study investigating acute β-antagonism in normal rats with ISO-stimulated lipolysis.

Design: Effects of long-term β-antagonism in obese rats

The β-antagonist with the most profound acute antilipolytic effect (β_x) was to be identified and used in chronic studies of normal and fat-fed rats. Forty-eight pairs of rats were assigned to 1 of 6 groups: normal chow; normal chow + placebo pellet; normal chow + β_x-antagonist; high-fat chow; high-fat chow + placebo pellet; high-fat chow + β_x-antagonist. For each treatment, one group of rats was used for serum and tissue sampling, and a separate group used for

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measurement of endothelium-dependent vasodilation. To minimize handling and stress-induced increases in adrenergic tone, placebo and β_x-antagonist treatments were administered by SC slow-release pellets (Innovative Research of America, Sarasota, FL). Pellets were in place during the final 4 weeks of the 16-week dietary intervention period (Figure 3.2). Instrumentation and measurements were identical to those used in acute studies.

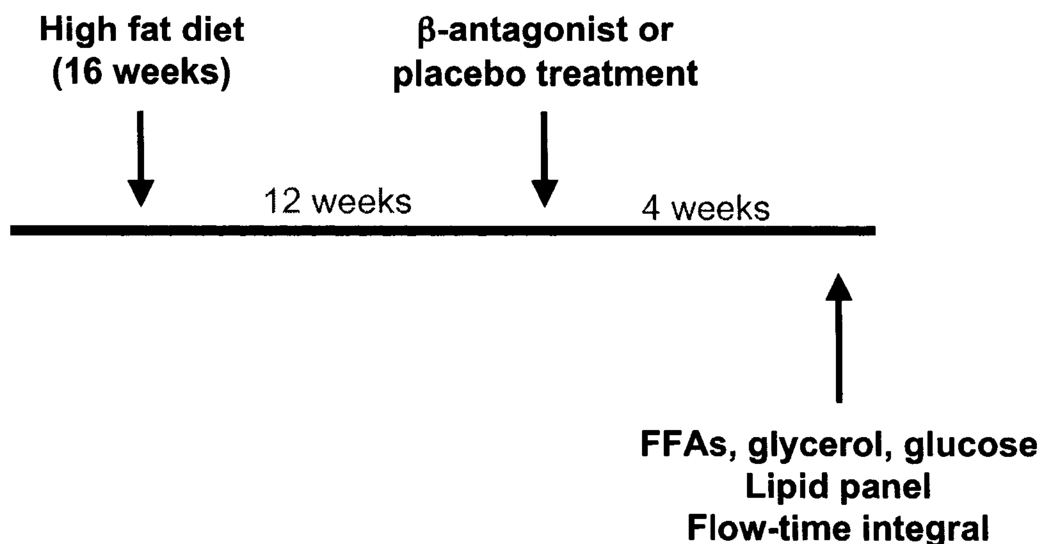

Figure 3.2 Experimental design of study investigating long-term β-antagonism in dietary obese rats.

AIM 2: MEASURE MARKERS OF INFLAMMATION AND OXIDATIVE STRESS IN NORMAL AND FAT-FED RATS WITH AND WITHOUT β-ANTAGONISM

Rationale/strategy

Serum FFAs may induce endothelial dysfunction by inflammation or oxidative stress, or both. Examining markers of inflammation and oxidative stress

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concomitantly with endothelium-dependent vasodilation was used to define the dominant pathologic process in FFA-mediated endothelial dysfunction. Further, it was determined whether reduced FFAs, and any improvement in endothelial function attributable to β-antagonism, were directly correlated with attenuation of inflammation, oxidation or both. Inflammation was assessed by serum CRP, IL-6, and/or TNF-α. Oxidative stress was assessed by reduced femoral artery nitrotyrosine, an indicator of peroxynitrite activity. Serum thiobarbituric acid reactive substances (TBARS), a measure of lipid peroxidation, used to assess oxidative stress.

Design: Measurement of serum and tissue markers of inflammation and oxidation in normal rats with acute β-antagonism and fat-fed rats with chronic β-antagonism

Treatment groups, anesthesia, and instrumentation were identical to Aim 1. The same group of rats used for investigation of Aim 1 was used for study of Aim 2, in that blood procured for Aim 1 serum parameters was used for measurement of CRP, IL-6, TNF-α and TBARS. Immediately after exsanguination, the left femoral artery (not cannulated in rats used for serum assessment only) was harvested for nitrotyrosine localization by immunohistochemistry (IHC) and quantitation by slot blot analysis (Figure 3.3).

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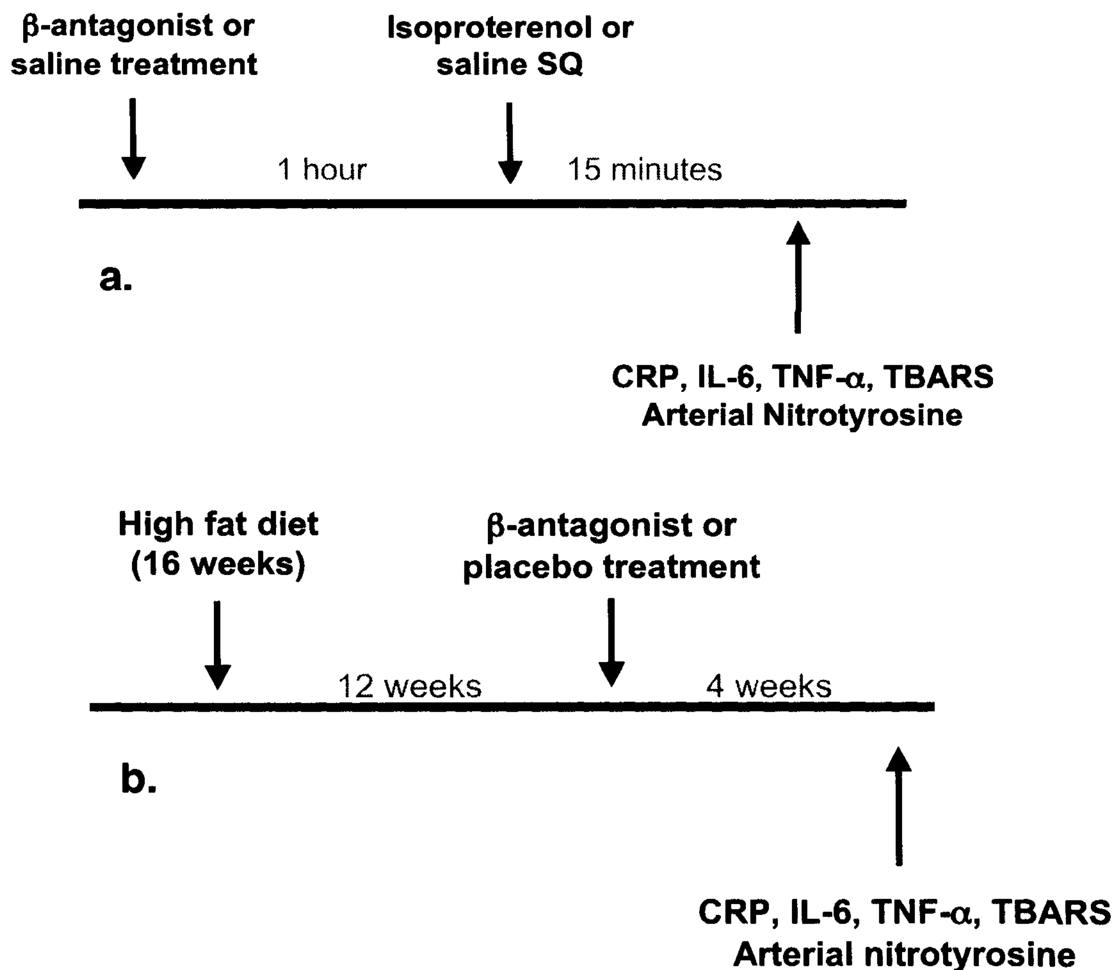

Figure 3.3 Experimental design of the study of oxidative stress and inflammation in response to β-antagonism in normal rats under conditions of acute ISO-stimulated lipolysis (a) and in dietary obese rats (b).

RESEARCH METHODS

Animals

Adult male SD rats were studied. During 2-weeks of acclimation after arrival, they were fed a normal rodent diet and observed for illness or maladjustment. Thereafter, the rats were observed for malaise or inappetence

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twice weekly. Animals were housed in environmentally controlled rooms with 12-hour light/dark cycles, and were weighed at the end of the study period.

Dietary composition

All rats received ad libitum dietary intervention (normal or high-fat chow) for 16 weeks, a period chosen due to published data showing increased plasma FFA and impaired endothelial function in rats after eleven weeks of high-fat feeding.[257] Feed intake was recorded as grams consumed in preliminary studies. Diets supply sufficient minerals and vitamins to meet estimated requirements for rats.

The pelleted high-fat diet (Harlan Teklad, Madison, WI) consists of 19.8% fat (15.8% lard, 4% soybean oil), 42.1% carbohydrates (including 10% sucrose), and 24.5% protein. Lard fatty acid constituents: 0.1% capric, 0.1% lauric, 1.5% myristic, 0.2% pentadecanoic, 24.8% palmitic, 3.1% palmitoleic, 0.5% margaric, 0.3% margaroleic, 12.3% stearic, 45.1% oleic, 9.9% linoleic, 0.1% linolenic, 0.2% arachidic, 1.3% gadoleic, 0.1% eicosadienoic, and 0.4% arachidonic. Soybean oil fatty acid constituents: 0.1% myristic, 11% palmitic, 0.1% palmitoleic, 4% stearic, 23.4% oleic, 53.2% linoleic, 7.8% linolenic, 0.3% arachidic, 0.1% behenic.

The diet does not contain vitamin C. Vitamin E concentrations were minimized to approximate the minimal nutrient requirements, in IU/kg diet, for rats so benefits of exogenous antioxidant administration on endothelial function were minimized.[258]

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The control diet (Harlan Teklad, Madison, WI) was formulated to meet growth and maintenance nutrient requirements for laboratory rodents, with the following composition: protein 24%, fat 4.4 %, fiber 4%, nitrogen-free extract 47%.

Implantation of subcutaneous pellets

Rats were placed in a sealed rodent anesthesia device containing an isofluorane-impregnated gauze. After confirmation of surgical anesthesia, the animals were placed upon a clean surgical field and the interscapular space clipped, then scrubbed with Hibiclens and wiped dry with a clean gauze. Cold-sterilized instruments were used, and care was taken to dry them prior to contact with pellets. The loose interscapular skin was grasped with forceps, and a 0.25-0.5 cm full-thickness incision made with a #12 blade. Using forceps, the pellets were placed in the SC space approximately 3 cm caudal to the incision site. Where two pellets were placed (i.e. rats that received β_x-antagonists), one pellet was positioned on the right side of the animal, and the other on the left side to avoid interaction of pellet materials. Any bleeding was minimal and controlled with local pressure. After recovery from anesthesia, the animals were examined daily for two days, and twice weekly thereafter.

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Procurement and processing of serum samples

Normal baseline serum parameters were established in separate animals so that test animals would not require multiple samplings, thus minimizing the effect of hypovolumia on sympathetic tone. To obtain sufficient serum for sampling, a medial sternotomy was performed and the left ventricle punctured with an 18-ga, 1.5-inch needle attached to a 5 cc syringe. The maximum amount of blood was gently aspirated to avoid hemolysis of red blood cells. The animals expired shortly thereafter, never having regained consciousness after initial anesthetic induction. Whole blood samples were immediately placed in a serum separator tube and allowed to clot at room temperature for 2 hours. Blood was then centrifuged at 20,000 RPM for 15 minutes, and the serum aspirated. The serum was divided into aliquots for each test, then stored at -80 °C.

Measurement of serum free fatty acids

Serum FFA determination was conducted using an enzymatic calorimetric kit (Wako NEFA-C, Wako Chemicals USA, Richmond, VA).[259] Serum FFAs form acyl-CoA when combined with acyl-CoA synthetase, Co-A, and magnesium. Oxidation of the acyl-CoA results in production of hydrogen peroxide. Addition of peroxidase permits oxidative condensation, and a purple adduct is formed. The optical density of the adduct can be spectophotometrically measured at 550 nm, thus the concentration of FFAs estimated. Vitamin C present in the serum

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sample is oxidized by ascorbate oxidase added by the manufacturer and thus eliminated.

Measurement of serum glycerol

Relative changes in the rate of lipolysis were estimated by measuring serum glycerol in conjunction with FFA concentrations.[260] Serum glycerol concentrations were determined by the University of Colorado General Clinical Research Center Laboratory, using the Cobas Miral Plus Chemistry Analyzer (Roche Molecular Systems, Inc., Alameda, CA). Glycerol is first phosphorylated by ATP to L-glcerol-3-phosphate and adenosine diphoshate (ADP). Phosphoenolpyruvate converts the ADP back to ATP + pyruvate. The pyruvate is subsequently reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with resultant NADH oxidation. The amount of oxidized NADH is proportional the amount of glycerol in the sample. Absorption is read at 340 nm, and glycerol concentrations derived from the standard curve.

Measurement of serum glucose

Fasting serum glucose was measured at the University of Colorado Clinical Laboratory by oxygen depletion enzymatic methodology. Whole blood was centrifuged and separated promptly to minimize erythrocyte consumption of glucose.

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Determination of the lipid profile (total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides)

Components of the lipid profile were measured at the University of Colorado Clinical Laboratory. Total cholesterol was measured enzymatically. After exposure to cholesterol esterase and oxidase, the absorbency of the chromagen-coupled adduct was measured spectrophotometrically, and the total cholesterol concentration derived.

The HDL cholesterol is measured after surfactants are applied to remove non-HDL lipoproteins. Subsequently, HDL is exposed to cholesterol esterase and oxidase, a chromagen is added, and the resulting product measured spectrophotometrically.

The LDL cholesterol is derived from the following calculation: LDL = total cholesterol – HDL cholesterol – VLDL cholesterol (or 20% TGs).

Serum TGs were measured by enzymatic glycerol-blanked methodology. After TG hydrolysis by LPL, the resultant glycerol is phosphorylated, then catalyzed by glycerol oxidase to form dihydroxyacetone phosphate and hydrogen peroxide, the latter of which is exposed to peroxidase with subsequent chromagen formation. Measured absorbance is proportional to TG concentration.

Measurement of serum leptin

Leptin concentrations were measured by the University of Colorado General Clinical Research Center Laboratory using a Rat Leptin

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Radioimmunoassay Kit (Linco Research, Inc., Saint Charles, MO). This kit incorporates ^{125}I-labeled rat leptin and a rat leptin antiserum using double antibody methodology.

Measurement of serum C-reactive protein

The Turbitex CRP Ultra (Biocon, Rockville, MD) is a high sensitivity immunoturbidimetric assay, which relies on anti-CRP antibodies binding to serum antigen. Once agglutination has occurred, the sample was evaluated turbidimetrically at 578 nm, and the mg/dl CRP concentration calculated from the standard curve.

Measurement of serum interleukin-6 and tumor necrosis factor-α

Both cytokines were quantitated by ELISA. The Rat IL-6 and Rat TNF-α commercial kits are solid phase sandwich ELISAs (BioSource International, Inc., Camarillo, CA).

Measurement of serum thiobarbituric acid-reactive substances

Serum contains lipid hydroperoxides and aldehydes, which increase with oxidative stress. These TBARS form an adduct with malondialdehyde (MDA). The OXI-TEK TBARS Kit (Zeptometrix Corporation, Buffalo, NY) was used to generate the TBARS-MDA adduct for spectrophotometric quantification.

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Localization and quantitation of nitrotyrosine residues in the femoral artery

Peroxynitrite is a potent oxidant formed when superoxide reacts with NO, and contributes to the pathogenesis of many conditions associated with early endothelial dysfunction.[7,8,25] Exposure of proteins to peroxynitrite causes nitration of tyrosine residues, thus nitrotyrosine is an indirect marker of peroxynitrite generation.

For localization of nitrotyrosine by immunohistochemistry, segments of femoral arteries were formalin fixed, paraffin embedded, and sectioned. Following deparaffinization, high temperature antigen retrieval was applied to reduce crosslinking and optimally expose nitrosylated residues. Slides were then incubated in hydrogen peroxide to consume endogenous peroxidase. Subsequent incubation with avidin allowed binding of endogenous biotin. Tissues were incubated overnight with a rabbit polyclonal anti-nitrotyrosine antibody (Upstate, Lake Placid, NY), then exposed to a goat anti-rabbit biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA). Finally, slides were incubated with avidin:biotinylated enzyme complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and exposed to 3,3'-diaminobenzidine (DAB) substrate to produce a brown coloration. Tissues were counterstained with haematoxylin to facilitate visualization of tissue morphology. Tissues were dehydrated, and slides mounted using toluene-based mounting media. Nitrotyrosine was identified as brown coloration under light microscopy. For negative controls, primary antibody was incubated with 10 mM 3-nitrotyrosine solution (Sigma, St. Louis, MO) for 1 hour prior to application to tissues. For

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positive controls, tissue tyrosine resides were nitrated by incubation of tissues with peroxynitrite (Upstate, Lake Placid, NY) diluted 1:5 in phosphate-buffered saline.

For quantitation of nitrotyrosine residues by slot blot analysis, femoral artery segments were flash frozen and stored at -80°C. Tissues were homogenized using buffer and protease inhibitor (Halt Protease Inhibitor Cocktail Kit, Pierce, Rockford, IL). Total homogenization time was 3 minutes, with 15-second periods of alternating grinding and storage on ice. Samples were centrifuged at 4°C at 10,000 g for 30 minutes. The supernatant was collected and aliquots were frozen at -80°C. Sample protein concentrations were measured using the calorimetric bovine serum assay (BCA Protein Assay, Pierce, Rockford, IL). Samples were run in triplicate, and read by microplate analysis at 570 nm. Volumes required for delivery of 5 μg and 20 μg were subsequently calculated.

Tris buffered saline 10X (87.66 gms NaCl + 100 mls 1M tris, pH 7.4) was diluted to 1X and mixed with tween 20 detergent (polyoxyethylene-sorbitan monolaurate, Sigma, St Louis, MO). This solution (TBST) was used to dilute protein samples so that a total volume of 400 μl was achieved. The polyvinylidene fluoride (PVDF) membrane was prepared by immersion in full strength methanol for 5 minutes, followed by a 5-minute immersion in western transfer buffer (2.5 mls NuPAGE Transfer Buffer, Invitrogen, Carlsbad, CA + 10 mls methanol + 37.5 mls deionized water). The 400 μl protein samples were applied to a vacuum-assisted dot-blot apparatus. Once wells were depleted of

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sample, an additional 300 μl volume of TBST was instilled, and this was repeated so that 2 washes were applied. The membrane was removed with the vacuum engaged and immediately put into blocking solution (5% dry milk in TBST). After 30 minutes in blocking solution, the membrane was incubated overnight at 5°C in a 1:1000 solution of rabbit polyclonal 3-nitrotyrosine antibody (Upstate, Lake Placid, NY) and blocking solution. After washing the membrane in blocking solution, goat anti-rabbit horseradish peroxidase-conjugated antibody (Vector Laboratories, Burlingame, CA) in a 1:5000 concentration with blocking solution was applied to the membrane and incubated for 1 hour at room temperature. Washes were then repeated as described, with a final wash in TBST. To create a chemiluminescent signal, Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was applied to the membrane for 1 minute. The PVDF was then encased in plastic wrap and secured in a radiographic cassette. Films were developed after 30-second, 60-second, and 5-minute exposures to the PVDF membrane. Quantification of nitrosylated tyrosine residues was estimated by evaluating signal strength.

Measurement of endothelium-dependent vasodilation

Endothelium-dependent vasodilation is one measure of endothelial function, and can be characterized by evaluating changes in flow in response to vasoactive substances. On exposure to Ach, vascular endothelial cells generate NO, prostacyclin, and EDHF, resulting in relaxation of the underlying smooth

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muscle and dilation of the vessel. If pressure remains constant, vessel dilation increases flow. A less marked dilator response to Ach was interpreted as decreased endothelium-dependent vasodilation, and thus impaired endothelial function.

Measurements of endothelium-dependent vasodilation were conducted in a set of animals distinct from those used for serum analysis, eliminating the effects of volume depletion on flow. Treatment groups were identical to those described above (Figures 3.1 and 3.2). After induction of general anesthesia, the left carotid artery was catheterized using PE 10 tubing attached to a pressure transducer and data analysis software (Biopac Systems Inc., Goleta, CA) for recording of mean arterial pressure (MAP). During preliminary studies, the distance of insertion necessary for the catheter tip to rest in the proximal carotid was established, and the same length of tubing was advanced in each animal. The distal aorta was cannulated via retrograde advancement of heparinized PE 50 tubing through the right femoral artery. As with the carotid catheter, preliminary studies were conducted to establish the length of tubing needed for insertion so that the catheter tip rested at the aortic bifurcation. Because the catheter was of sufficient diameter to effectively occlude the artery, drugs necessarily flowed into the right femoral artery at the origin off the distal aorta, and proximal to the flow probe. A microport was connected to the distal end of this catheter and used for drug infusion.

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Blood flow was measured by laser Doppler flowmetry in vivo (Transonic Systems Inc., Ithaca, NY). Use of flow probes or sensors is the most commonly employed method of *in vivo* determination of organ flow.[261] Laser Doppler flowmetry relies on moving objects (i.e. red blood cells) reflecting laser light at a wavelength distinct from that sent to the blood from the probe.[261] The reflected signal represents the product of the velocity and concentration of cells.[262] If the laser light is placed so as to span the short axis of the vessel, the vessel may be left undisturbed and function-altering manipulation avoided.[261] This method of flow measurement has been applied to the brain, renal, and tail circulation in rats.[262-265]

A 0.5 mm laser Doppler flow probe was placed on the right femoral artery. This vessel was chosen for study for ease of atraumatic isolation and because of a lack of end-organ feedback systems regulating flow. Endothelium-dependent vasodilation is primarily mediated by NO in this vessel, thus the contribution of EDHF and prostacyclin to endothelium-dependent dilation is minimized.[146] MAP was measured in the carotid artery, and resistance calculated (Resistance = MAP/Flow).

Drugs used to alter vasoreactivity included Ach; N_{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthetase; and sodium nitroprusside, a NO donor and determinant of endothelium-independent vasodilation. At 10-minute intervals, increasingly larger doses of Ach were injected (0.25, 0.75, 2.5 μg/kg). Dilator responses were defined as the area under the flow curve (flow-time

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integral or FTI in mLs) from the point of injection to the time at which the flow reached a plateau, defined as the point at which flow remained unchanged for 90 seconds or more (Figure 3.4). To confirm that Ach-induced dilations were mediated by NO, the same experiment was conducted 10 minutes after preadministration of 10 mg/kg L-NAME, administered as an infusion over 10 minutes. After a second equilibration period, to verify intact endothelium-independent vasodilation, the artery was infused with 5.2 mg sodium nitroprusside and the change in flow determined (Figure 3.5).

The portion of the vasodilatory response attributable to NO may be approximated by identifying that which is abolished by administration of the NOS inhibitor L-NAME. To quantify this and compare the relative NO-mediated response among chronic treatment groups, FTI in response to Ach was measured before and after L-NAME administration. The difference in these two FTI responses (Δ FTI) was calculated and used to approximate the NO-mediated FTI response. Additionally, resistance was calculated prior to and after administration of L-NAME, and the difference analyzed so that the proportion of baseline resistance attributable to NO could be estimated and compared between groups. Similarly, resistance was calculated prior to and after administration of nipride, and the difference analyzed so that the proportion of resistance attributable to endothelium-independent vasodilation could be estimated.

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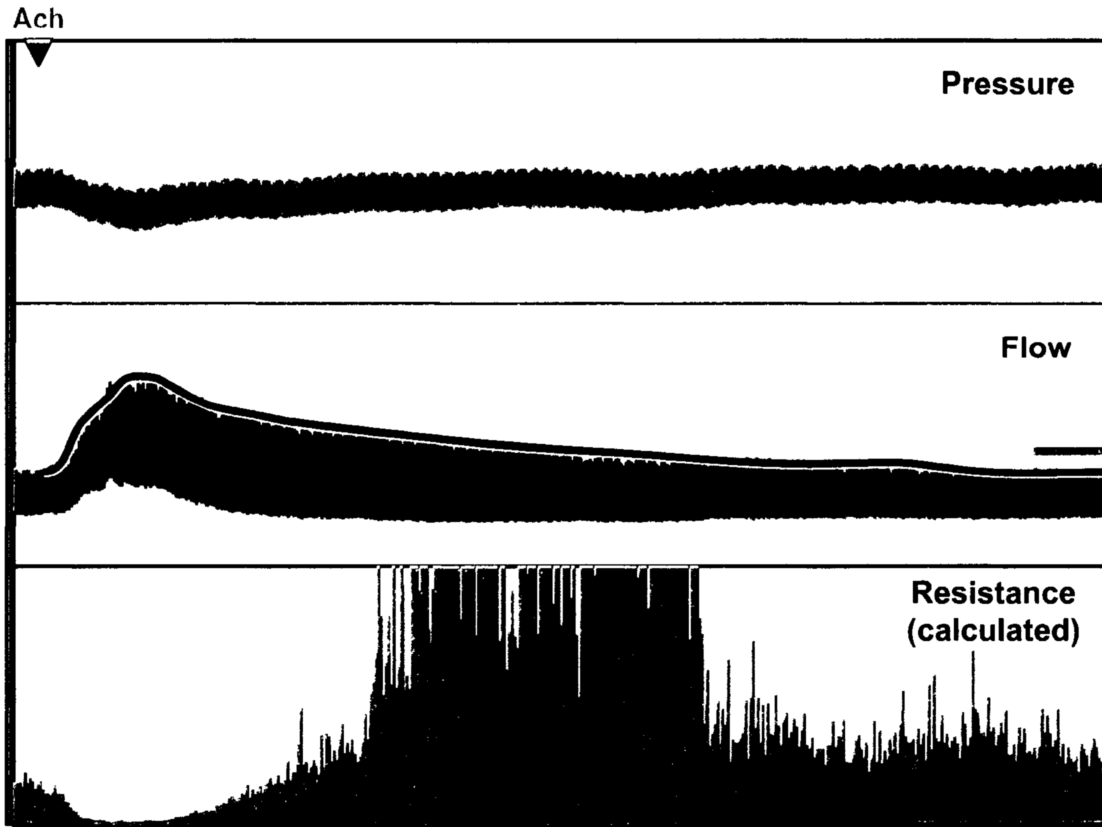

Figure 3.4 Biopac-generated data showing pressure, flow, and resistance (calculated as pressure/flow) in response to a single injection of Ach (top left red arrow). Red line represents plateau in flow curve. Black line over flow curve represents the area used to determine flow-time integral.

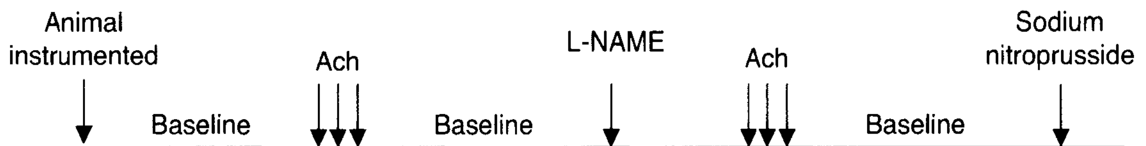

Figure 3.5 Sequence and frequency of vasoactive drug administration for determination of endothelium-dependent vasodilation.

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DATA ANALYSIS

Data analyses were conducted using Prism 4.0 for Macintosh (Graphpad Software, Inc., San Diego, CA). The D'Agostino and Pearson omnibus normality test was used to establish normal distribution of data. When the distribution was not normal, transformation of data was carried out. In most cases, logarithmic transformation was sufficient for establishing normality. When data could not be normalized with transformation, the Mann-Whitney (unpaired t-test equivalent) or Kruskal-Wallis (1-way ANOVA equivalent) nonparametric tests were used. The F-test was used to identify unequal variation in the data, and the Welch's correction used where appropriate.

To compare more than two population means, one-way analysis of variance (ANOVA) was applied. If the overall p-value was < 0.05, the Tukey method of multiple comparisons was used determine significant differences between individual means. Additionally, unpaired t-tests were performed where appropriate, to eliminate the effect of multiple factors. For example, in acute studies, the β_2-antagonist was soluble in NS, while the β_1- and β_3-antagonists were only soluble in DMSO. Because of this, these two groups were compared to the appropriate controls of NS + ISO and DMSO + ISO, respectively. It was therefore valid to gain an overall sense of significance using the ANOVA, then use unpaired t-tests to compare treatment and respective control groups.

Paired t-tests were used to determine significance in serum FFAs when pre- and post-ISO values were being compared in the same animals. Pearson's

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correlation was used to define the linear association between two data sets that were normally distributed. Data that was not normally distributed was transformed. If data transformation did not confer normality, the Spearman correlation test was used.

Data measured in response to progressively larger doses of Ach (i.e. FTI) were analyzed using 2-way ANOVA for repeated measures, so that significant changes in the overall response could be determined. The Bonferroni post-test was used to compare individual means. Data is expressed as mean +/- SE. Two-tailed tests were used for all analyses. Results were considered significant at values of $p < 0.05$.

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CHAPTER IV

RESULTS

Prior to the description of results directly related to specific aims of the study, preliminary data leading to validation of experimental design is presented. Subsequently, research results are described in accordance with study aims. The following abbreviations are used throughout the RESULTS section:

μ = mean

n = sample size

SE = standard error

PRELIMINARY STUDY

Effects of high-fat diet, isoproterenol, and β-antagonism on body weight and serum free fatty acids

Rats fed the high fat diet for 16 weeks had increased body weight despite decreased food intake. Fat-fed rats gained an average of 170 gms, while rats that received normal chow gained 113 gms (n=9). Serum FFAs were measured in normal rats and those fed the high fat diet for 16 weeks. Rats that received

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high fat dietary treatment had higher serum FFA concentrations than those rats that consumed normal rat chow (Table 4.1).

	n	μ FFA (meq/L)	SE
Normal	14	.5417	.0380
Fat fed	18	.6989	.0450

Table 4.1 Mean serum FFA concentrations in normal and fat-fed rats.

Serum FFAs were higher ($p = 0.02$) than baseline values after administration of ISO (Figure 4.1).

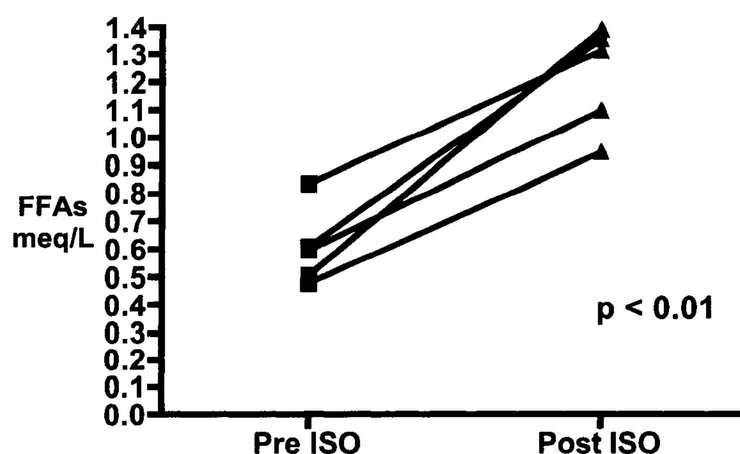

Figure 4.1 Rat serum FFA concentrations are increased after receipt of 0.05 mg/kg isoproterenol (ISO) SC (pre-ISO: μ = .6040, SE = .0626; post-ISO: μ = 1.221, SE = .0859).

Premedication with atenolol attenuated the ISO-induced rise in serum FFAs ($p = 0.03$) (Table 4.2).

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	n	μ FFA (meq/L)	SE
DMSO + ISO	6	1.299	.1142
β_1 + ISO	6	.9333	.0852

Table 4.2 Mean serum FFA concentrations in fat-fed rats given ISO 0.05 mg/kg SC. Administration of the β_1-antagonist atenolol attenuated the ISO-induced rise in FFAs when compared to control animals that received carrier only (DMSO).

Further evidence for the efficacy of β-antagonist agents in reducing elevated FFAs attributable to ISO was seen when the same experiment was conducted on an additional group of normal female adult rats, with the addition of the β_3-antagonist SR59230A to the β_1-antagonist atenolol. Rats exposed to combined β_1- and β_3-antagonists had reduced serum FFAs when compared to those receiving DMSO only ($p < 0.001$) (Table 4.3).

	n	μ FFA (meq/L)	SE
DMSO + ISO	7	1.0769	.0549
$\beta_1\beta_3$ + ISO	7	.7573	.0411

Table 4.3 Mean serum FFA concentrations in normally fed female rats given ISO 0.05 mg/kg SC. Administration of combined β_1- and β_3-antagonists attenuated the ISO-induced rise in FFAs when compared to control animals that received carrier only (DMSO).

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Effects of acetylcholine, L-NAME on flow-time integral

The FTI that was measured in response to all Ach doses was greater than that of NS alone, and increasingly larger doses of Ach produced sequentially larger increases in the FTI (Figure 4.2). Though these responses were not significantly different, this pattern of response was appreciated, to a variable extent, in all treatment groups. The most common deviation was a failure of the highest Ach dose to maximally augment the FTI, and this was attributed to reduced MAP.

The flow response was attenuated after administration of L-NAME (Figure 4.3a). Though administration of L-NAME did not significantly attenuate the FTI in response to NS and Ach, this pattern is distinctly different from that derived when NS was given in place of L-NAME (Figure 4.3b). Comparison of these data suggest that the attenuation in flow measured in response to L-NAME was not a temporal decline, and that it is distinct from the relatively unchanged FTI response measured after NS administration.

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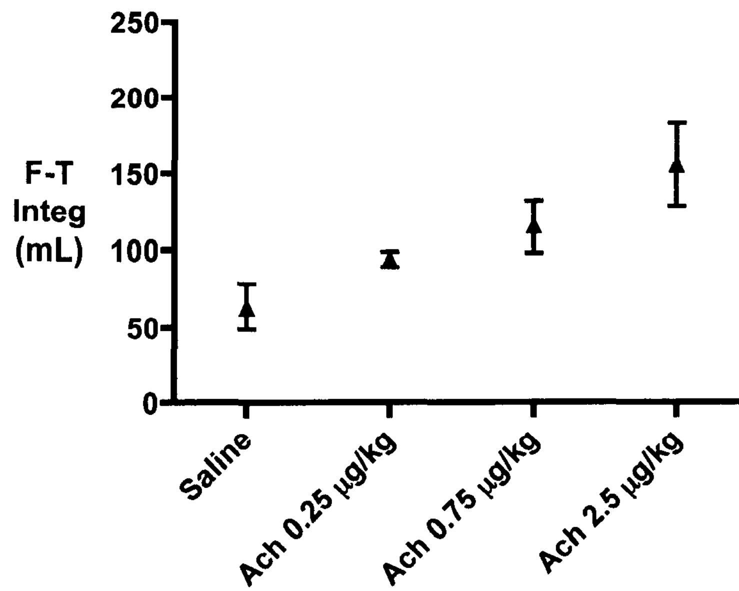

Figure 4.2 Graphical representation of the FTI calculated in response to NS and increasingly larger doses of Ach in fat-fed rats. Note that Ach produced a larger response than NS, and that increasingly larger doses of Ach produced incrementally larger increases in the flow-time integral (n = 6).

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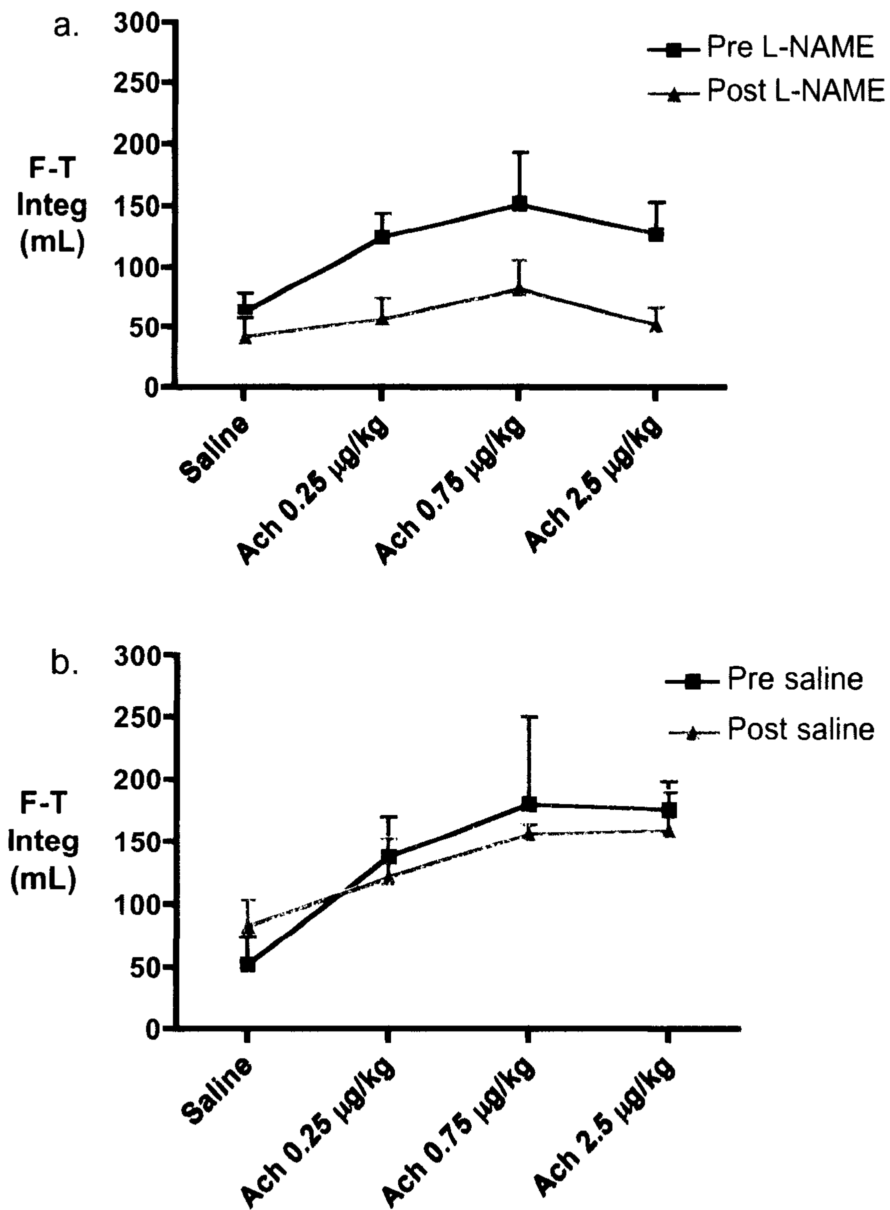

Figure 4.3 a. FTI in response to NS and progressively larger doses of Ach, before (in blue) and after (in red) administration of L-NAME (n = 7). **b.** FTI in response to the same agonists, but with NS given in place of L-NAME. Attenuation of the FTI is less profound in this group (n = 4).

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SPECIFIC AIMS

To improve clarity and minimize redundancy, treatment groups will be referenced in the following manner:

Normal = rats fed normal rat chow for the 12 week dietary treatment period

Fat-fed = rats that received high-fat dietary treatment for the 12 week dietary treatment period

Untreated/baseline = rats that received neither placebo nor $\beta_1\beta_3$-antagonist pellets (i.e. no pharmacologic treatment)

Placebo = inert SC pellet

$\beta_1\beta_3$-antagonism or **$\beta_1\beta_3$-pellets** = 1 pellet containing the β_1-antagonist atenolol and 1 pellet containing the β_3-pellet SR59230A

It should also be noted that the β_1- and β_3-antagonists were soluble in DMSO, while the β_2-antagonist was soluble in NS. Therefore, during data analysis, the ISO-stimulated rats that received β_1- and/or β_3-antagonists were compared to the DMSO + ISO control group, and the rats that received the β_2-antagonist were compared to the NS + ISO control group.

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Aim 1: Measure FFAs and in vivo endothelial function in normal and fat-fed rats with and without β-antagonism.

Serum free fatty acids during acute β-antagonism in isoproterenol-stimulated rats

Table 4.4 summarizes the descriptive statistics relevant to serum FFAs in isoproterenol-stimulated rats.

	n	μ meq/L	SE
NS + NS	8	.4724	.0331
NS + ISO	6	.8227	.0367
DMSO + ISO	7	.7700	.0343
β_1 + ISO	8	.7718	.0746
β_2 + ISO	8	.7019	.0487
β_3 + ISO	8	.8300	.0456
$\beta_1\beta_3$ + ISO	8	.6891	.0416

Table 4.4 Sample size, mean, and standard error of serum FFA concentrations measured in control (NS + NS, NS + ISO, DMSO + ISO) and β-antagonist treated (β_1, β_2, β_3, $\beta_1\beta_3$) rats.

When compared to rats that received NS as a negative control for both the β-antagonist and ISO, rats that were given NS + ISO had higher serum FFAs ($p < 0.001$) (Figure 4.5). To assure that DMSO alone did not alter FFA concentrations, serum FFAs were compared in rats that received NS as a negative control for β-antagonism to those animals that received DMSO. Serum FFAs were similar in the two groups (Figure 4.4).

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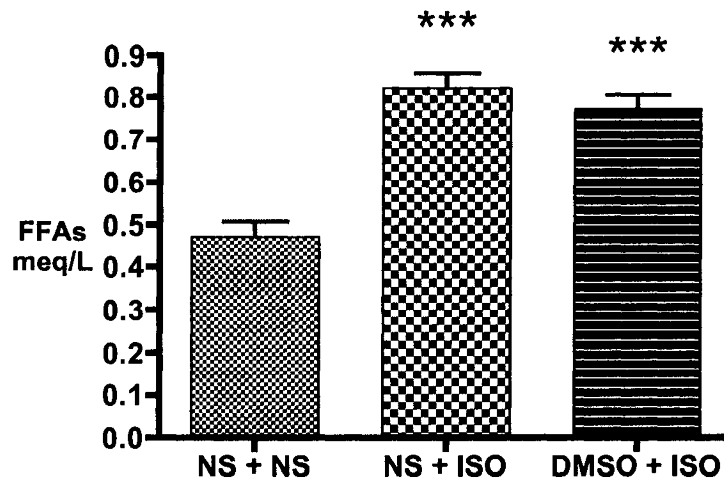

Figure 4.4 Comparison of serum FFA concentrations in control rats used for acute experiments. Rats that received ISO had increased FFAs when compared to those rats that received NS only (*** = $p < 0.001$). There was no difference in serum FFAs when the two carriers, NS and DMSO, were compared.

Serum FFAs from rats given β-antagonists specific for a single subtype were not different than ISO controls (Figure 4.5). Based on known receptor density in rat adipose and data from preliminary studies, a combined $\beta_1\beta_3$-antagonist was given to an additional group of ISO-stimulated rats. Absolute serum FFA values from these animals were lowest of all groups that received β-antagonists, but the attenuation in ISO-stimulated FFAs remained statistically insignificant.

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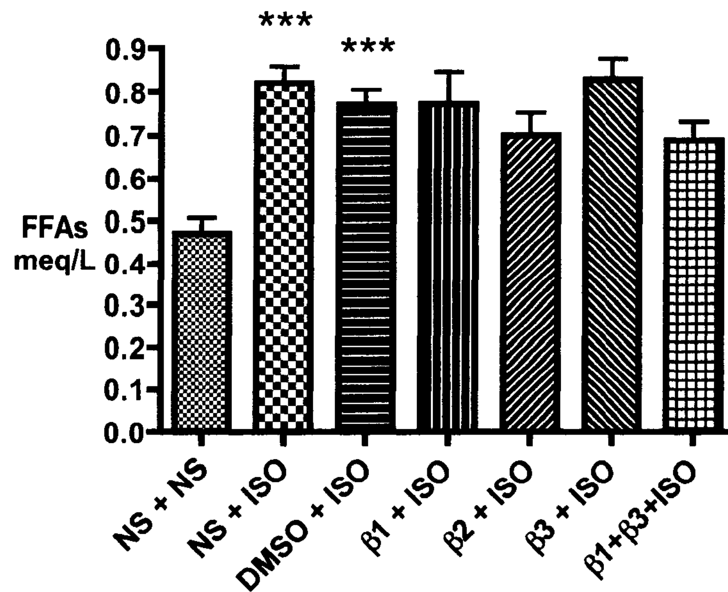

Figure 4.5 Serum FFA concentrations in control (NS + NS, NS + ISO, DMSO + ISO) and β-antagonist treated (β_x + ISO) rats. Asterisks denote significance when compared to NS + NS (*** = $p < 0.001$). Treatment with β-antagonists did not significantly attenuate the ISO-stimulated rise in serum FFAs.

Serum free fatty acids during chronic β-antagonism in fat-fed rats

Sixteen weeks of high-fat feeding did not increase serum FFAs in untreated rats ($p = 0.06$). There was no difference in serum FFAs when normal untreated rats were compared to normal animals that received placebo pellets (Figure 4.6). In contrast to normal and fat-fed baseline measurements described above, serum FFAs were higher in fat-fed rats with placebo pellet implantation when compared to those fed normally (Figure 4.6).

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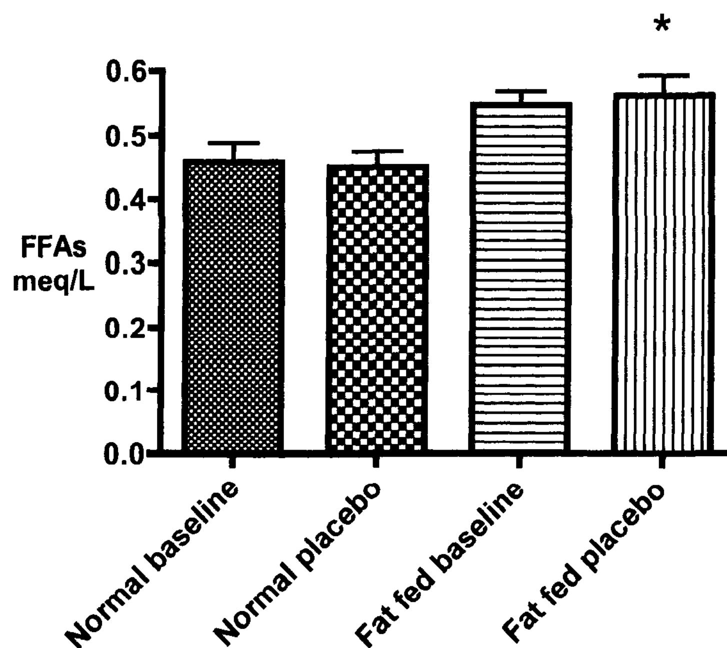

Figure 4.6 Serum FFA concentrations in normal and fat-fed rats, comparing values in untreated rats with those measured in rats that received placebo pellets. Serum FFAs in the normal animals were similar; high-fat feeding increased FFAs in placebo-treated rats (* = $p < 0.05$). Asterisk denotes significance when compared to normal placebo group.

Serum concentrations were compared in fat-fed animals with SC placebo pellets and those with implantation of pellets containing $\beta_1\beta_3$-antagonists (Figure 4.7). In normal and fat-fed rats, chronic $\beta_1\beta_3$-antagonism resulted in higher serum FFAs ($p < 0.01$). A summary of FFA data generated from chronic studies is provided in Table 4.5.

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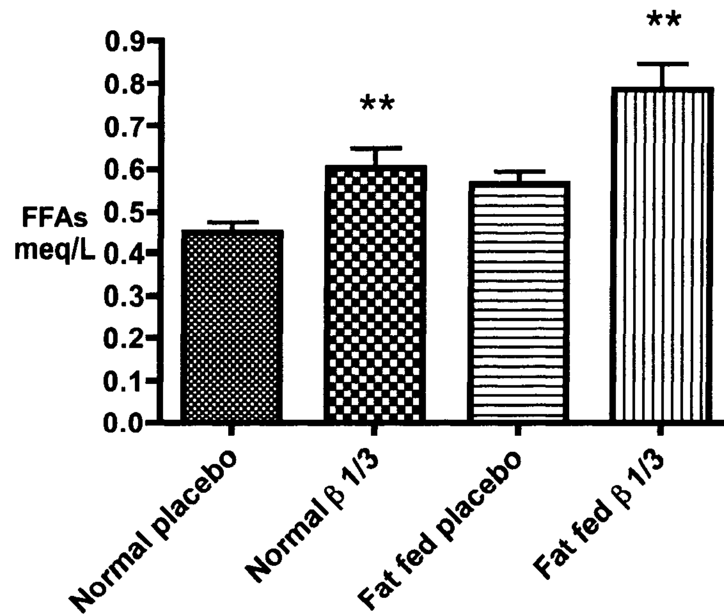

Figure 4.7 Serum FFA concentrations in normal and fat-fed rats, with and without chronic administration of $\beta_1\beta_3$-antagonists by slow release SC pellets. Both groups had increased serum FFAs with chronic $\beta_1\beta_3$-antagonism (** = $p < 0.01$). Asterisk denotes significance when compared to similarly fed placebo group.

	n	μ meq/L	SE
Normal baseline	9	.4555	.0323
Normal placebo	8	.4480	.0259
Normal $\beta_1\beta_3$	8	.5634	.0299
Fat-fed baseline	6	.5468	.0218
Fat-fed placebo	9	.6024	.0434
Fat-fed $\beta_1\beta_3$	9	.7845	.0609

Table 4.5 Sample size, mean, and standard error of serum FFA concentrations measured in normal and fat-fed rats at baseline (untreated), implanted with placebo pellets, and in those given $\beta_1\beta_3$-antagonist pellets.

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Serum glycerol during acute β-antagonism in isoproterenol-stimulated rats

Treatment with ISO did not change serum glycerol (Table 4.6). Samples from ISO-stimulated rats that received selective β-antagonists were compared to respective saline and DMSO ISO-stimulated control animals. Rats that received the β_3-antagonist, alone or in combination with the β_1-antagonist atenolol, had higher serum glycerol than those in the DMSO + ISO control group. Though there was a trend towards higher serum glycerol in other antagonist-treated groups, none of these changes were significant (Figure 4.8).

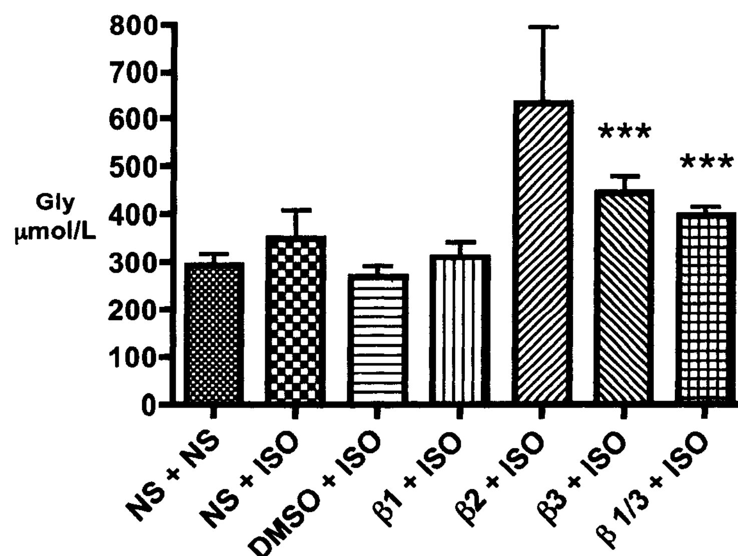

Figure 4.8 Serum glycerol concentrations in control and ISO-stimulated rats with subsequent application of control (NS, DMSO) and β-antagonist treatment. Rats that received β_3-antagonist treatment alone, or combined with the β_1-antagonist treatment, had higher serum glycerol than control animals (*** = $p < 0.001$). Asterisks denote significance when compared to DMSO + ISO control group.

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	n	μ μmol/L	SE
NS + NS	8	293	25
NS + ISO	7	350	58
DMSO + ISO	8	269	22
β_1 + ISO	8	311	31
β_2 + ISO	8	631	164
β_3 + ISO	8	446	32
$\beta_1\beta_3$ + ISO	8	397	19

Table 4.6 Sample size, mean, and standard error of serum glycerol concentrations measured in control (NS + NS, NS + ISO, DMSO + ISO) and β-antagonist treated (β_1, β_2, β_3, $\beta_1\beta_3$) rats.

Serum glycerol during chronic β-antagonism in fat-fed rats

Serum glycerol concentrations in normal untreated rats were not different from those measured in fat-fed untreated rats. Implantation of placebo pellets did not alter serum glycerol from those values measured in untreated rats. High-fat feeding was associated with higher serum glycerol in those rats that received placebo pellets ($p < 0.01$), in contrast to the negligible effect of high-fat feeding on untreated animals (Figure 4.9). Chronic $\beta_1\beta_3$- antagonism did not change serum glycerol concentrations. Table 4.7 summarizes glycerol data obtained from chronic studies.

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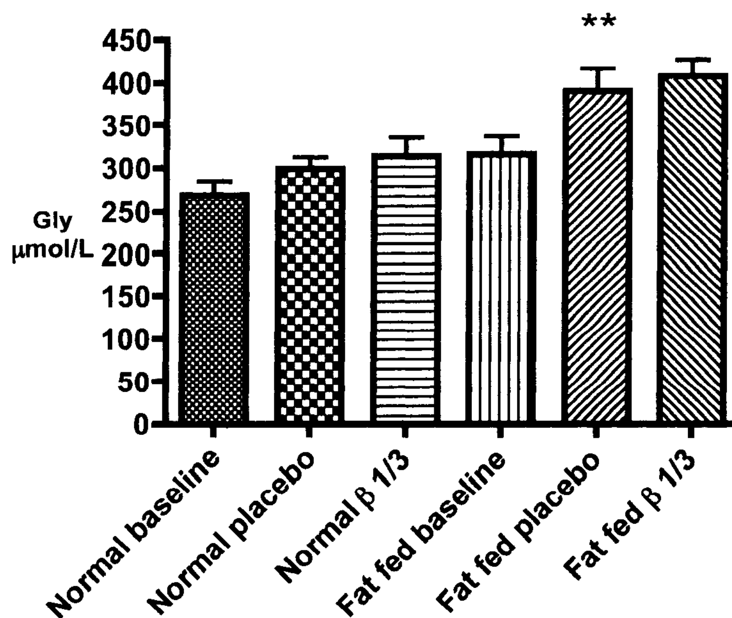

Figure 4.9 Serum glycerol concentrations in normal and fat-fed rats at baseline, with placebo pellet implantation, and with $\beta_1\beta_3$-antagonist pellet implantation. Pelleting alone did not alter serum glycerol. Rats fed a high-fat diet had higher serum glycerol values than respective controls, and this was a significant difference in rats with placebo pellets (** = $p < 0.01$). Asterisk denotes significance when compared to normal placebo animals.

	n	µ µmol/L	SE
Normal baseline	9	269	16
Normal placebo	8	299	14
Normal $\beta_1\beta_3$	9	315	20
Fat-fed baseline	6	318	19
Fat-fed placebo	8	391	26
Fat-fed $\beta_1\beta_3$	9	408	19

Table 4.7 Sample size, mean and standard error of serum glycerol concentrations measured in normal and fat-fed rats that were untreated, implanted with placebo pellets, and given $\beta_1\beta_3$-antagonist treatment.

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Serum glycerol and FFA measurements from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a weak positive correlation between variables (Figure 4.10).

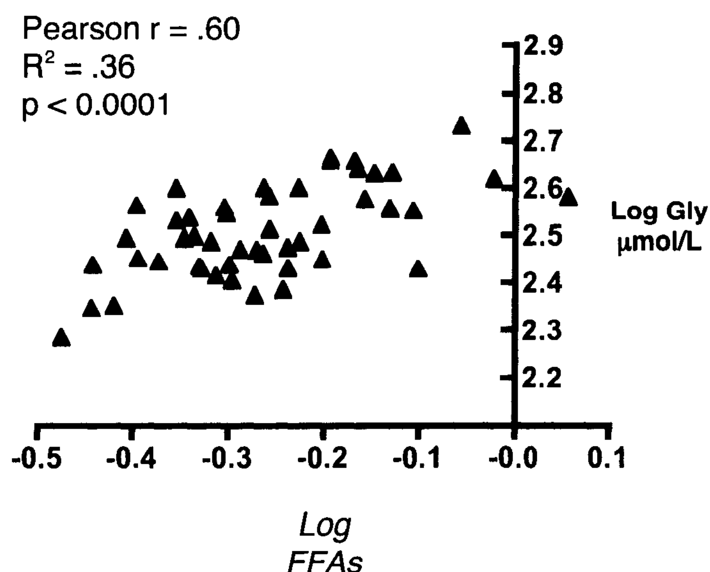

Figure 4.10 Correlation between serum FFAs and glycerol (log transformed for normality) in pooled dietary control and chronic pelleted treatment groups (n = 49).

Additional measured parameters

Body Weight

To evaluate body weights, data from rats used for hemodynamic studies were pooled with those from rats used for serum and tissue sampling. Mean weights were not different between untreated normal and fat-fed rats. Subsequently, normal and fat-fed rats that received placebo pellets were

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compared. The fat-fed, pelleted rats had higher body weights when compared to normal pelleted rats (Figure 4.11).

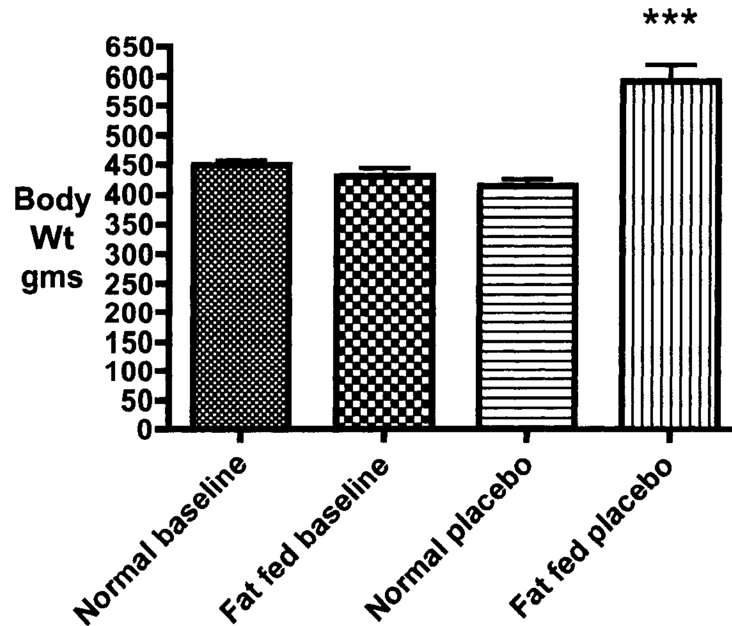

Figure 4.11 Comparison of body weights between rats that were untreated (baseline) and those implanted with placebo pellets. High-fat feeding resulted in higher body weights in pelleted rats, but not in untreated animals (*** = $p < 0.001$, compared to normal placebo).

In both normal and fat-fed treatment groups, rats that received chronic $\beta_1\beta_3$-antagonism had higher body weights when compared to rats that received placebo pellets (Figure 4.12). Body weight data is summarized in Table 4.8.

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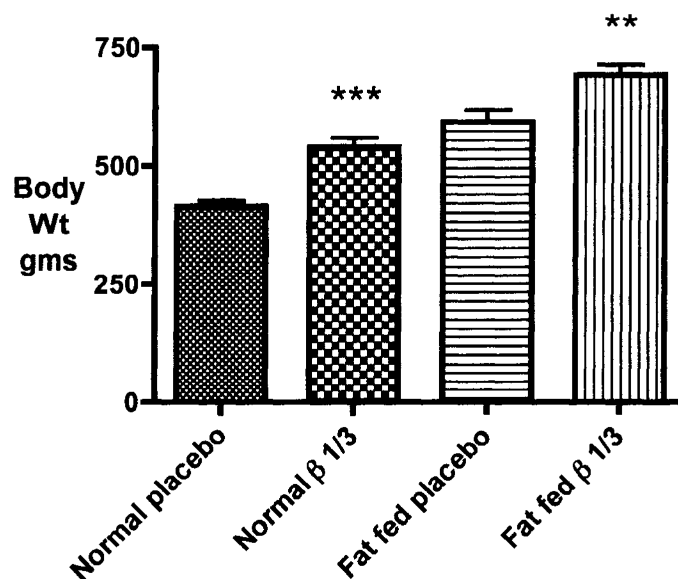

Figure 4.12 Effect of chronic $\beta_1\beta_3$-antagonism on body weight in normal and fat-fed rats. Animals that received β-antagonist treatment had higher body weights than those animals that received placebo treatment. This effect was present in both normal and fat-fed rats (*** = $p < 0.001$; ** = $p < 0.01$).

	n	μ gms	SE
Normal baseline	25	448	8
Normal placebo	15	413	12
Normal $\beta_1\beta_3$	19	536	21
Fat-fed baseline	12	430	13
Fat-fed placebo	17	592	26
Fat-fed $\beta_1\beta_3$	19	692	21

Table 4.8 Sample size, mean and standard error of body weights in normal and fat fed animals under baseline (untreated) conditions, with implantation of placebo pellets, and with chronic $\beta_1\beta_3$-antagonist pellets.

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Serum FFA concentrations and body weights from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a weak positive correlation between variables (Figure 4.13).

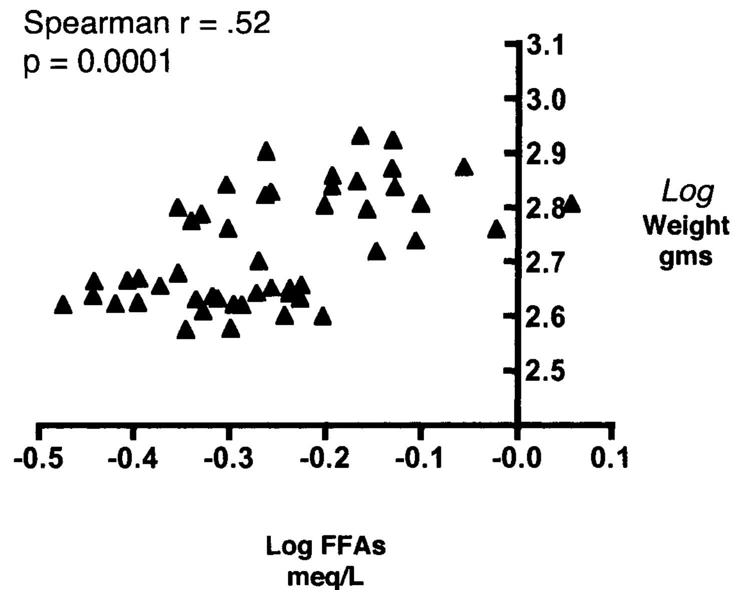

Figure 4.13 Correlation between serum FFAs and body weight (log transformed for normality) in pooled dietary control and chronic pelleted treatment groups (n = 49).

Glucose

Treatment with ISO did not alter serum glucose concentrations. Rats exposed to β_2-antagonism had increased serum glucose values ($p < 0.05$) (Figure 4.14).

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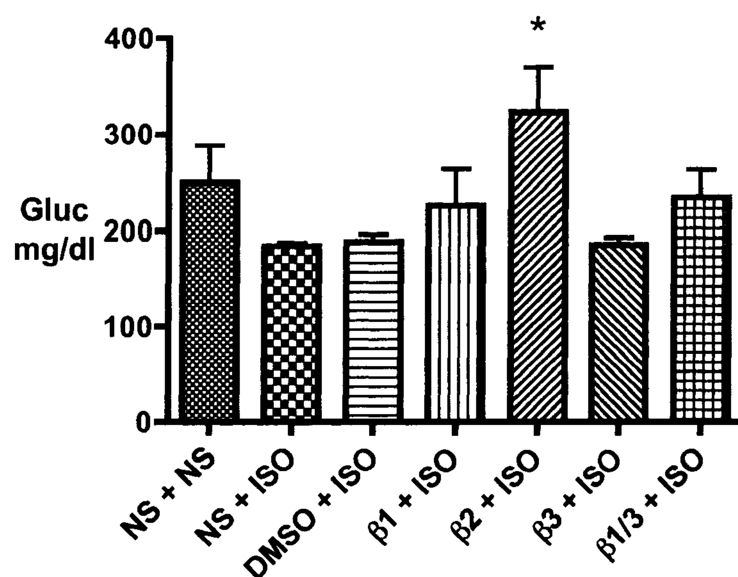

Figure 4.14 Serum glucose concentrations in control and ISO-stimulated rats with subsequent application of control (NS, DMSO) and β-antagonist treatment. Rats that received the β_2-antagonist butoxamine had higher serum glycerol than the NS + ISO control animals (* = $p < 0.05$).

There were no differences in serum glucose concentrations when normal and fat-fed untreated rats were compared. Similarly, in chronic studies, normal and fat-fed placebo animals had similar serum glucose levels. Chronic $\beta_1\beta_3$-antagonism did not result in differences when compared to animals with placebo pellets. Table 4.9 summarizes serum glucose data.

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	n	μ mg/dl	SE
NS + NS	8	250	37
NS + ISO	6	184	4
DMSO + ISO	7	188	8
β_1 + ISO	8	226	38
β_2 + ISO	8	323	47
β_3 + ISO	8	186	7
$\beta_1\beta_3$ + ISO	8	235	28
Normal baseline	9	205	28
Normal placebo	8	201	23
Normal $\beta_1\beta_3$	9	243	18
Fat-fed baseline	6	177	15
Fat-fed placebo	7	273	14
Fat-fed $\beta_1\beta_3$	9	301	17

Table 4.9 Sample size, mean and standard error of serum glucose concentrations measured in all experimental groups.

Serum FFA and glucose concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a weak positive correlation between variables (Pearson $r = 0.59$; $R^2 = 0.34$; $p < 0.001$).

Lipid Panel

Triglycerides

Changes in serum TG in response to acute interventions include the reduction ($p < 0.05$) in concentrations with administration of ISO (Figure 4.15). Rats that received the β_3-antagonist, whether alone or in combination with β_1-

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antagonism, had the largest increase in serum TG, but this change lacked significance.

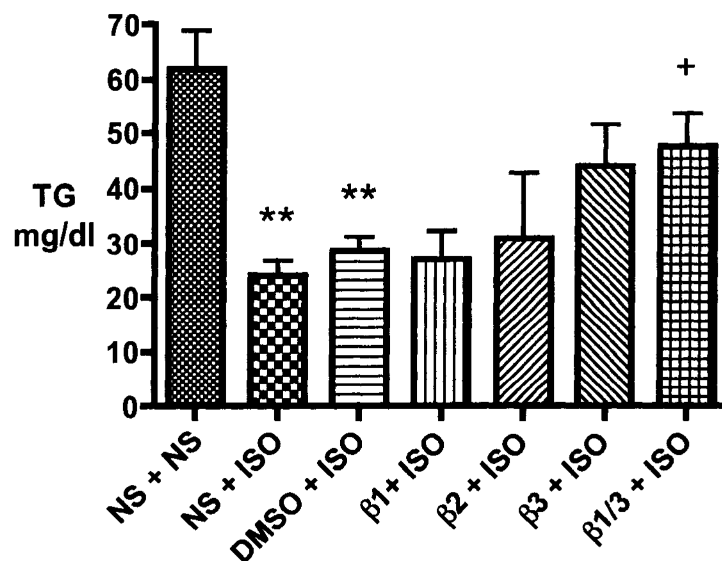

Figure 4.15 Serum TG concentrations in control (NS + NS, NS + ISO, DMSO + ISO) and β-antagonist treated (β_x + ISO) rats. Treatment with ISO, in the absence of β-antagonists, resulted in lowered serum TG (** = $p < 0.01$). Rats treated with combined $\beta_1\beta_3$-antagonists had increased serum TGs (+ = $p < 0.05$) compared to respective controls. Asterisk denotes significance when compared to NS + NS; plus sign denotes significance when compared to the DMSO + ISO control group.

In contrast to untreated rats, those with implanted placebo pellets had higher serum TG when exposed to high-fat dietary treatment ($p < 0.001$). Treatment with chronic $\beta_1\beta_3$-antagonists did not alter the elevated serum values (Figure 4.16). Table 4.10 summarizes serum TG data.

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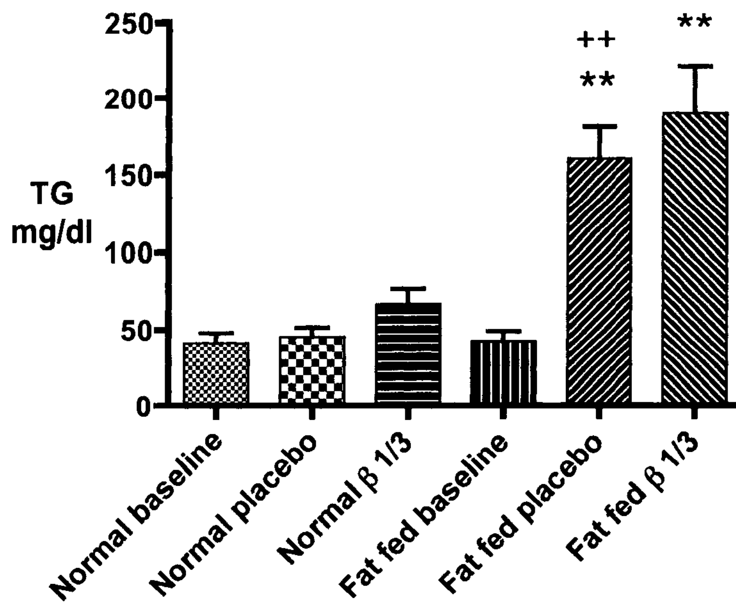

Figure 4.16 Serum TG concentrations in untreated rats (normal and fat baseline) and those that received chronic placebo or β-antagonist treatment. While high-fat feeding did not alter serum TG in baseline animals, it did increase serum TG in those rats with placebo pellets when compared to the normal placebo group (++ = $p < 0.01$). Chronic $\beta_1\beta_3$-antagonism did not increase TG beyond the level achieved with high-fat feeding. Asterisks denote significance in relation to fat-fed baseline rats (** = $p < 0.01$).

Serum FFA and TG concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a very weak significant positive correlation between variables (Pearson r = 0.53; R_2 = 0.28; p = 0.0001).

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	n	μ mg/dl	SE
NS + NS	8	61.9	7.0
NS + ISO	5	24	2.8
DMSO + ISO	7	28.7	2.5
β_1 + ISO	8	27.1	5.2
β_2 + ISO	7	31.0	11.7
β_3 + ISO	8	44	7.5
$\beta_1\beta_3$ + ISO	8	47.5	6.3
Normal baseline	8	40.9	7.0
Normal placebo	8	45.1	6.4
Normal $\beta_1\beta_3$	9	66.7	9.9
Fat-fed baseline	6	42.3	6.5
Fat-fed placebo	7	160.0	21.2
Fat-fed $\beta_1\beta_3$	9	190.0	30.6

Table 4.10 Sample size, mean and standard error of serum TG concentrations measured in all experimental groups.

Cholesterol

Treatment with ISO did not affect serum cholesterol. Acute treatment of ISO-stimulated rats with the β_2-antagonist butoxamine was associated with a decrease in cholesterol ($p = 0.05$). High-fat dietary treatment did not alter serum cholesterol concentrations in untreated or placebo pelleted rats. Serum cholesterol was higher in fat-fed rats with placebo and $\beta_1\beta_3$-antagonist pellet implantation when compared to fat-fed, untreated animals ($p < 0.05$). This increase was quantitatively similar in the two pelleted groups. Table 4.11 summarizes serum cholesterol data.

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	n	μ mg/dl	SE
NS + NS	8	35.9	1.4
NS + ISO	5	40.2	3.7
DMSO + ISO	7	37.0	3.0
β_1 + ISO	8	37.9	2.5
β_2 + ISO	7	29.3+	3.2
β_3 + ISO	8	41.3	2.8
$\beta_1\beta_3$ + ISO	8	41.1	2.9
Normal baseline	8	38.9	2.5
Normal placebo	8	39.9	2.1
Normal $\beta_1\beta_3$	9	38.8	2.2
Fat-fed baseline	6	33.8	1.7
Fat-fed placebo	7	46.4*	4.1
Fat-fed $\beta_1\beta_3$	9	46.2*	3.3

Table 4.11 Sample size, mean and standard error of serum cholesterol concentrations measured in all experimental groups. Plus sign denotes significance at $p = 0.05$ when compared to NS + ISO control group. Asterisk denotes significance at $p < 0.05$ when compared to fat-fed baseline group.

High-density lipoprotein

Serum HDL concentrations were statistically similar across all treatment groups. Treatment with the β_2-antagonist butoxamine was associated with the greatest change in HDL in ISO-stimulated rats, but this increase was not significant. Table 4.12 summarizes HDL data.

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	n	μ mg/dl	SE
NS + NS	8	31.9	1.1
NS + ISO	5	32.2	2.2
DMSO + ISO	7	35.6	2.4
β_1 + ISO	8	34.4	2.0
β_2 + ISO	7	29.3	2.6
β_3 + ISO	8	34.3	1.4
$\beta_1\beta_3$ + ISO	8	35.9	2.4
Normal baseline	8	34.4	2.3
Normal placebo	8	37.4	1.5
Normal $\beta_1\beta_3$	9	37.0	2.1
Fat-fed baseline	6	35.2	1.2
Fat-fed placebo	7	38.4	2.1
Fat-fed $\beta_1\beta_3$	9	41.2	2.0

Table 4.12 Sample size, mean and standard error of serum HDL concentrations measured in all experimental groups.

Low-density lipoprotein

LDL cholesterol is calculated as follows: **LDL = cholesterol - HDL - VLDL (equal to 20% of TG)**. Mean LDL values were negative, except in the NS + ISO group. This group also had the smallest mean TG concentration (see above). LDL values were deemed physiologically irrelevant due to the likely contribution of higher TG concentrations resulting in negative calculated LDL.

Leptin

Neither ISO nor β-antagonist treatment altered leptin levels from those measured in saline-treated controls. Data is presented in Table 4.13.

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High-fat feeding was associated with elevated serum leptin concentrations in untreated rats ($p < 0.001$) (Figure 4.17). High-fat feeding in rats with implanted placebo pellets also resulted in a profound increase in serum leptin, and this was far greater than that measured in pharmacologically untreated animals ($p < 0.001$) (Figure 4.17). Chronic $\beta_1\beta_3$-antagonism did not alter these values. All leptin data is summarized in Table 4.13.

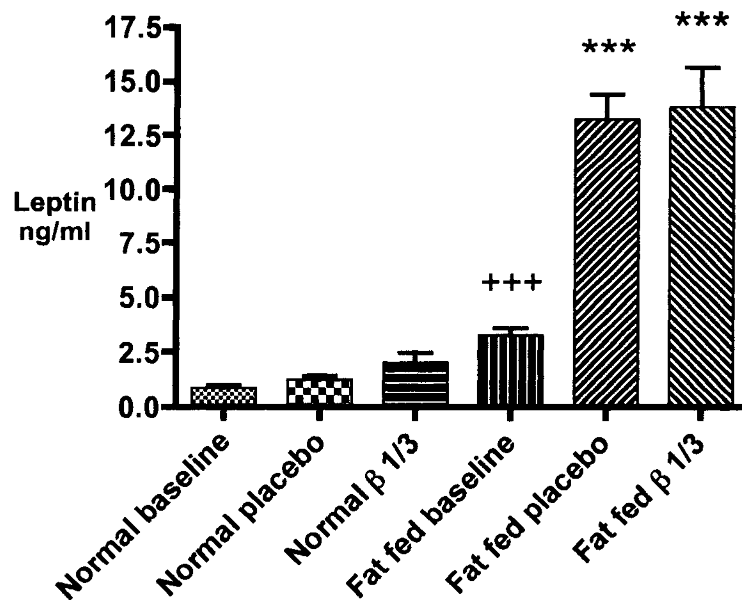

Figure 4.17 Serum leptin concentrations in untreated rats (normal and fat baseline) and those that received chronic placebo or $\beta_1\beta_3$-antagonist treatment. Fat-fed baseline rats had higher serum leptin concentrations than normal baseline animals (+++ = $p < 0.001$). This graph demonstrates the profound increase in leptin levels that was measured in response to high-fat feeding in placebo-pelleted animals compared to untreated fat-fed controls (*** = $p < 0.001$). Chronic $\beta_1\beta_3$-antagonism did not increase leptin concentrations further.

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	n	µ mg/dl	SE
NS + NS	8	.930	.158
NS + ISO	6	.772	.188
DMSO + ISO	7	.824	.169
β_1 + ISO	8	.941	.166
β_2 + ISO	8	1.275	.289
β_3 + ISO	8	.780	.065
$\beta_1\beta_3$ + ISO	8	1.371	.276
Normal baseline	9	.876	.128
Normal placebo	8	1.243	.160
Normal $\beta_1\beta_3$	9	2.011	.453
Fat-fed baseline	6	3.232	.317
Fat-fed placebo	8	13.22	1.156
Fat-fed $\beta_1\beta_3$	9	13.80	1.798

Table 4.13 Sample size, mean, and standard error of serum leptin concentrations measured in all experimental groups.

Serum FFA and leptin concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a very weak positive correlation between variables (Pearson $r = 0.54$; $R^2 = 0.29$; $p < 0.0001$).

Body weight and serum leptin concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a moderate positive correlation between variables (Figure 4.18). Body weights were more closely correlated with leptin than were serum FFAs.

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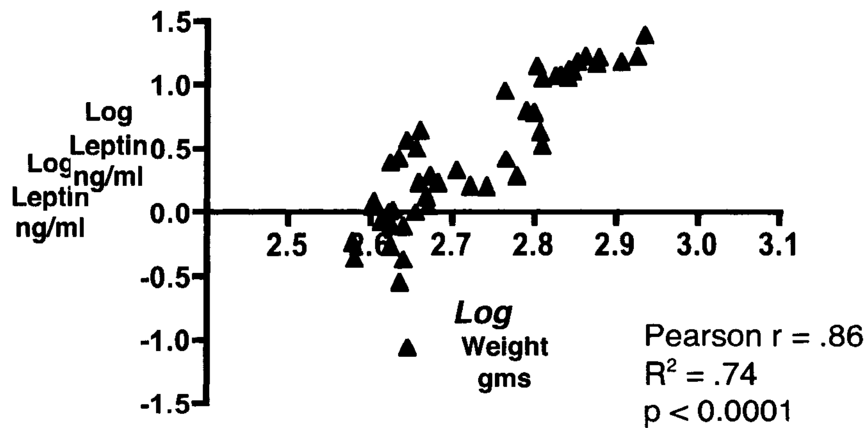

Figure 4.18 Correlation between body weight and serum leptin (log transformed for normality) in pooled dietary control and chronic pelleted treatment groups (n = 49).

Endothelium-dependent vasodilation during acute β-antagonism in isoproterenol-stimulated rats

Baseline MAP, flow, resistance, and heart rate (HR) were measured in normally fed animals 1 hour after injection of control solutions (NS, DMSO) or a β-antagonist but prior to receipt of ISO. Baseline MAP, flow, and resistance were not altered by β-antagonist treatment when compared to control values. Rats that received β_3-antagonist treatment had higher HR than control (DMSO) animals ($p < 0.05$); HRs were otherwise unchanged by β-antagonist treatment (Table 4.14).

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	n	MAP mm Hg	Flow ml/min	Resis mmHg X min/ml	HR bpm
NS + ISO	9	92/8	.88/.07	115/17	202/6
DMSO + ISO	7	90/5	.68/.09	150/22	210/11
β_1 + ISO	8	91/8	.90/.16	115/14	194/7
β_2 + ISO	9	87/4	.74/.13	156/31	212/6
β_3 + ISO	8	80/5	.82/.15	147/56	246/7
$\beta_1\beta_3$ + ISO	10	78/4	.55/.10	154/24	208/6

Table 4.14 Sample size and baseline mean arterial pressure, flow, resistance and heart rate (beats per min) in normal rats premedicated with placebo solutions (NS, DMSO) or β-antagonists. Data are shown as mean/SE.

ISO lowered MAP ($p < 0.001$), elevated HR ($p < 0.01$-0.001), increased flow ($p < 0.05$-0.001), and decreased resistance ($p < 0.05$) when compared to saline alone. Premedication with the β_1-antagonist atenolol, whether alone or in combination with β_3-antagonism, attenuated the ISO-associated rise in HR ($p < 0.01$-0.001) (Figure 4.19). There were no changes in post-ISO MAP, flow, or resistance with β-antagonism. Of note however, is the observation that, consistent with the role of β_2-receptor stimulation in peripheral vasodilation, rats that received butoxamine had the lowest flow values and highest resistance when compared to all other β-antagonist treated groups. Table 4.15 summarizes post-ISO hemodynamic data.

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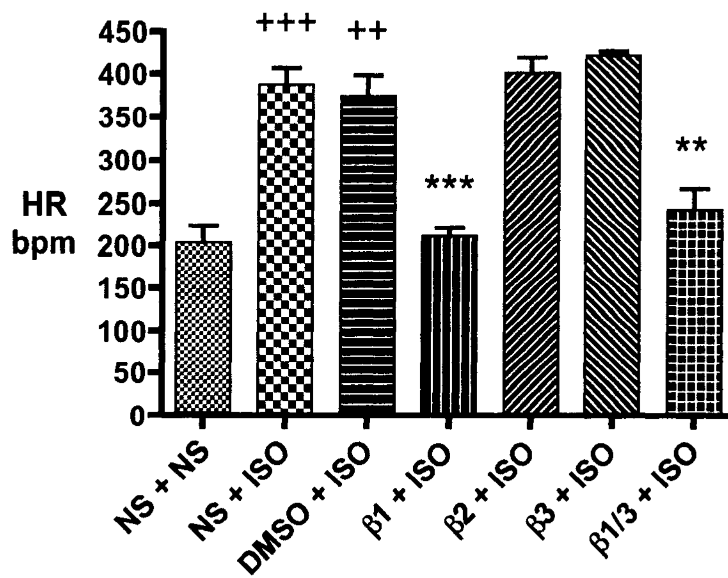

Figure 4.19 Heart rates measured in pharmacologically untreated (normal and fat-fed baseline), control (NS + NS, NS + ISO, DMSO + ISO), and β-antagonist treated (β + ISO) rats. ISO was associated with higher HR (+++ = $p < 0.001$; ++ = $p < 0.01$). Rats treated with β_1-antagonism, whether alone or in combination with β_3-antagonism, had attenuation of ISO-induced tachycardia (*** $p < 0.001$; ** = $p < 0.01$).

	n	MAP mm Hg	Flow ml/min	Resis mmHg X min/ml	HR bpm
NS + NS	7	92/5	.79/.10	136/28	204/19
NS + ISO	9	49/2	1.50/.22	39/6	387/19
DMSO + ISO	8	53/2	1.75/.15	32/4	374/24
β_1 + ISO	8	45/3	1.27/.17	42/9	211/9
β_2 + ISO	9	55/2	1.06/.15	62/10	401/19
β_3 + ISO	8	51/2	1.60/.12	34/4	423/5
$\beta_1\beta_3$ + ISO	10	50/2	1.96/.27	34/9	243/23

Table 4.15 Sample size, mean arterial pressure, flow, resistance, and heart rate in normal rats premedicated with placebo solutions (NS, DMSO) or β-antagonists, and subsequently administered ISO. Data are shown as mean/SE.

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The data for FTI was not normally distributed in all groups, so was log transformed. The FTIs were compared in rats that received NS only to those that received ISO with one of the β-antagonist control solutions (NS or DMSO) (Figure 4.20). Treatment with DMSO + ISO increased the FTI ($p < 0.01$) when compared to saline-treated animals, but did not change the FTI when compared to the NS + ISO group, the more appropriate control.

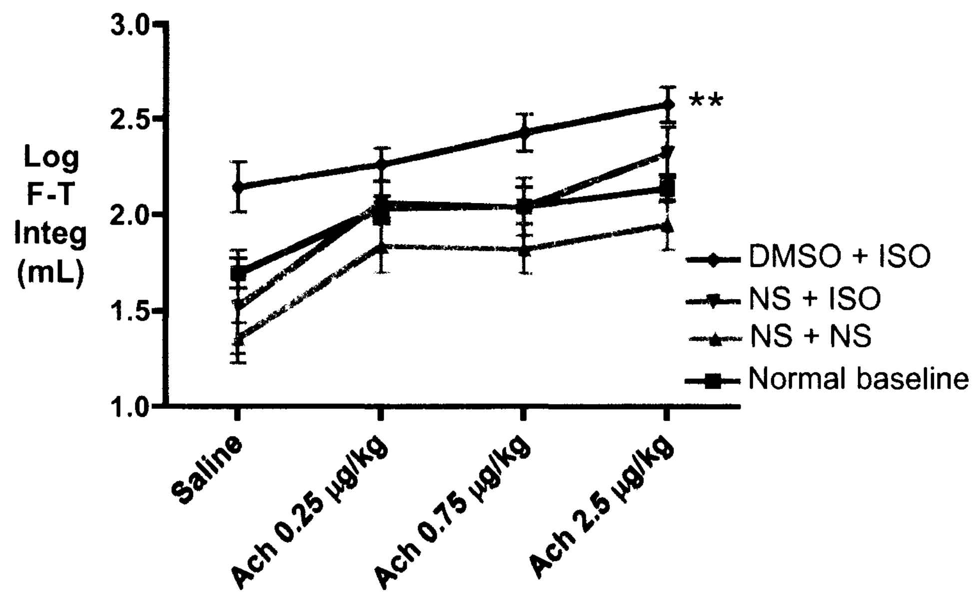

Figure 4.20 Femoral artery FTI measured in response to NS and increasingly larger doses of intra-arterial Ach in untreated rats, those that received NS only, and those that received ISO with one of the β-antagonist control solutions (NS or DMSO). DMSO + ISO increased FTI when compared to NS control animals (** = $p < 0.01$). There was a significant ($p < 0.001$) effect attributable to Ach dose.

There were no differences in FTI attributable to β-antagonism in acute studies of isoproterenol-stimulated rats (Figure 4.21).

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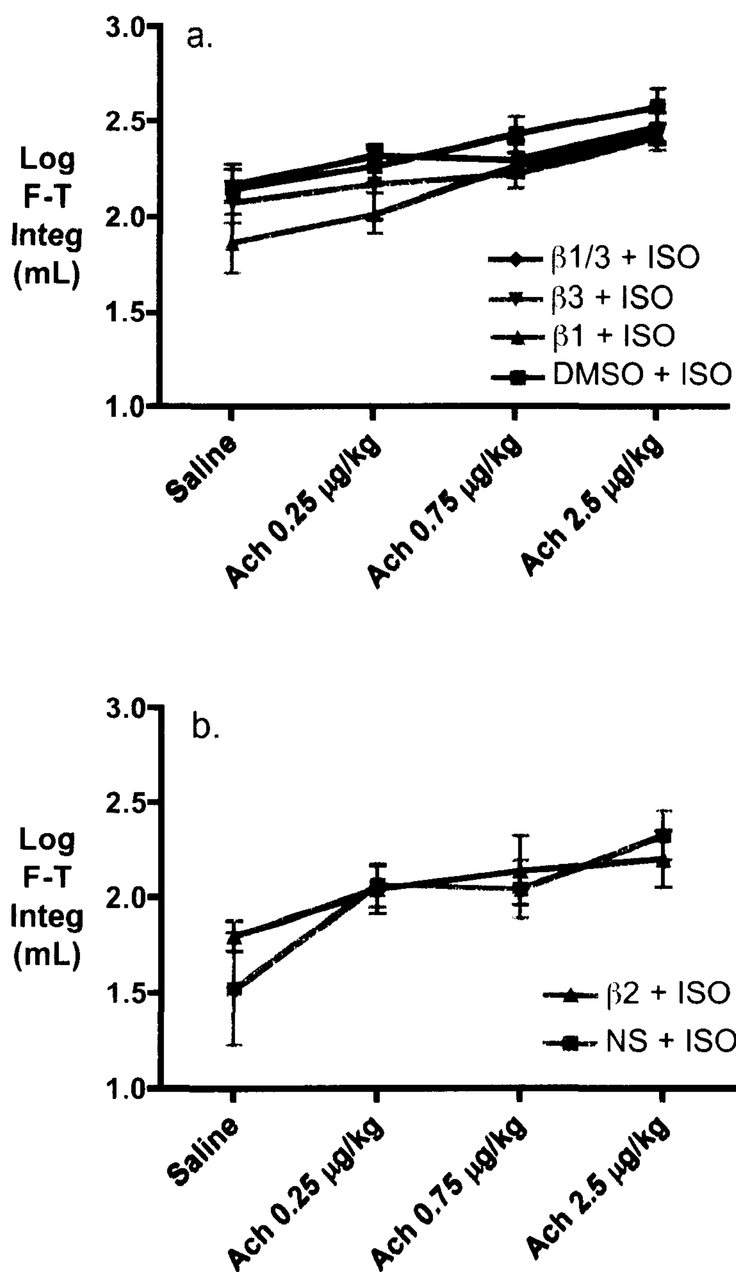

Figure 4.21 Graphic representation of femoral artery FTI in response to NS and increasingly larger doses of intra-arterial Ach in rats treated with β-antagonists compared to respective control animals. None of the β-antagonist treatments changed FTI when compared to respective controls. There was a significant ($p < 0.001$) effect attributable to Ach dose.

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Endothelium-dependent vasodilation during chronic β-antagonism in fat-fed rats

Basal MAP, flow, resistance, and HR were compared in all treatment groups. There were no differences in MAP or flow. When fat-fed treatment groups were compared, rats that were treated with the $\beta_1\beta_3$-antagonist pellets had lower resistance ($p < 0.05$) and higher HRs ($p < 0.001$) than those with placebo pellets. A summary of basal hemodynamic data is provided in Table 4.16.

	n	MAP mm Hg	Flow ml/min	Resis mmHg X min/ml	Log (Resis)	HR bpm
Normal baseline	17	87/4	.59/.06	178/21	2.20/.05	204/6
Normal placebo	7	93/11	.79/.14	129/17	2.09/.05	199/7
Normal $\beta_1\beta_3$	10	85/3	.80/.07	113/10	2.04/.04	213/18
Fat-fed baseline	6	89/4	.72/.09	132/16	2.10/.05	198/5
Fat-fed placebo	9	96/6	.76/.10	137/14	2.12/.04	188/7
Fat-fed $\beta_1\beta_3$	10	98/4	1.03/.14	99/12	1.97/.05*	233/5***

Table 4.16 Sample size, mean arterial pressure, flow, resistance, and heart rate in normal and fat-fed rats at baseline, with placebo pellet implantation, and with combined $\beta_1\beta_3$-antagonist pellets. Data is displayed as mean/SE. Asterisks denote significance when compared to fat-fed placebo group (*** = $p < 0.001$; * = $p < 0.05$).

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There was no effect of placebo pellet implantation on femoral artery FTI; thus, for simplicity, the normal and fat baseline FTI data will be omitted from the remainder of the results discussion, and placebo pelleted rats used as controls.

High-fat feeding did not alter FTI in rats with placebo pellet implantation (Figure 4.22).

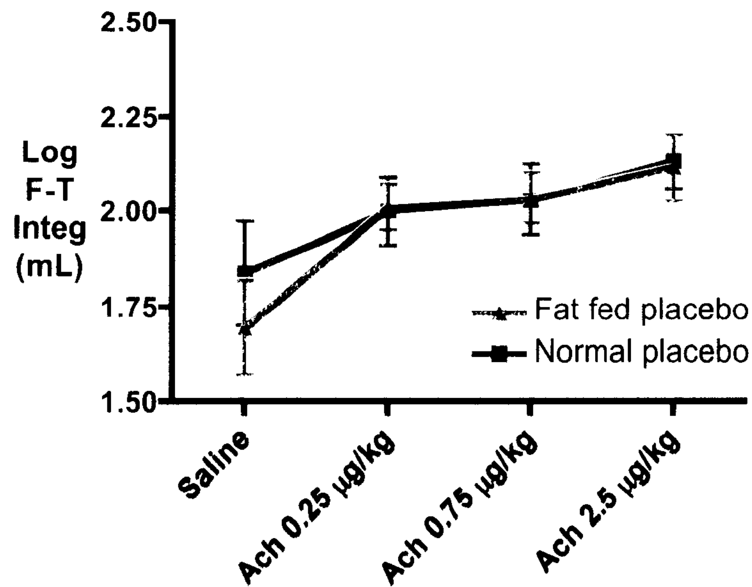

Figure 4.22 Femoral artery FTI in response to NS and increasingly larger doses of intra-arterial Ach. Normal and fat-fed rats that received placebo pellet implantation are compared. High-fat feeding did not alter FTI. A significant Ach dose effect was present ($p < 0.001$).

In both normal and fat-fed rats, chronic treatment with combined $\beta_1\beta_3$-antagonists did not change the FTI, though a trend toward improved FTI at all treatment points was observed (Figure 4.23).

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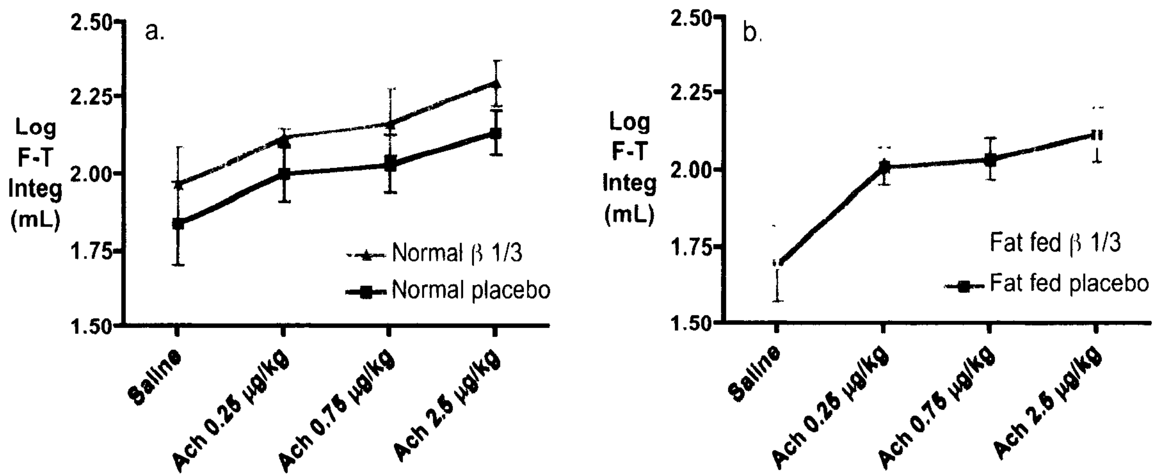

Figure 4.23 Femoral artery FTI in response to NS and increasingly larger doses of intra-arterial Ach. Normal (a) and fat-fed (b) rats that received placebo pellet implantation are compared to those that received $\beta_1\beta_3$-antagonist pellets. Chronic $\beta_1\beta_3$-antagonism did not significantly alter FTI, though a trend toward improved FTI is observed. A significant Ach dose effect was present (a: $p < 0.01$ and b: $p < 0.001$).

Neither high-fat feeding nor chronic $\beta_1\beta_3$-antagonism induced a significant change in the Δ FTI. However, both normal and fat-fed rats that received $\beta_1\beta_3$-antagonist treatment had a trend toward greater Δ FTI when compared to placebo-pelleted controls (Figure 4.24).

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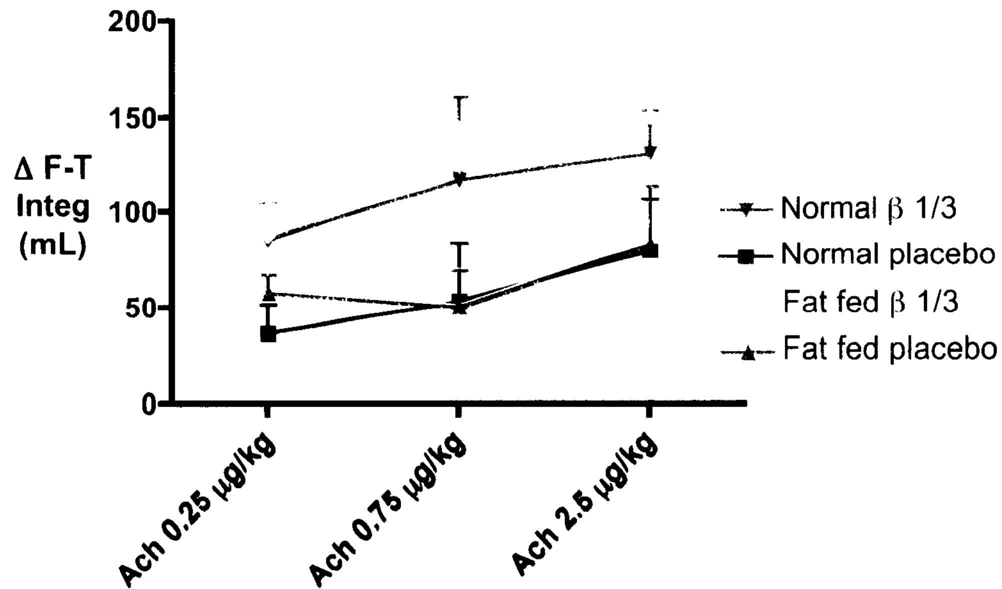

Figure 4.24 Quantitation of endothelium-dependent dilatory response attributable to NO, measured as the difference in pre- versus post- L-NAME FTI with increasingly larger doses of Ach. Data from normal and fat-fed rats with placebo or $\beta_1\beta_3$-antagonist pellet implantation are shown. The larger NO-mediated FTI response attributable to chronic $\beta_1\beta_3$-antagonism was not significant.

In both normal and fat-fed animals, the difference between pre- and post-L-NAME resistance values was less in those animals that received chronic $\beta_1\beta_3$-antagonist treatment; however, this difference was not significant (Figure 4.25). No differences emerged when change in resistance was analyzed as a percentage of baseline resistance.

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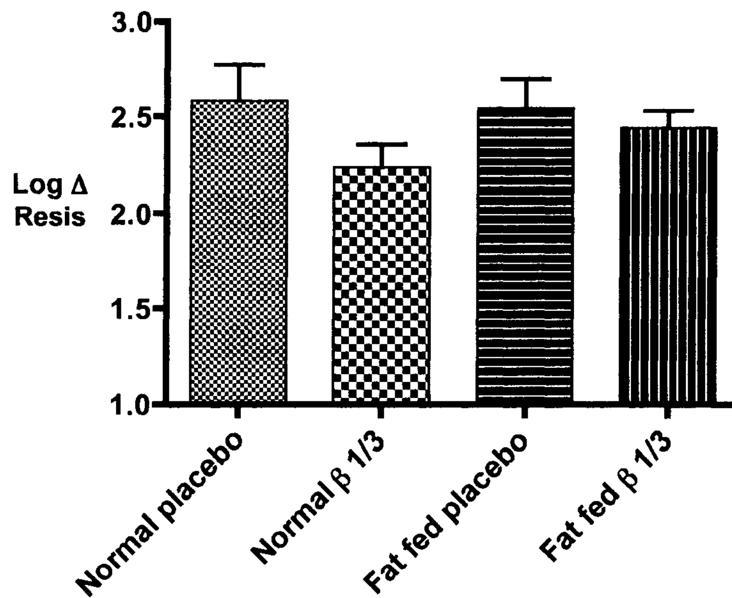

Figure 4.25 Graphical representation of the portion of endothelium-dependent dilation attributable to NO, approximated by calculating the difference between pre- and post-L-NAME resistance. While a trend in reduced NO-dependent vasodilation was observed in rats that received chronic $\beta_1\beta_3$-antagonist treatment when compared to placebo animals, this was not a significant change.

There were no differences in the absolute resistances associated with inhibition of endothelium-independent vasodilation. Differences in resistance remained insignificant when analyzed as a percentage of baseline resistance.

Data were collected from untreated normal and fat-fed rats, and from all rats that received chronic placebo and $\beta_1\beta_3$-antagonist treatment. Body weights were analyzed for correlation with FTI in response to saline and each dose of Ach, as well as with the total FTI response. There was no correlation between body weight and FTI.

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Aim 2: Measure markers of inflammation and oxidative stress in normal and fat-fed rats with and without β-antagonism.

Inflammation

C-Reactive Protein

Neither ISO nor β-antagonist treatment altered CRP concentrations in acute studies.

There were no differences in serum CRP when normal and fat-fed, untreated animals were compared. Placebo pellet implantation alone was associated with elevations in serum CRP in normal ($p < 0.001$), but not fat-fed rats. This phenomenon was not observed in fat-fed rats with placebo pellets, resulting in lower serum CRP in this group when compared to normal placebo rats ($p < 0.001$). Chronic $\beta_1\beta_3$-antagonism reduced CRP levels in normal and fat-fed rats when compared with placebo pelleted controls ($p < 0.001$ and $p < 0.05$, respectively) (Figure 4.26). A summary of CRP data is presented in Table 4.17.

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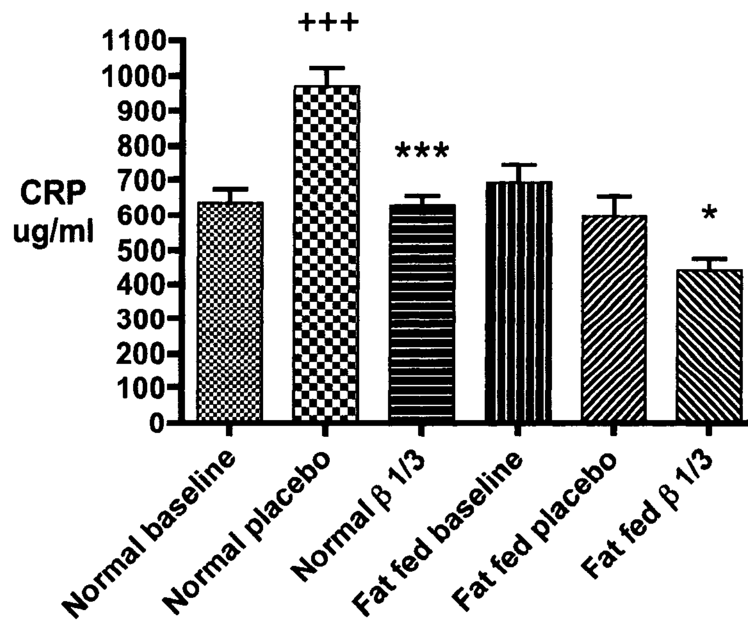

Figure 4.26 Serum CRP concentrations in untreated rats (normal and fat baseline) and those that received chronic placebo or β-antagonist treatment. Placebo pellet implantation was associated with larger CRP levels in normal rats when compared with normal baseline animals (+++ = $p < 0.001$). Fat-fed rats with placebo pellets did not display this phenomenon. Normal and fat-fed rats given chronic $\beta_1\beta_3$-antagonists had reduced CRP concentrations compared to respective placebo controls (*** = $p < 0.001$; * = $p < 0.05$).

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	n	μ ug/dl	SE
NS + NS	8	707.6	45.6
NS + ISO	6	672.1	39.6
DMSO + ISO	7	643.0	52.36
β_1 + ISO	8	819.1	42.6
β_2 + ISO	8	714.2	21.3
β_3 + ISO	8	692.4	52.3
$\beta_1\beta_3$ + ISO	8	703.2	54.0
Normal baseline	9	635.8	37.1
Normal placebo	8	969.7	48.8
Normal $\beta_1\beta_3$	9	628.8	25.3
Fat-fed baseline	6	690.4	51.3
Fat-fed placebo	8	597.4	55.12
Fat-fed $\beta_1\beta_3$	9	441.1	34.3

Table 4.17 Sample size, mean and standard error of serum CRP concentrations measured in all experimental groups.

Serum FFA and CRP concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a weak negative correlation between variables (Figure 4.27).

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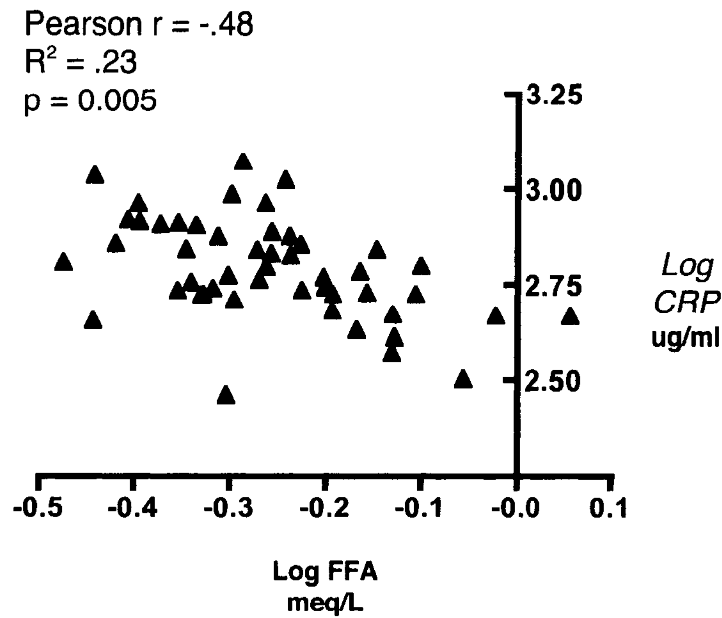

Figure 4.27 Correlation between serum FFAs and CRP concentrations (log transformed for normality) in pooled dietary control and chronic pelleted treatment groups (n = 49).

Interleukin-6

Treatment with ISO did not affect serum IL-6 in acute studies. Animals treated with β_1- and β_2-antagonists had higher IL-6 when compared to respective control animals. Figure 4.28 graphically illustrates these observations.

High-fat feeding did not alter serum IL-6 concentrations in untreated animals. Studies of fat-fed rats and chronic $\beta_1\beta_3$-antagonism revealed an increase in IL-6 with placebo pellet implantation alone. In both normal and fat-fed rats, this augmentation was reduced by $\beta_1\beta_3$-antagonism, and this effect was significant in the normal animals (Figure 4.29). Table 4.18 summarizes all IL-6 data.

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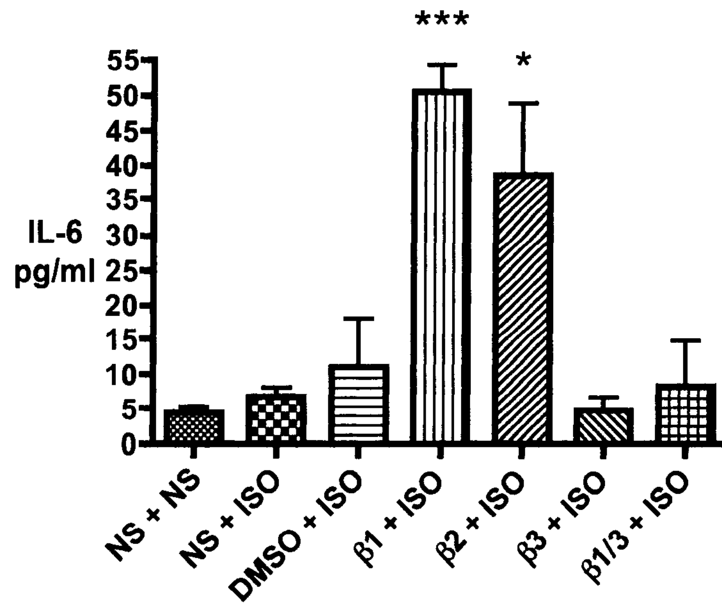

Figure 4.28 Serum IL-6 concentrations in untreated (normal and fat-fed baseline), control (NS + NS, NS + ISO, DMSO + ISO), and β-antagonist treated (β + ISO) rats. Treatment with β_1- and β_2-antagonists elevated serum IL-6 levels in ISO-stimulated rats when compared with DMSO + ISO and NS + ISO controls, respectively (*** = $p < 0.001$; * = $p < 0.05$).

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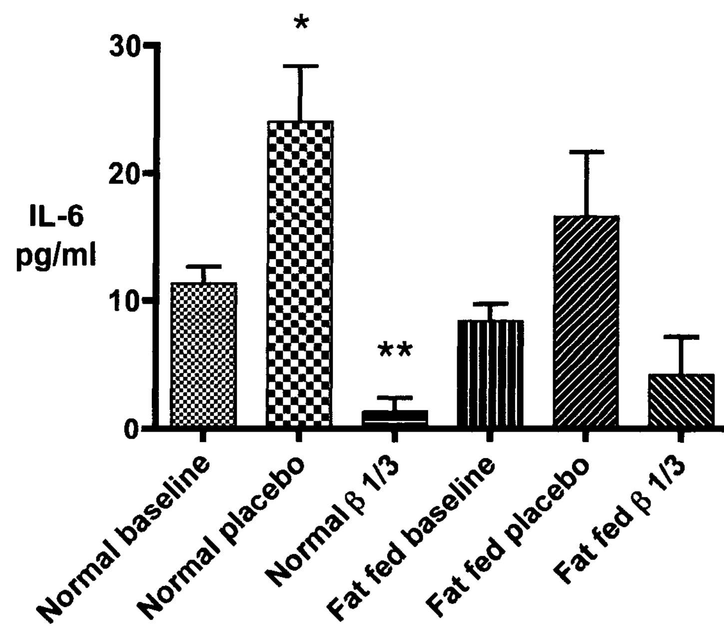

Figure 4.29 Serum IL-6 concentrations in untreated rats (normal and fat baseline) and those that received chronic placebo or $\beta_1\beta_3$-antagonist treatment. Placebo pellet implantation was associated with increased IL-6 in normally fed rats (* = $p < 0.05$). Chronic $\beta_1\beta_3$-antagonist treatment reduced IL-6 in both groups, with the change being significant in normally fed animals (** = $p < 0.01$).

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	n	μ pg/ml	SE
NS + NS	8	4.42	.80
NS + ISO	6	6.79	1.24
DMSO + ISO	7	11.05	7.00
β_1 + ISO	8	50.51	3.78
β_2 + ISO	7	38.63	10.27
β_3 + ISO	8	4.78	1.87
$\beta_1\beta_3$ + ISO	8	8.24	6.56
Normal baseline	8	11.30	1.32
Normal placebo	8	24.08	4.26
Normal $\beta_1\beta_3$	8	1.35	1.01
Fat-fed baseline	6	8.37	1.35
Fat-fed placebo	8	16.51	5.06
Fat-fed $\beta_1\beta_3$	9	4.17	2.92

Table 4.18 Sample size, mean and standard error of serum IL-6 concentrations measured in all experimental groups.

Serum FFA and IL-6 concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was no correlation between variables (Pearson r = -.12; R^2 = .28; p = 0.49).

Tumor necrosis factor-α

Treatment with ISO did not alter serum TNF-α values when compared to NS controls. Rats that received β_1-antagonism had higher TNF-α concentrations when compared to DMSO controls ($p < 0.05$) (Figure 4.30).

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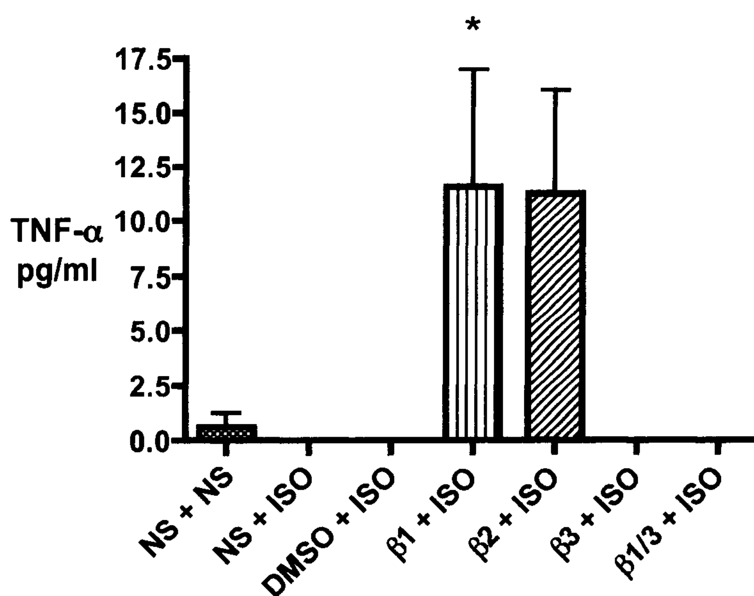

Figure 4.30 Serum TNF-α concentrations in control (NS + NS, NS + ISO, DMSO + ISO) and β-antagonist treated (β + ISO) rats. ISO treatment did not alter TNF-α concentrations. Treatment with the β_1-antagonist atenolol increased TNF-α when compared to the DMSO + ISO control group (* = $p < 0.05$).

Serum TNF-α concentrations were lower in fat-fed untreated rats when compared with normally fed controls ($p < 0.05$). In both normal and fat-fed rats, implantation of placebo pellets were associated with increased TNF-α that was not significant. Chronic $\beta_1\beta_3$-antagonism decreased TNF-α levels in both groups, and this was significant in the normal dietary group ($p < 0.01$) (Figure 4.31). Table 4.19. summarizes acute and chronic TNF-α data.

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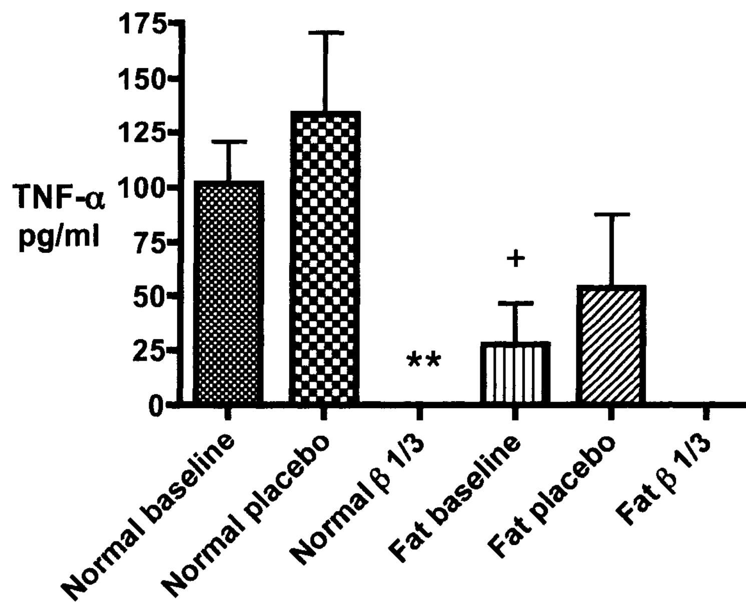

Figure 4.31 Serum TNF-α concentrations in untreated rats (normal and fat baseline) and those that received chronic placebo or $\beta_1\beta_3$-antagonist treatment. In untreated animals, high-fat feeding was associated with lower TNF-α when compared to normally fed controls (+ = $p < 0.05$). The increase in TNF-α that was measured in normal and fat-fed placebo pelleted rats was not significant. Chronic $\beta_1\beta_3$-antagonism decreased concentrations in both dietary treatment groups, and this was a significant reduction in the normally-fed rats (** = $p < 0.01$).

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	n	μ pg/ml	SE
NS + NS	8	.64	.64
NS + ISO	6	0	0
DMSO + ISO	7	0	0
β_1 + ISO	8	11.56	5.43
β_2 + ISO	8	11.28	4.72
β_3 + ISO	8	0	0
$\beta_1\beta_3$ + ISO	8	0	0
Normal baseline	9	101.60	18.86
Normal placebo	8	134.00	36.52
Normal $\beta_1\beta_3$	9	0	0
Fat-fed baseline	6	27.59	18.96
Fat-fed placebo	8	53.53	34.48
Fat-fed $\beta_1\beta_3$	9	0	0

Table 4.19 Sample size, mean and standard error of TNF-α serum concentrations measured in all experimental groups.

Serum FFA and TNF-α concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was no correlation between variables (Pearson r = -.07; R^2 = .01; p = 0.75).

Oxidative stress

Thiobarbituric acid-reactive substances

In acute studies, rats treated with DMSO + ISO had lower serum TBARS than rats treated with NS + ISO ($p < 0.01$). Treatment with β-antagonists was associated with similar absolute serum TBARS concentrations. Because of the

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reduced levels measured in the DMSO + ISO group, the values measured in β_1- and β_3-antagonist treated rats were not different from this control. Only the β_2-antagonist treatment was reduced when compared to the appropriate NS + ISO control (Figure 4.32).

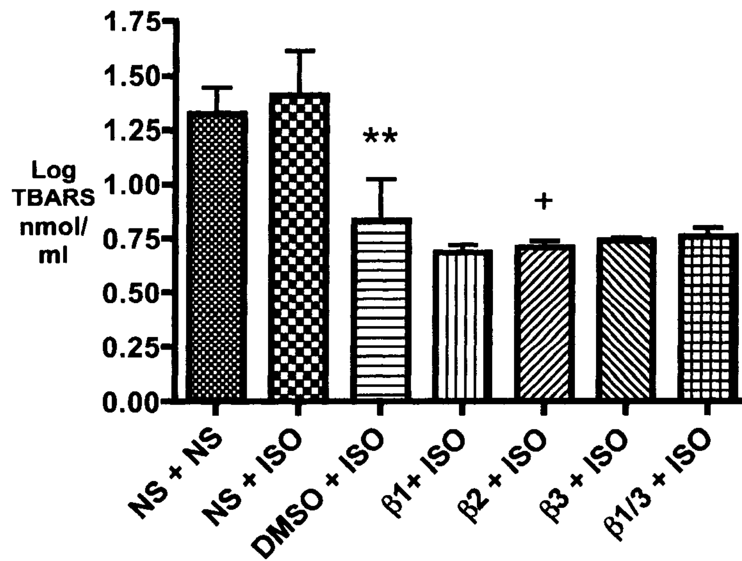

Figure 4.32 Serum concentrations of TBARS in control (NS + NS, NS + ISO, DMSO + ISO) and β-antagonist treated (β + ISO) rats. Rats treated with DMSO + ISO had reduced TBARS when compared to rats that received NS + ISO ($p < 0.01$). The collective $\beta_1\beta_3$-antagonist response was not significantly decreased from the DMSO + ISO control animals. Rats that received β_2-antagonist treatment did have reduced serum TBARS when compared to the NS + ISO control group.

Serum concentrations of TBARS were not altered by high-fat feeding in untreated or placebo pelleted rats. In normally-fed rats, TBARS were increased by placebo pellet implantation; concentrations were similarly elevated with chronic $\beta_1\beta_3$-antagonism. Placebo pellet implantation did not increase serum

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TBARS in fat-fed rats; however, fat-fed rats that received chronic $\beta_1\beta_3$-antagonist treatment had elevated serum TBARS when compared to baseline and placebo controls ($p < 0.001$) (Figure 4.33). All TBARS data is summarized in Table 4.20.

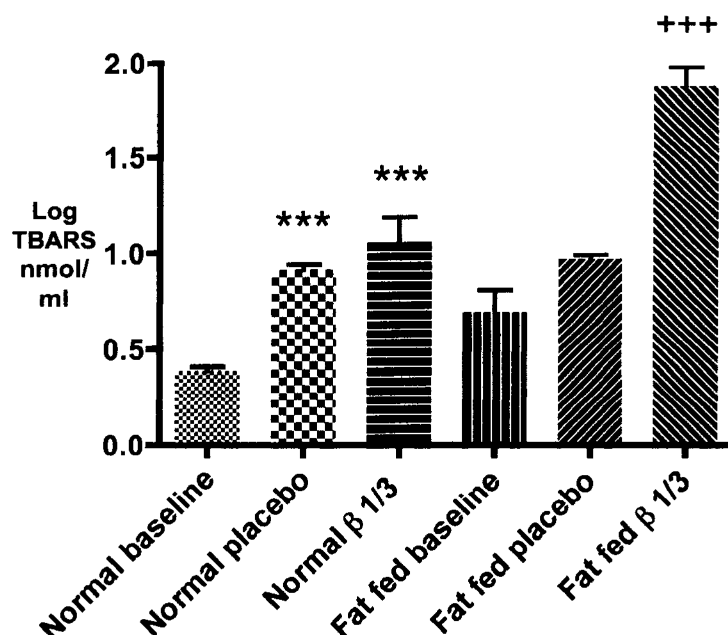

Figure 4.33 Serum concentrations of TBARS in untreated rats (normal and fat baseline) and those that received chronic placebo or $\beta_1\beta_3$-antagonist treatment. Placebo pellet implantation alone increased TBARS in normal animals; this was not increased further by $\beta_1\beta_3$-antagonism (*** = $p < 0.001$ when compared to normal baseline rats). Chronic treatment of fat-fed rats with $\beta_1\beta_3$-antagonists increased serum TBARS when compared to fat-fed baseline and placebo controls (+++ = $p < 0.001$).

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	n	μ pg/ml	SE
NS + NS	8	25.72	5.53
NS + ISO	6	39.71	14.75
DMSO + ISO	7	18.10	13.78
β_1 + ISO	8	4.93	.44
β_2 + ISO	7	5.16	.34
β_3 + ISO	8	5.51	.16
$\beta_1\beta_3$ + ISO	8	5.88	.56
Normal baseline	9	2.47	.14
Normal placebo	8	8.34	.54
Normal $\beta_1\beta_3$	9	16.65	5.42
Fat-fed baseline	6	6.09	2.02
Fat-fed placebo	8	9.41	.52
Fat-fed $\beta_1\beta_3$	9	89.00	13.22

Table 4.20 Sample size, mean and standard error of serum concentrations of TBARS measured in all experimental groups.

Serum FFA and TBARS concentrations from the 2 dietary baseline and 4 chronic treatment groups were pooled and paired to determine correlation. There was a weak positive correlation between variables (Figure 4.34). Further correlative analyses were performed investigating the relationship between TBARS and serum glucose, TGs, and leptin. A summary of correlation data is presented in Table 4.21.

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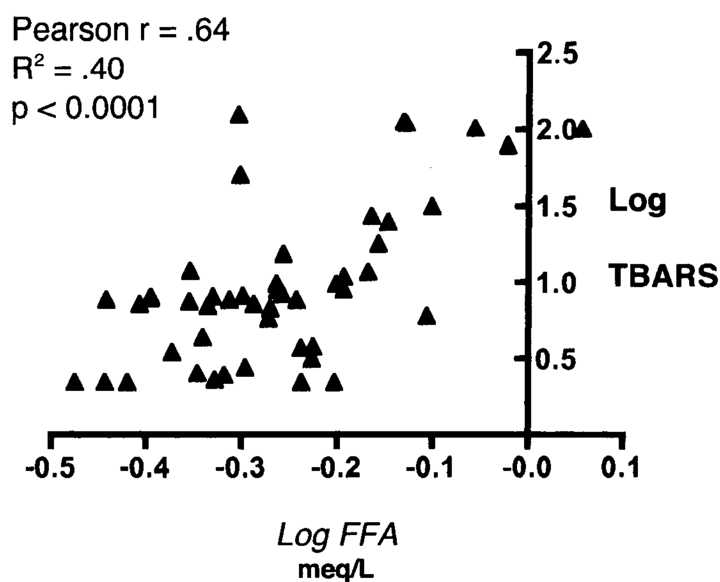

Figure 4.34 Correlation between serum FFAs and TBARS concentrations (log transformed for normality) in pooled dietary control and chronic pelleted treatment groups (n = 49).

	Pearson r	p-value	R^2
FFAs	0.64	<0.0001	0.40
Glucose	0.55	< 0.0001	0.30
Triglycerides	0.69	< 0.0001	0.47
Leptin	0.62	< 0.0001	0.38

Table 4.21 Correlation data describing the relationship between TBARS and each of the factors listed, including FFAs, glucose, TGs, and leptin.

Localization of femoral artery nitrotyrosine residues by immunohistochemistry

Immunohistochemistry revealed the most robust nitrotyrosine staining to be in the endothelium. Focal areas of staining were also present in the subendothelial and medial regions (Figure 4.35). Differences in pattern or

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intensity of staining between groups were difficult to discern; thus, immunohistochemistry was used for nitrotyrosine localization only.

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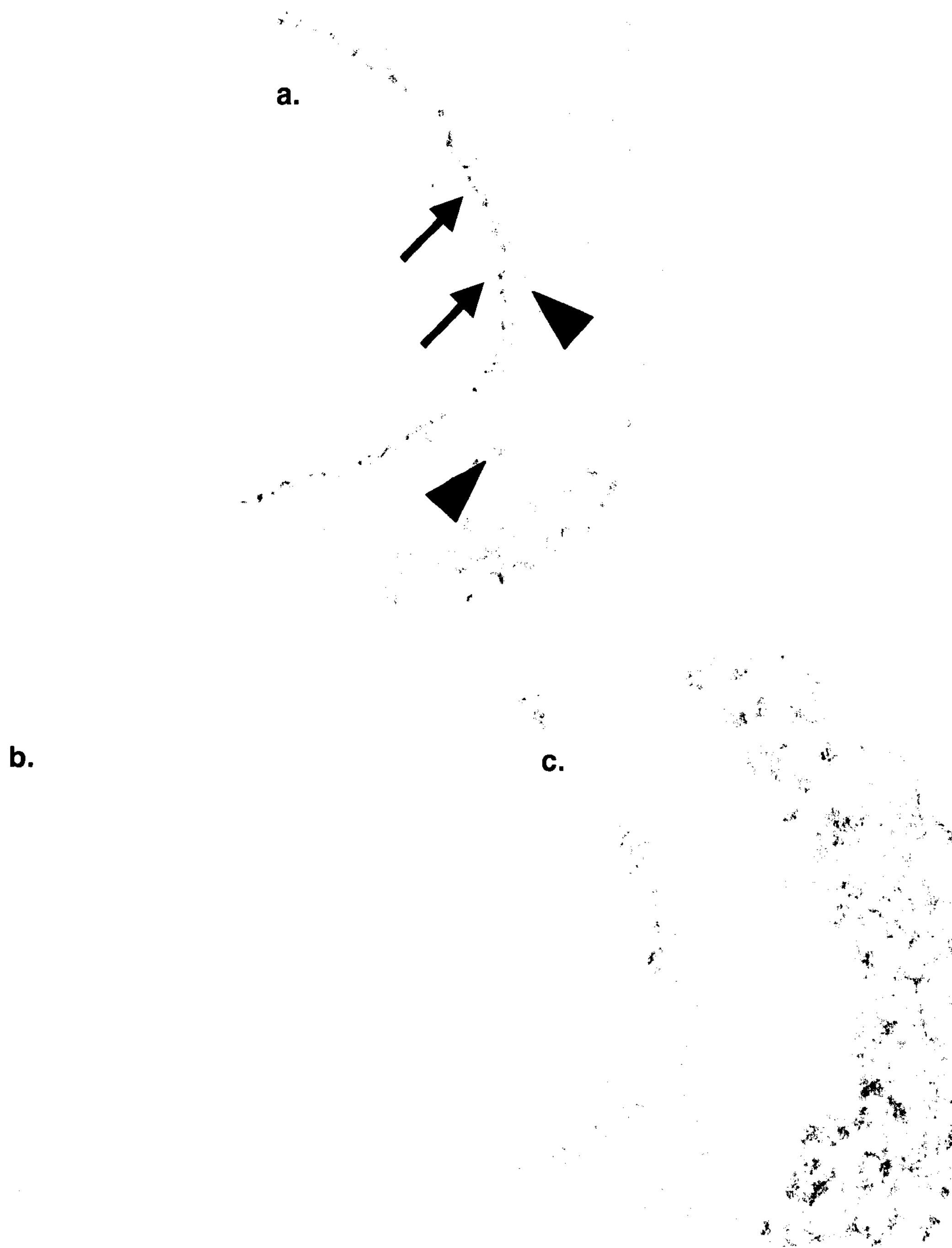

Figure 4.35 Immunohistochemical detection of nitrotyrosine residues in a femoral artery segment from a fat-fed rat that received $\beta_1\beta_3$-antagonist treatment (a). Nitrotyrosine segments stain brown, with the darkest staining present in the endothelial cells lining the vascular lumen (arrows). Focal areas of less intense staining are seen in the subendothelial and medial spaces (arrowhead). Negative (b) and positive (c) controls are shown.

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Quantitation of femoral artery nitrotyrosine residues by slot blot analysis

With installation of 20 μg of protein per well, slot blot analysis of femoral artery segments from chronically treated rats revealed a stronger signal in animals that received $\beta_1\beta_3$-antagonist treatment. This trend was observed in both normal and fat-fed rats, with the strongest signal observed in animals that received combined dietary and β-antagonist treatment (Figure 4.36). These results were not repeatable with 5 μg of protein per well.

Figure 4.36 Detection of arterial nitrosylated protein residues (nitrotyrosine) by slot blot analysis. In both normal and fat-fed animals, the signal is stronger in arteries from rats that received $\beta_1\beta_3$-antagonist treatment. Arterial segments from fat-fed, β-antagonist-treated rats appear to have the largest quantity of nitrosylated protein.

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CHAPTER V

DISCUSSION

As in RESULTS, treatment groups will be referenced in the following manner:

Normal = rats fed normal rat chow for the 12 week dietary treatment period

Fat-fed = rats that received high-fat dietary treatment for the 12 week dietary treatment period

Untreated/baseline = rats that received neither placebo nor $\beta_1\beta_3$-antagonist pellets (i.e. no pharmacologic treatment)

Placebo = inert SC pellet

$\beta_1\beta_3$-antagonism or **$\beta_1\beta_3$-pellets** = 1 pellet containing the β_1-antagonist atenolol and 1 pellet containing the β_3-pellet SR59230A

There is increased risk of hypertension, dyslipidemia, insulin resistance, and coronary artery disease in those who are overweight or obese.[1,2] Obesity and associated comorbidities are characterized by endothelial dysfunction. The etiology of obesity-induced attenuation of endothelial function has yet to be

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elucidated.[266] This study was undertaken to determine whether serum FFAs, commonly elevated in the obese state, contribute to impaired endothelium-dependent vasodilation. Further, this study was designed to determine whether β-antagonist treatment could be used to inhibit lipolysis and reduce serum FFAs to the extent that any associated endothelial dysfunction would be attenuated. Finally, the mechanism of FFA-mediated endothelial dysfunction was investigated by measuring markers of inflammation and oxidative stress in serum and arterial segments.

EFFECTS OF HIGH-FAT DIET, ISOPROTERENOL AND β-ANTAGONISM ON BODY WEIGHT AND METABOLISM

Free fatty acids

Serum FFAs were significantly increased by ISO ($p < 0.001$), reaching concentrations of .8227 meq/L in the NS + ISO group, and .7700 meq/L in the DMSO + ISO group. Serum FFAs reached a higher concentration in preliminary studies ($\mu = 1.299$ meq/L in fat-fed males; $\mu = 1.0769$ meq/L in normal females). The augmented response to ISO in fat-fed animals, if representative of the population, lies in contrast to evidence of impaired adipocyte β-receptor responsiveness in the obese state.[77-80] The apparently enhanced lipolytic response may also be attributable to increased visceral adipose mass in fat-fed animals, which is more sensitive to the lipolytic catecholamines.[83,118,119] and more resistant to the antilipolytic effects of insulin.[121,122] The higher concentrations

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observed in female rats is consistent with enhanced responsiveness to β-receptor agonists has been observed in female rat adipose.[267,268]

In preliminary studies of normal female and fat-fed male rats, administration of the β_1-antagonist atenolol, alone and in combination with the β_3-antagonist SR59230A, resulted in attenuation of the ISO-associated rise in serum FFAs. Using the same protocol with a larger sample size, administration of β-antagonists did not significantly attenuate the ISO-induced increase in FFAs in study rats. In a study investigating the antilipolytic effects of various β-antagonists in male Wistar rats, premedication with atenolol at 1 mg/kg SC produced > 90% inhibition of ISO-stimulated lipolysis (ISO 0.1 mg/kg SC).[256] This is consistent with the established role of β_1- and β_3-receptors as the primary subtypes by which lipolysis is mediated in rodent white adipocytes.[67,68] Administration of ISO produced a 94% increase in serum FFAs in the Wistar rats, compared to a more modest mean increase of 41% observed in this study. Differences in LPL and hepatic lipase activity between Wistar and Sprague-Dawley rat strains have been characterized,[269] and this may partially account for the varied responses observed. Additionally, there was a 0.05 mg/kg difference in ISO dose between these two studies. In dogs, in vivo stimulation of the β_3-receptor requires a higher dose of ISO than that needed for agonism at β_1- and β_2-subtypes.[270] It is possible that in these rats, the lower dose of ISO resulted in recruitment of β_2-receptors in mediating lipolysis. This proposed mechanism is supported by the observed attenuation of lipolysis by β_2-antagonism that was

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nearly equal to that achieved with β_3-antagonism in absolute measures, and surpassed that of β_3-antagonism when measured as % change from respective ISO controls (15% with β_2, 11% with β_3).

The lack of significant attenuation of ISO-stimulated lipolysis by β_1-and β_3-antagonism was unexpected. In addition to current knowledge of β-receptor density and function described above, it has been shown that over 90% of β-receptors present on the surface of rodent adipocytes are those of the β_3-subtype.[271] The dose of the β_3-antagonist SR59230A used in this study was the same dose used for parenteral administration in Wistar rats to elicit recovery of restraint-induced small intestinal ileus,[272] and in dogs to attenuate the high-dose ISO-induced rise in serum FFAs and glycerol.[270] While there are reports of SR59230A specificity for the β_3-receptor,[273,274] agonist activity of SR59230A at atypical β-receptors of the porcine gastrointestinal tract[275] and pleiotropic signaling at murine β_3-receptors[276] have subsequently been described. Thus, the lack of significant attenuation of ISO-stimulated lipolysis by β_3-antagonism in this study may be attributable to inadequate serum and tissue concentrations for this model, differences between in vitro and in vivo binding and functional effect, species/strain distinctions, pleiotropic signaling, and/or activity at atypical β-receptors.

As noted, the β_2-induced attenuation of lipolysis was similar to that achieved with β_3-antagonism in absolute measures, and surpassed β_3-mediated inhibition when measured as % change from respective ISO controls. Though not

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significant, rats exposed to β_2-antagonism had the greatest attenuation of the ISO-induced increase in femoral artery flow. Because β_2-receptors do not directly contribute to control of in vitro lipolysis in Sprague-Dawley rats,[68] it is possible that the effect on serum FFAs in mediated indirectly by this hemodynamic alteration. Administration of ISO to rats causes increased adipose blood flow in vivo, and associated augmentation of local adipose and serum glycerol concentrations.[277] Preservation of adipose blood flow prevents accumulation of FFAs derived from lipolysis, and preserves normal efflux of FFAs from adipocytes.[252] In contrast, in vivo studies in men have demonstrated inhibition of lipolysis by angiotensin II, possibly partly attributable to vasoconstriction and reduction in adipose blood flow with resultant local accumulation of FFAs.[278] Additionally, decreased blood flow may inhibit catecholamine-induced lipolysis by reducing distribution of the agent to the adipose tissue.[279] It is therefore possible that β_2-antagonism, by increasing resistance, reduced white adipose flow to the extent that indirect attenuation of lipolysis occurred.

As noted in the discussion of high-fat feeding and body weight (please see "Body weight", below), serum FFAs were unexpectedly unchanged by dietary treatment in untreated (baseline) animals. Proposed explanations for this observation have been outlined above. In contrast, serum FFAs were significantly elevated in the fat-fed rats that received placebo pellets when compared to normally-fed pelleted control animals. Thus, in this group of

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animals, high-fat dietary treatment effectively induced the desired increase in serum FFAs.

Unexpectedly, rats with chronic exposure to combined $\beta_1\beta_3$-antagonist pellets had significantly higher serum FFAs than placebo controls. This is in contrast to rats exposed to β-antagonists acutely. One explanation, described above, is the inhibition of lipolysis and FFA clearance from adipose that occurs with vasoconstriction. Though β_2-receptors are traditionally associated with vascular relaxation and were not blocked in these rats, there is evidence of regional β_3-mediated vasodilation in the rat and dog.[270,280] There is also evidence of atypical β-receptors in rat mesenteric arteries.[281] However, when compared to placebo controls, normal and fat-fed rats that received $\beta_1\beta_3$-antagonists did not have significantly different femoral artery flow, which suggests that hemodynamic alterations that did occur (i.e. increased HR, decreased resistance) were not sufficient to promote accumulation of substrate and inhibition of lipolysis. These study results also fail to lend support to the possibility of impaired NO-mediated dilation attributable to increased FFAs.[176]

The possibility of β-receptor upregulation with chronic β-antagonism, and subsequently enhanced lipolysis, exists. Though little can be extrapolated from murine transgenic models, it is worth noting that mice lacking β_3-receptors demonstrated upregulation of β_1-mRNA, but not β_2-mRNA.[282] Diabetic rats demonstrated a higher adipocyte β-receptor density that was unchanged by $\beta_1\beta_2$-antagonism with propranolol.[283] In contrast, propranolol induced a higher

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adipocyte β-receptor density in control animals. Investigation of the long-term effects of β-antagonism on β-receptor density and sensitivity is lacking.

While both β_1- and β_3-antagonist pellets were implanted in the study rats, the lack of attenuation of MAP, resistance, and HR is inconsistent with the reported effects of long-term β_1-antagonist treatment with atenolol in rats.[284] However, in acute studies, it was the response to ISO, not the baseline hemodynamic indices, that were predictably altered by β-antagonists. As discussed below, SR59230A pellets were found within encapsulated abscesses in 10 of the 18 rats treated with the active pellets. Postmortem examination revealed that the encased pellets had almost completely dissolved. While there was not evidence of incomplete absorption of the β_1-antagonist, it is possible that the SR59230A pellets were not fully absorbed, and provided only incomplete antagonism of adipose β_3-receptors. This may have allowed considerable β_3-mediated lipolysis, potentially augmented by β-receptor upregulation.

In humans and rats, treatment with the β-antagonist propranolol either decreased or did not change, respectively, LPL activity.[285,286] It is therefore unlikely that chronic $\beta_1\beta_3$-antagonism augmented serum FFAs through enhanced LPL activity and subsequent inhibition of FFA uptake into adipocytes. This is consistent with the finding that $\beta_1\beta_3$-antagonism did not alter serum TGs.

It is possible that the augmentation of serum FFAs with chronic $\beta_1\beta_3$-antagonism was due to activation of alternative lipolytic pathways. The adenylyl cyclase-PKA pathway recruited for β-mediated lipolysis is stimulated

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(adrenocorticotropic hormone, glucagons, thyroid stimulating hormone, parathyroid hormone) and inhibited (adenosine, prostaglandin, nicotinic acid) by other factors to alter lipolytic generation of FFAs.[59] Additionally, genetically altered mice lacking adipose β-receptors demonstrated increased lipolysis with fasting when compared to wild type mice,[60] suggesting that alternative pathways of intracellular SNS control of lipolysis exist. Moreover, there is evidence of a β-mediated, PKA-independent lipolytic cascade characterized by activation of the ERK/MAP kinase pathway.[61] The extent of alternative lipolytic systems, and the degree to which compensatory upregulation in response to β-inhibition may occur, remains to be elucidated.

The most likely explanation for the observed increase in serum FFAs with chronic $\beta_1\beta_3$-antagonism involves the relationship between β-adrenergic receptors and brown fat thermogenesis. In response to stimulation of β_1- and β_3-receptors in brown adipose, there is an increase in local blood flow and enhanced FFA uptake. These fatty acids serve as the primary substrate for mitochondrial oxidative metabolism, which maintains ATP stores in the face of increased demand.[58,287] Administration of the potent antilipolytic β_3-agonist CL316243 to obese Zucker rats decreased body weight and white adipose tissue mass, consistent with the expected effects of enhanced lipolysis in the white adipose.[88] However, a paradoxical reduction in serum FFAs was observed, and attributed to the profound increase in fatty acid oxidation in the brown adipose.[88] Administration of CL316243 to obese rats resulted in hypertrophy of brown fat,

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accompanied by increased factors associated with fatty acid oxidation.[288] Treated rats had higher resting metabolic rates and overall energy expenditure. In contrast, administration of the β-antagonist propranolol to dogs with heart failure was associated with decreased fatty acid transport into myocardial mitochondria and reduced fatty acid oxidation.[289] Consistent with these observations, chronic exposure to $\beta_1\beta_3$-antagonists may have induced inhibition of white adipose lipolysis, accompanied by a more profound inhibition of fatty acid uptake and oxidation in the brown adipose. The net result would be consistent with the observed increase in serum FFAs.

Glycerol

Serum glycerol concentrations have been used, together with FFA values, to estimate whole body lipolytic activity,[260,290] and significant correlation between serum FFA and glycerol has been observed.[260] Glycerol, unlike FFAs, does not have the potential for reuptake by adipocytes after release, but rather is used as a precursor for gluconeogenesis in the liver.[291] During glycolysis, only a small fraction of plasma glycerol comes from blood glucose, further providing evidence for its valid use as a measure of body lipolysis.[292] Underestimation of lipolysis by glycerol measurement may occur when the glycerol released does not enter the systemic circulation, as occurs with lipolysis within visceral adipocytes.[293] For this reason, measuring local glycerol release by adipose microdialysis or the rate of glycerol appearance in the systemic circulation, is often utilized.[292-294]

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Because of cost and equipment limitations, serum glycerol measurements were obtained as an estimate of whole body lipolysis. There was a weak positive correlation between glycerol and FFA concentrations. Treatment with ISO failed to augment serum glycerol, while FFAs were significantly elevated. This may reflect enhanced visceral lipolysis, with subsequent uptake of glycerol into the liver.[293] Rats treated with β-antagonists, especially the β_3-antagonist, had higher serum glycerol concentrations than those that received ISO with saline or DMSO only. As noted, rats treated with ISO + β-antagonists had similar serum FFAs when compared to the ISO + NS/DMSO groups. Administration of propranolol has been reported to inhibit compensatory hepatic glucose production during hypoglycemia;[295] however, propranolol has also been associated with an increased delivery of blood to the liver (as % cardiac output).[296] Given these seemingly opposing study results, it is difficult to predict whether impaired hepatic blood flow or changes in gluconeogenesis contributed to the increase in serum glycerol.

Serum glycerol concentrations were increased in rats implanted with placebo pellets, a pattern similar to that seen with serum FFAs. Unlike FFAs, glycerol values were not increased with chronic $\beta_1\beta_3$-antagonism. This may reflect basal lipolysis of visceral adipocytes with subsequent β-mediated inhibition of FFA uptake for oxidation, but without knowing the effect of chronic β-antagonism on hepatic artery flow and gluconeogenesis, it is difficult to predict this with assurance. Given the weak correlation with serum FFA and the

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unknown fate of both FFAs and glycerol in this study, it is acknowledged that local measurement of adipose glycerol and FFA efflux would have improved the understanding of lipolytic generation and subsequent fate of these substrates.

Body weight

In preliminary studies, rats fed the high-fat diet for 16 weeks had increased body weight. This effect of high-fat dietary treatment was not observed in untreated dietary control animals. However, when dietary treatment was applied to rats with implanted SC placebo pellets, an increased body weight was observed in fat-fed animals. This blunting of the effects of high-fat feeding that was observed in the group of untreated animals, compared to placebo pelleted animals, is unlikely to be caused by pellet-induced inflammation as serum CRP, IL-6, and TNF-α were higher in normally fed animals. The increased body weight and lack of inappetence or malaise in pelleted animals further suggests that pelleting alone did not contribute to the observed differences. Finally, serum FFAs, glycerol, TGs, cholesterol, and leptin also followed this pattern of attenuation in fat-fed untreated animals when compared to the fat-fed placebo group. The difference is most likely due to differences in age and time of dietary treatment. Though all animals were adults, rats in the normally fed, untreated group were approximately 14 days younger than fat-fed counterparts. Additionally, all untreated animals were 30 days younger and had 30 days less dietary intervention than pelleted rats. Because of this, it would seem prudent to

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view placebo-pelleted rats as the control animals in chronic studies of metabolic parameters.

Both normal and fat-fed rats that received $\beta_1\beta_3$-antagonist treatment had higher body weights than placebo control animals. This is consistent with the observation that long-term use of β-antagonists for hypertension in humans causes increased body weight, with the majority of gain occurring in the first months of treatment.[240] This has been attributed to a decrease in resting metabolic rate,[242] increased fatigue and diminished anxiety which reduces baseline thermogenesis,[240] and decreased post-meal thermogenesis.[297] The direct effect of β-antagonism on inhibition of lipolysis is also considered a potential mechanism by which these drugs promote weight gain.[240]

Glucose

Because endothelial dysfunction can occur as a result of hyperglycemia, and β-antagonists contribute to insulin resistance, serum glucose was measured in the hope that glucose might be differentiated from FFAs as contributors to any changes to endothelium-dependent dilation. Acute β_2-antagonism increased serum glucose when compared to ISO + NS rats. This is unlikely to have occurred due to changes in substrate delivery and tissue uptake, as flow and resistance measurements were not significantly altered by β_2-antagonism. High fat feeding and chronic $\beta_1\beta_3$-antagonism both increased serum glucose levels, but these changes were not significant. Others have reported a lack of change in

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glucose in rats with obesity induced by 12-16 weeks of high-energy or high-fat feeding,[216,298] thus, the findings of this study are consistent with published reports. Given this, hyperglycemia was eliminated as a remarkable contributor to changes in endothelium-dependent vasodilation that were measured in chronically treated animals.

Triglycerides

There is controversy as to which moiety, FFAs or the parent TG, contributes most significantly to endothelial dysfunction in obese individuals. Multiple investigators have attributed endothelial dilator dysfunction to elevated serum TGs,[217-219] while others have observed correlations with co-existing metabolic aberrancies such as insulin resistance, including elevated FFAs.[220] Hypertriglyceridemic patients with impaired LPL activity do not demonstrate attenuated endothelial dilator function suggesting that it is the product of TG hydrolysis (i.e. FFAs), and not the TGs per se, that contribute to the endothelial dysfunction in these patients.[221] Here, both lipid moieties were evaluated so that the relative change and correlation with endothelial function might be differentiated.

Serum TGs were reduced by ISO treatment. This, together with significantly increased serum FFAs, is consistent with enhanced lipolysis. Rats treated with the β_3-antagonist SR59230A + ISO had the highest TG values when compared to those that received ISO + DMSO, and this difference was significant

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when β_1- and β_3-antagonists were given concomitantly. The fact that the negative correlation between FFAs and TGs was lost with β-antagonist treatment points to the possibilities of incomplete inhibition of ISO-stimulated lipolysis by β-antagonism, and post-lipolytic diversion of FFAs (i.e. to brown and white adipose as substrate for oxidation within mitochondria).

High-fat feeding increased serum TGs in placebo-pelleted rats, and chronic $\beta_1\beta_3$-antagonism did not increase TG concentrations. The increase in TG values observed in response to high-fat feeding was statistically more significant than the rise in serum FFAs, which may indicate that LPL is not fully functional. This observation is consistent with reports that adipose LPL is resistant to postprandial insulin-stimulated activity in obese rats,[299] and that high-fat feeding reduces skeletal muscle LPL protein and reduces TG clearance in the fasting rat.[300] The moderate rise in serum TGs, compared to the significant increase in FFAs, in response to $\beta_1\beta_3$-antagonism lends support to the idea that chronic β-antagonist treatment may inhibit oxidation (use) of FFAs out of proportion to lipolytic generation of FFAs.

Lipid profile

Serum cholesterol was reduced by acute β_2-antagonism. Cholesterol is the sum of LDL, HDL, and VLDL (VLDL = 20% TGs). Serum HDL and VLDL/TGs were unchanged. LDL values were deemed invalid for use in this study due to calculated negative values. Given that β_2-antagonism did not reduce serum

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FFAs, it seems unlikely that the decreased cholesterol reflects a reduction in FFA substrate to the liver. While there are reports of favorable metabolic sequelae to β_2-receptor agonism,[301,302] the role of the receptor in human lipolysis prevents the generalization of human data to animals in this study. High-fat feeding resulted in higher serum cholesterol values in pelleted rats, but the increase was not significant when compared to pelleted dietary controls. Given the incomplete knowledge of β-receptor mediation of rat lipolysis, the invalid LDL values partially derived from cholesterol levels, and the more prominent role of TGs and FFAs in endothelial dysfunction, it would seem prudent to avoid speculation and eliminate this data from the analysis.

Leptin

Leptin secretion by rat white adipose is inhibited by β-receptor stimulation.[105] Overweight rats have increased leptin secretion from adipocytes that is attenuated by treating the animals with a β_3-agonist.[106] In the present study, serum leptin concentrations were unchanged by ISO. In humans given ISO by continuous infusion, plasma leptin levels were reduced, but only after 30+ minutes of exposure.[303] This suggests that the 15-minute period between ISO administration and sample procurement in the present study was likely an insufficient amount of time for changes in leptin levels to be observed. Neither acute ISO + β-antagonist treatment nor chronic $\beta_1\beta_3$-antagonism altered leptin concentrations. Reduced ob gene expression occurs concomitantly with β_3-

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mediated reduction in plasma leptin,[107,108] an effect measured by 4 hours in lean mice. Extrapolating to the use of antagonists, it is possible that leptin levels remained unchanged in these acute studies due to inadequate time for the effects of altered leptin gene expression to be manifested in serum concentrations. Patients with essential hypertension and overweight individuals with hypertension and dyslipidemia had lower plasma leptin after 1 month and 12 months, respectively, of treatment with β-antagonists.[304,305] In contrast, cachectic patients with chronic heart failure exhibited a greater increase in plasma leptin with 6 months of β-antagonist treatment when compared to noncachectic controls.[306] This suggests that underlying pathology and body composition may modulate the effect of chronic β-antagonists on leptin concentrations. It is likely that any modest effects of chronic $\beta_1\beta_3$-antagonism were minimized by the large increase in leptin attributable to the dietary-induced obesity.

High-fat dietary treatment was associated with elevated leptin values in both untreated (baseline) and placebo-pelleted controls; absolute concentrations and percent change were more profound in pelleted animals (see "Body weight" for related discussion). There was a moderate positive correlation between body weight and serum leptin. These data are consistent with the observation that serum leptin concentrations are proportional to adipose mass, and that obese humans have higher serum leptin concentrations.[100] This suggests that obesity is characterized by leptin resistance,[100] a phenomenon that is widely accepted and the subject of much investigation.[101]

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EFFECTS OF ISOPROTERENOL AND ACUTE β-ANTAGONISM ON HEMODYNAMIC INDICES AND ENDOTHELIUM-DEPENDENT VASODILATION

Hemodynamic indices

Baseline MAP, flow, and resistance were unchanged by treatment with any of the β-antagonists in normal rats prior to ISO administration. Rats treated with the β_3-antagonist SR59230A had higher HRs than control animals. This finding is unexpected, as β_3-agonism causes NO-dependent vasorelaxation in rat aortic rings.[280] Further, dogs given high doses of ISO displayed increased HR and decreased MAP values that were abolished with SR59230A.[270] Because MAP and flow were not different relative to rats that received other β-antagonist treatments, the idea of a compensatory elevation in HR lacks validity. The possibility exists that the β_3-mediated effect on HR in these rats is attributable to yet unknown β_3-receptor activity in the rat SA node or alternative vascular beds, but given the defined role of β_1-antagonism in reducing heart rate[307] that was not observed in these rats, this is unlikely.

In contrast to baseline values, there were differences in response to ISO that were consistent with the known hemodynamic effects of specific β-antagonists, suggesting that in vivo concentrations of drugs were adequate to achieve systemic effects. Treatment with ISO had the anticipated effect of decreasing MAP and increasing flow, thus lowering resistance. Rats also had marked increases in HR after ISO treatment. The rats exhibited these

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hemodynamic responses within seconds of ISO administration SC. Rats premedicated with the β_1-atagonist atenolol, whether alone or in combination with β_3-antagonism, had significant attenuation of the ISO-induced increase in HR, consistent with known β_1-receptor density in the SA node. Though not statistically significant, rats premedicated with the β_2-antagonist butoxamine had the lowest flow, and highest resistance measured among treated rats, consistent with the role of the β_2-receptor agonism in peripheral dilation.

Flow-time integral

There were no differences in FTI attributable to ISO despite increased serum FFAs in ISO-stimulated rats. β-antagonist treatments did not alter FTI when compared to respective control animals. Rats that received DMSO + ISO had greater FTI than NS control animals, but the response was not significantly greater than that of the NS + ISO rats, the more appropriate comparison group. Though DMSO has been reported to have antioxidant properties,[308] and oxidative stress contributes to endothelial dysfunction, this effect is unlikely to have been significant in this study. DMSO + ISO treatment did not significantly alter serum glucose, TG, cholesterol, or leptin values.

These acute studies reveal that FTI was unchanged or increased by ISO treatment, and this occurred despite elevated serum FFAs. Additionally, no significant change in FTI or serum FFAs was associated with β-antagonist treatment. Neither the hyperglycemia present with β_2-antagonism, nor the

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hypertriglyceridemia observed with combined $\beta_1\beta_3$-antagonism, altered the FTI in these normal animals. The FTI was also unchanged by the lowered serum cholesterol measured in response to β_2-antagonism. Characteristic hemodynamic responses to the activity of ISO and β-antagonists on cardiovascular receptors likely were of sufficient magnitude that any alterations attributable to metabolic parameters were not discernable. While the acute studies were helpful in defining trends in serum FFAs in response to β-receptor modulation, FTI and hemodynamic data reflect known cardiovascular effects of β-antagonism, rather than changes in endothelial dilator function attributable to metabolic alterations. Given that the aim of these studies was to investigate the role of β-antagonists in altering endothelial function through changes in serum FFAs, and that chronic β-antagonism is more physiologically relevant, it would seem reasonable to extract lipid and glucose data from these acute studies, and use the chronic model for investigation of endothelium-dependent dilation.

EFFECTS OF HIGH-FAT DIET AND CHRONIC $\beta_1\beta_3$-ANTAGONISM ON HEMODYNAMIC INDICES AND ENDOTHELIUM-DEPENDENT VASODILATION

Hemodynamic indices

The effects of high-fat feeding and pelleting (i.e. placebo and $\beta_1\beta_3$-antagonists) on baseline parameters revealed no differences attributable to high-fat feeding or placebo pellet implantation. Rats treated with chronic $\beta_1\beta_3$-

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antagonists had lower resistance and higher HRs than placebo-pelleted controls, and these differences were significant in the fat-fed group. The elevated HR with β_3-antagonism is consistent with trends observed in acute, normal animals, and is not explicable given current knowledge of rat cardiovascular β_3-receptors. Unexpectedly, the β_1-antagonist atenolol did not lower HR. In acute studies, however, baseline parameters were not different, though the doses of β-antagonists were sufficient to impact the response to ISO in a predictable way. This suggests that the β-antagonist may still exert a physiologically relevant effect without changing baseline hemodynamic parameters. The lower resistance calculated in rats treated with chronic $\beta_1\beta_3$-antagonists is primarily due to increased flow measured in these animals. Though the augmented flow was not significant, it is possible that, with increased sample size, $\beta_1\beta_3$-antagonism might emerge as a treatment that improves baseline flow, thereby reducing resistance, in fat-fed rats.

Flow-time integral

Unexpectedly, high-fat feeding did not alter the FTI in response to Ach. It is possible that, if elevated serum FFAs are accepted to be a contributor to endothelial dysfunction in obesity, impaired endothelium-dependent vasodilation may be present in the postprandial, but not the fasting state. Many theories have been put forth regarding the etiology of obesity-induced elevations in serum FFAs. These include greater β_3- and cAMP-mediated lipolysis,[86] insulin

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resistance,[91,309,310] and impaired uptake of FFAs by the adipocytes.[311] Because of evidence pointing to inappropriate lipolysis in both the fasting and postprandial state,[28,87,311] it is likely that multiple mechanisms contribute to elevated FFAs in obesity. If the argument for impaired adipocyte β-receptor responsiveness[77-80] is accepted, the postprandial elevation in FFAs, attributable to insulin resistance and impaired FFA trapping, may be more prominent than that observed during fasting, and less affected by β-adrenergic tone. In this study, measurement of serum FFAS after food intake may have revealed a more profound rise in serum FFAs, and association with impaired endothelium-dependent vasodilation. Because serum TGs and glucose may also be elevated postprandially, these moieties would also need to be considered as contributors to any endothelial dysfunction measured during this time.

The possibility of compensatory augmentation in EDHF activity in response to impaired NO-mediated vasodilation was considered as a cause of similar FTI responses in normal and fat-fed rats. Studies have validated the idea that EDHF serves as a "back-up" in states of reduced NO activity, such as hypercholesterolemia and hypertension.[143,147,148] The portion of the vasodilatory response attributable to NO is considered to be that which is abolished by administration of the NOS inhibitor L-NAME. To quantify this and compare the relative NO-mediated response among groups, both FTI in response to Ach, and baseline resistance were analyzed before and after L-NAME. Neither high-fat feeding nor chronic $\beta_1\beta_3$-antagonism induced change in the mean difference of

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FTI response pre- and post-L-NAME. However, both normal and fat-fed rats that received $\beta_1\beta_3$-antagonist treatment had a greater FTI response attributable to NO activity when compared to placebo-pelleted controls. It is possible, with larger sample size, the trend of β-antagonist-induced augmentation of NO-mediated dilation might prove to be significant.

In the same way, baseline vascular resistance was calculated before and after L-NAME administration so that the change in vascular tone attributable to NO might be approximated. In contrast to the aforementioned FTI response, the change in resistance attributable to NOS inhibition was reduced in rats treated with $\beta_1\beta_3$-antagonists, though this difference was not significant. Together, these data support the idea that different mechanisms mediate baseline tone versus the endothelium-dependent response to agonists. Given the consistent trends in the data, it is possible that larger sample sizes would add power to the analysis and reveal a significant difference in the contribution of NO-mediated vasodilation in the baseline and stimulated states. Additionally, the small- and intermediate-size K^+ channel inhibitors apamin and charybdotoxin, established inhibitors of EDHF activity, could be incorporated into the study design to further quantitate the contribution of EDHF to endothelium-dependent vasodilation, and reveal the magnitude of residual dilatory activity that might be attributable to yet another factor. Lastly, it is possible that a greater concentration of or increased sensitivity to vasoconstrictors, rather than a paucity of vasodilators, characterizes the defect in endothelium-dependent dilation in this model. In obese humans, endothelin

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contributes to increased basal vascular tone, as well as the attenuated dilator response to agonists.[312] Use of isolated vessel rings or perfused vessel segments would assist in identifying this mechanism, as the constrictor response to endothelin or phenylephrine could be quantitated without the deleterious side effects of in vivo constrictor administration encountered by this investigator. There were no differences in the change in baseline resistance prior to and after receipt of the NO donor nipride, when compared among the 4 chronic treatment groups. This suggests that the portion of baseline resistance attributable to endothelium-independent vasodilation did not change as a result of dietary or $\beta_1\beta_3$-antagonist treatment.

While the described analysis of pre-and post-L-NAME FTI and resistance do not assist in elucidating the lack of effect of high-fat feeding on FTI response to Ach, it is evident that chronic treatment with $\beta_1\beta_3$-antagonists may induce changes in the NO:EDHF ratio. These trends lend credibility to the observation that FTI response to Ach was increased, though insignificantly, in animals exposed to chronic $\beta_1\beta_3$-antagonism. Of all the measured serum parameters, FFAs are the only metabolic moiety that reflected the same pattern of response to $\beta_1\beta_3$-antagonism in both normal and fat-fed animals. Unexpectedly, the relationship between the insignificant improvement in FTI and the significant rise in serum FFAs is a positive correlation. A conservative interpretation of this data would suggest that endothelium-dependent vasodilation is not impaired by the elevated serum FFAs present in rats treated with $\beta_1\beta_3$-antagonists. A bolder

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analysis is that chronic $\beta_1\beta_3$-antagonism may improve endothelium-dependent vasodilation in the face of elevated serum FFAs, and this would be observed as a significantly improved FTI if sample sizes were larger and study power strengthened. Regardless, one must consider whether elevated serum FFAs did not attenuate endothelial function in these animals, or whether FFAs had the potential to inhibit dilator function, but that β-antagonist treatment protected the endothelium from deleterious sequelae. Given that numerous studies have linked serum FFAs with endothelial dysfunction, it is proposed that the latter theory is correct, and that $\beta_1\beta_3$-antagonism sustains or even improves the endothelium-dependent flow response by means other than reduced serum FFAs.

In contrast to known correlations of dietary obesity with ex vivo endothelial dysfunction in rats,[216] rats that received chronic $\beta_1\beta_3$-antagonist treatment exhibited the aforementioned preservation of in vivo endothelium-dependent vasodilation in the face of increased body weight. It is known that in humans, the amount of visceral, relative to subcutaneous, adipose is more relevant in predicting the presence of cardiovascular risk factors and endothelial dysfunction than overall body weight.[1,113-115,313] Less is known about the contribution of visceral:subcutaneous ratio in the rat; however, given that the proposed pathology of visceral adipose is likely consistent among species (i.e. increased FFAs directed to the liver via the portal circulation[123]), it is expected that body fat distribution is relevant in rodents. The study could have been strengthened by measuring fat pad masses, so it could be determined whether the fat-fed rats had

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a diffuse increase in adipose mass, or whether the visceral:subcutaneous ratio was altered.

Summary

In relation to Aim 1 of this study, acute β-antagonism appears to minimally inhibit the ISO-induced rise in serum FFAs, and the direct effects of β-antagonists on cardiovascular β-receptors inhibit discernment of any FFA-mediated alteration in endothelium-dependent vasodilation. Though no single β-antagonist reduced serum FFAs in ISO-stimulated rats, the combination of $\beta_1\beta_3$-antagonism was chosen for chronic studies based on known rat adipose β-adrenergic receptor density, and the effect of this combination in best attenuating the rise in serum FFAs without suspected change in adipose or skeletal muscle tissue flow. Rats administered chronic $\beta_1\beta_3$-antagonist treatment had paradoxically higher serum FFAs, and a trend toward improved endothelium-dependent vasodilation. The former phenomenon might best be explained by considering the concomitant attenuation of white adipose lipolysis and more profound inhibition of brown adipose fatty acid uptake and oxidation, favoring a net increase in serum FFAs. Additionally, activation of, or compensation by, alternative lipolytic pathways may have contributed to enhanced lipolysis in the face of β-antagonism. The lack of significant change in FTI in response to high-fat feeding or chronic $\beta_1\beta_3$-antagonism may be attributable to insufficient power due to low sample size for these in vivo flow studies, or alterations in the

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NO:EDHF ratio that maintained quantitatively similar overall FTI responses. Regardless of the mechanism, rats that received chronic $\beta_1\beta_3$-antagonist treatment had a preserved FTI response in the face of elevated serum FFAs. This suggests that β-antagonism confers protection from FFA-mediated endothelial dysfunction, or enhances endothelium-dependent vasodilation, by means other than a reduction in serum FFAs.

EFFECTS OF HIGH-FAT DIET, ISOPROTERENOL AND β-ANTAGONISM ON INFLAMMATION AND OXIDATIVE STATE

Inflammation

Inflammatory cytokines, including CRP, IL-6 and TNF-α are elevated in overweight and obese individuals.[11-13] Specifically, elevated FFAs contribute to inflammation in obesity, being highly associated with increased IL-6, TNF-α, and CRP.[183] The mechanism of FFA-induced inflammation may be partly due to protein emulsification by FFA detergent activity.[10] Additionally, the induced cytokines can be toxic to the endothelium. CRP induces expression of monocyte chemoattractant peptide,[184] and increases expression of adhesion molecules in human endothelial cells.[185] The cytokine IL-6 increases endothelial permeability in cultured monolayers.[22] Elevated serum FFAs further contribute to inflammation by activating the transcription factor NF-κB, which promotes the expression of a number of genes for chemokines, adhesion molecules, and immune receptors.[24] In endothelial and vascular smooth muscle cells, impaired NO activity has also

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been attributed to CRP.[23,186,187] Elevated serum CRP, IL-6, and TNF-α have been correlated with increased risk for cardiovascular events in healthy individuals and those with existing atherosclerosis.[15,19,20] These data suggest that inflammation may be a principle mechanism by which FFAs contribute to endothelial dysfunction in obesity. To investigate this idea, serum markers of inflammation were measured and correlated with serum FFAs and endothelial dysfunction.

Treatment with ISO did not alter serum TNF-α. This is inconsistent with the effect of ISO in augmenting human adipose TNF-α measured by microperfusion.[294] The augmented cytokine concentration was measured in the adipose one hour after ISO perfusion, however, so it is possible that procuring a serum sample 15 minutes after ISO administration introduced dilution and temporal differences such that elevations in serum TNF-α were not detectable. Consistent with published data is the observation that serum IL-6 remained unchanged by ISO. In response to injury, circulating CRP increases over a period of hours,[314,315] thus it was not surprising that in this study, CRP levels were unchanged by acute interventions.

Rats given ISO after treatment with β_1- and β_2-antagonists showed a rise in serum TNF-α and IL-6. This trend was dissimilar to that of serum FFAs or any other metabolic moiety, suggesting that the β-antagonists did not modulate cytokine levels indirectly by altering metabolic factors. Data published on the association of β-receptor modulation and inflammatory cytokines describe an anti-inflammatory effect of β-agonism, especially at the β_2-receptor.[316-318] The anti-

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inflammatory properties, however, are defined as attenuation of a proinflammatory stimulus such as endotoxemia or IgE stimulation of mast cells. There is little information on the effects of β-receptor modulation on cytokines in the baseline state. While the concomitant increase in TNF-α and IL-6 with β-antagonism might be anticipated given the effects of β-agonism in reducing cytokine concentrations, much remains to be elucidated regarding baseline versus stimulated state, β-receptor specificity, in vivo effects, and agonism versus antagonism.

Due to the inconsistencies in age and duration of dietary treatment between untreated (baseline) and placebo-pelleted rats already outlined, it would seem prudent to view placebo pelleted rats as the control animals in chronic studies of inflammatory and oxidative parameters. Data from the baseline group will be described, however, so that effects of placebo pellet implantation alone might be approximated.

Rats fed the high-fat diet did not have elevated CRP, Il-6, or TNF-α, and in most cases, fat-fed animals had lower concentrations. This is unexpected given the aforementioned effects of obesity on inflammatory cytokines in humans. Additionally, rats with obesity from varied etiologies display upregulation of TNF-α mRNA in adipose, increased TNF-α protein in tissues, and greater TNF-α serum concentrations.[319,320] It is not clear whether postprandial sampling may have produced different results. It is unlikely that the lack of change in serum TNF-α is due to the unaltered FFAs measured in response to high-fat dietary

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treatment, as the two indices diverge with chronic $\beta_1\beta_3$-antagonist treatment. No other metabolic parameters measured reflected a pattern similar to that of the inflammatory cytokines.

Inflammatory cytokine concentrations were higher in normal and fat-fed rats with placebo pellet implantation when compared to untreated control rats (with the exception of CRP in fat-fed animals). In all cases, this response was greater in normally fed animals. Placebo pellets were implanted in normal and fat-fed rats within the same 4-hour period, and rats from the two dietary groups were alternated. The pellet matrix was guaranteed inert, and the pellets inserted using cold sterilization and clean technique. Despite this, well-encapsulated sterile abscesses developed in normal and fat-fed animals, principally associated with the SR59230A pellet. Interestingly, only one rat with placebo pellet implantation developed such a lesion, and it was small when compared to those observed in the SR59230A-pelleted group. The abscesses were not correlated with increased serum inflammatory cytokines. Thus, the increased cytokine concentrations in placebo-pelleted rats are inconsistent with physical observations. It is possible that pellet implantation increased inflammatory cytokines in all rats, but that the well-encapsulated abscesses in rats implanted with β-antagonist pellets confined the inflammatory focus so that systemic indices were not altered. This would appear unlikely given the absence of malaise or any observed local tissue reaction in the majority of rats with placebo pellets.

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Regardless of the etiology of the increased inflammatory cytokines with placebo pellet implantation, normal rats treated with chronic $\beta_1\beta_3$-antagonists had lower cytokine concentrations. Fat-fed rats exhibited a strong, similar trend, though only the reduced CRP was statistically significant. The apparent blunted effect in fat-fed rats may be attributable to the lower mean cytokine concentrations in pelleted animals (i.e. a lower absolute value would be required to achieve statistical significance). Two explanations for the observed reduction in cytokine values with chronic $\beta_1\beta_3$-antagonism are offered. First, it is possible that the $\beta_1\beta_3$-antagonist pellets incited a less profound inflammatory response than that of the placebo pellets. This is unlikely given the local tissue abscessation associated with the β_3-pellets but must be considered given the elevated inflammatory cytokines in the rats that received placebo pellets. The alternative explanation is that chronic $\beta_1\beta_3$-antagonism is anti-inflammatory. This is consistent with a report of reduced TNF-α gene expression and protein production in infarcted rat myocardium after metoprolol treatment,[321] and with the association of β-antagonism and reduced CRP concentrations in patients with angina and coronary artery disease.[50] Treatment with β-antagonists has also been observed to reduce CRP and TNF-α in humans with cardiovascular disease.[50,322] If this latter theory is accepted, then the reduction in concentrations of inflammatory cytokines with $\beta_1\beta_3$-antagonist treatment occurs in the face of elevated serum FFAs. This view is supported by the observed lack of positive correlation of FFAs with any of the inflammatory markers. Thus, if $\beta_1\beta_3$-

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antagonism attenuates inflammation, it does not exert this effect through reduced serum FFAs.

Oxidative stress

Markers of systemic oxidative stress are positively correlated to waist-to-hip ratio.[189] Serum FFAs are elevated in individuals with increased waist-to-hip ratio, and induce oxidative stress.[24,191] Elevated serum FFAs promote acute and chronic endothelial vasodilator dysfunction,[3-6] and antioxidants attenuate endothelial dysfunction attributable to high-fat feeding in humans and rodents.[26,27] Elevated serum FFAs promote a pro-oxidant state through generation of ROS and depletion of endogenous antioxidants.[24] Excess ROS directly damage proteins, lipids, and DNA. Additionally, accumulated superoxide reacts with NO to form peroxynitrite, thereby depleting NO and interfering with NO-mediated endothelial functions.[7,8,25] Elevated CRP promotes expression of iNOS RNA, which is postulated to favor peroxynitrite formation and increased oxidative stress.[184] Another mechanism of FFA- and ROS-mediated oxidant endothelial dysfunction is activation of the transcription factor NF-κB, the implications of which have been described. Finally, FFAs induce insulin resistance and hyperglycemia. Elevated blood glucose increases oxidative stress and contributes to endothelial dysfunction.[213-215] These observations suggest that endothelial dilator dysfunction in obese individuals may partly be attributable to the direct and indirect pro-oxidant effects of circulating FFAs.

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Serum TBARS have been used as a standard by which to quantitate systemic oxidative stress in the rat.[323] In these acute studies, rats treated with DMSO + ISO had lower serum TBARS than rats treated with NS + ISO, suggesting that DMSO may indeed have inherent antioxidant properties in these rats. Treatment with β-antagonists resulted in similar absolute serum TBARS concentrations; however, the values were significantly reduced only in the group that received β_2-antagonist treatment (compared to the NS + ISO control). Because of the reduced TBARS in the rats treated with DMSO + ISO, the values measured in β_1- and/or β_3-antagonist treated rats were not different from this control. Due to similar TBARS concentrations in rats treated with NS only and those given NS + ISO, it does not appear that ISO increased TBARS, but that DMSO and β-antagonist treatment may reduce the constituitive oxidative stress in these rats. For these acute data to be valid, the serum concentrations of TBARS must be amenable to short-term fluctuation. This requisite rapid change in values would not be inconsistent with published reports.[324] The direction and magnitude of TBARS change were not reflective of alterations measured in serum FFAs, glucose, TGs, or leptin. These data suggest there is the potential for inherent antioxidant properties of β-antagonists, unrelated to any reduction in the pro-oxidant metabolic factors measured in this study.

Serum TBARS were not altered by high-fat feeding. This is in contrast to the known association between waist-to-hip ratio and markers of systemic oxidative stress.[189] It is possible that TBARS would have been significantly

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elevated in the postprandial state, when FFAs, glucose and TGs would be elevated and possibly exerting a pro-oxidant influence. Additionally, without measuring fat pad mass, the relative increase in visceral adipose was unknown, thus the predictive waist-to-hip ratio impossible to ascertain. It is conceivable that these rats are resistant to developing the large visceral-to-subcutaneous ratio that characterizes at-risk humans, or that the rat has adapted to the increased flux of FFAs to the liver, so that serum FFAs and other metabolic factors do not have the magnitude of deleterious effects observed in humans.

In normally-fed rats, TBARS were increased by placebo pellet implantation; however, placebo pellets did not markedly increase TBARS in fat-fed rats. The enhanced response in normal rats was similar to that observed in serum inflammatory markers. This is not unexpected, as oxidative injury directly damages proteins, lipids, and DNA. The lack of association between inflammatory indices and physical examination findings has been reviewed in the previous section. There is no evidence that the implantation procedure itself was different between the groups. It is therefore reasonable to consider that the normal rats may have had a qualitatively different response to the implanted foreign body. Aberrant immune function, characterized by abnormal leukocyte cell surface markers, has been observed in morbidly obese humans.[325] Given the complex interplay between cell-specific immune interactions and liberation of inflammatory cytokines, as well as the preexisting chronic inflammatory state of obese individuals, this observation must remain speculative at this time. Further

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studies investigating the inflammatory response to inert synthetic implants in normal and obese rats would verify the validity of the observed difference in serum markers.

Both normal and fat-fed rats that received chronic $\beta_1\beta_3$-antagonist treatment had elevated serum TBARS when compared to untreated baseline controls. The increased concentrations were significant compared to placebo-pelleted rats only in the fat-fed group, and the absolute value was markedly higher in these rats. The similar trends in inflammatory and oxidative markers observed between normal and fat-fed rats with placebo pellet implantation, then, diverge at this point when rats given $\beta_1\beta_3$-antagonists have higher TBARS and simultaneously reduced inflammatory markers. Correlation analyses of TBARS with pro-oxidant metabolic markers revealed similar significant, but weak positive correlations between TBARS and FFAs, glucose, TGs, and leptin. Though serum TGs were most closely correlated with TBARS (Pearson $r = 0.69$), it would not be valid to attempt to assign a principle effect of any single moiety.

In addition to the systemic oxidative state, femoral artery oxidative stress was described using nitrotyrosine immunohistochemistry localization, and slot blot analysis for quantitation of nitrotyrosine residues. Localization of nitrotyrosine primarily to the endothelium, and also to the vascular media, is consistent with published reports.[326,327] Slot blot analysis revealed increased arterial nitrotyrosine in both normal and fat-fed rats treated with β-antagonists, with the strongest signal derived from rats that received both dietary and β-antagonist treatment.

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These observations are consistent with trends in serum TBARS. This suggests that both systemic and arterial oxidative stress may be augmented with β-antagonist treatment, and that the effect is more profound when paired with high-fat dietary intake. It should be noted, however, that these results of the slot blot analysis were achieved only with use of high (20 μg) protein concentrations. These findings were not repeatable with use of lower (5 μg) concentrations. It is also important to consider the distribution of nitrotyrosine residues within the arteries as revealed by immunohistochemistry. Staining was most intense in the endothelium, which comprises a very small percentage (< 5%) of arterial tissue. The inconsistent results of nitrotyrosine quantitation observed with the smaller protein concentration may, then, be attributable to insufficient test sensitivity in detecting very small changes in total nitrosylated residues. Quantitation of nitrotyrosine, superoxide, or NADPH oxidase in cultured endothelial cells would more specifically identify oxidative changes in this cell population.

Because of the questions raised by slot blot quantitation of nitrotyrosine, it is not possible to correlate the preservation of endothelial dilator function with estimated arterial peroxynitrite concentrations in β-antagonist-treated rats. If arterial nitrotyrosine is indeed elevated with β-antagonism, it is possible that an NO-independent mechanism of endothelium-dependent vasodilation (i.e. EDHF) compensates for the depletion of NO by peroxynitrite, a phenomenon previously described.

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Summary

Rats treated with chronic $\beta_1\beta_3$-antagonists had preserved endothelium-dependent vasodilation in the face of increased serum FFAs and TBARS. This suggests that serum FFAs may principally exert pathology through oxidative, rather than inflammatory, pathways and that chronic $\beta_1\beta_3$-antagonism does not exert vascular protective effects through attenuation of FFA-mediated oxidative stress.

CONCLUSION

Investigation of the acute effects of selective β-receptor antagonism revealed minimal attenuation of the ISO-induced rise in serum FFAs. Rats treated with the β_2-antagonist butoxamine had the most marked reduction in serum FFAs when compared to the appropriate control group; however, use of combined $\beta_1\beta_3$-antagonists were chosen for use in chronic studies due to the most remarkable absolute reduction in FFAs, the known receptor $\beta_1\beta_3$-receptor density in rat adipose, the functional contribution of these subtypes to rodent lipolysis, and known effects of β_2-modulation of adipose blood flow. Hemodynamic and FTI data from the acute studies reflected known cardiovascular effects of β-receptor modulation (i.e. agonism and antagonism), and it is believed that changes in endothelial dilator function attributable to metabolic alterations would be overshadowed by the effects of activity at cardiovascular β-receptors.

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High-fat dietary treatment increased body weight, serum FFAs, TGs, and leptin concentrations. Chronic $\beta_1\beta_3$-antagonism increased body weight and serum FFAs. The strongest rationale for the augmented FFAs is inhibition of fatty acid oxidation in the brown adipose by the β-antagonists, an influence more profound than lipolytic inhibition. It is also possible that alternative lipolytic pathways were activated to compensate for inhibited β-mediated lipolysis. Despite emergence of elevated weight and FFAs, known risk factors for endothelial dysfunction, FTI was preserved. Trends toward improved FTI that were observed in these rats might have proved significant with increased sample size and reduced variability. Serum TGs were not increased with chronic $\beta_1\beta_3$-antagonist treatment, leading to the consideration that TGs may indeed be the more physiologically relevant lipid moiety in obesity-related endothelial dysfunction. Trends in serum FFAs were more consistent with serum TBARS than with markers of inflammation. This study revealed that chronic β-antagonist treatment may paradoxically increase serum FFAs, and that the protective effects of these agents in preserving endothelial function are unlikely to be related to inhibition of lipolysis and resultant attenuation of FFA-mediated pro-oxidant influences.

Principle areas in need of further investigation include identification of metabolic and hemodynamic indices in the postprandial versus fasting states, and the relative accumulation of visceral adipose in rodent dietary obesity, with implications thereof. In acute studies, treatment with the β_2-antagonist butoxamine resulted in significant metabolic alterations, including decreased

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serum FFAs, increased glucose, and reduced cholesterol. Of interest would be the identification of changes attributable to altered flow, and those due to atypical β_2-receptor function. Additional knowledge is needed regarding the relative contribution of NO, EDHF, prostacyclin, and unknown dilators to preservation of endothelium-dependent and -independent dilation in health and disease.

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