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NUTRITIONAL MODULATION OF BLOOD PRESSURE REGULATION BY THE AUTONOMIC NERVOUS SYSTEM

A Thesis in

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ABSTRACT

Cardiovascular function is modulated by changes in caloric intake and by metabolic factors in plasma such as free fatty acids (FFA). However, the mechanisms underlying the associated cardiovascular responses are unclear. The purpose of this series of experiments was to investigate the neural mechanisms underlying the altered control of blood pressure (BP) resulting from caloric/fat restriction and elevated FFA concentrations.

In the first study we investigated the impact of caloric restriction (CR) on cardiovascular function. Astronauts consume fewer calories during spaceflight and return to earth with an increased risk of orthostatic intolerance. Whether a caloric deficiency modifies orthostatic responses is not understood. Thus, we determined the effects of a hypocaloric diet (25% CR) during 6° head down bedrest (BR; an analog of spaceflight) on autonomic neural control during lower body negative pressure (LBNP). Nine healthy young men participated in a randomized crossover BR study, consisting of four (2 weeks each) interventions (normocaloric BR (NB), normocaloric ambulatory (NA), hypocaloric BR (HB), hypocaloric ambulatory (HA)), each separated by 5 months. Muscle sympathetic nerve activity (MSNA), heart rate (HR), and arterial pressure were recorded before, during, and after 3 consecutive stages (7 min each) of LBNP (-15, -30, -45 mmHg). Caloric and posture effects were compared using two-way ANOVA with repeated measures. Both BR and CR were associated with lower systolic BP during LBNP ($p<.01$); however, HR responses were directionally opposite (i.e., increase with BR, decrease with CR). Survival analysis revealed a significant reduction in orthostatic

tolerance (OT) following CR (p=0.03). Caloric restriction modifies autonomic responses to LBNP, which may decrease OT after spaceflight.

The second study employing combinations of BR and CR tested the hypothesis that a caloric deficit diminishes the pressor response to static exercise and noxious cold stress. Nine healthy young men participated in a randomized crossover BR study, consisting of four, two-week interventions (HA, HB, NA, NB), each separated by 5 months. HR, arterial pressure, and MSNA were recorded before, during, and after static handgrip HG (40% of maximum voluntary contraction to fatigue), post-exercise muscle ischemia (forearm occlusion), and the cold pressor test. Bedrest and nutritional combinations were compared using two-way ANOVA with repeated measures. HR, MSNA, and the change in systolic BP during HG were attenuated with CR. CR was associated with a higher diastolic BP during cold pressor; however, HR was directionally opposite (i.e., increase with BR, decrease with CR). Therefore, CR, but not BR, severely attenuates autonomic responses to static exercise.

The third study tested the hypothesis that acute elevation of FFA activates the sympathetic nervous system (SNS). Previous studies have shown that acute increases in plasma FFA raise systemic vascular resistance (SVR) and BP. However, these studies fail to distinguish between central nervous system (CNS) mechanisms that raise sympathetic activity and paracrine mechanisms that increase SVR directly independent of CNS involvement. On 2 days separated by at least 2 wks 17 lean, healthy volunteers (10M/7F; 22±1 yrs; BMI: 23±1 kg/m^2; mean±SEM) received a 4-hr iv infusion of Intralipid 20% or placebo (single-blind, randomized, balanced order). MSNA, HR, BP (brachial auscultation), and cardiac output ($\dot{Q}$, C_2H_2 rebreathing) were measured before

and throughout infusion. The change in HR (+8.2±1.0 vs. +2.4±1.2 beats/min), systolic BP (+14.0±1.6 vs. +3.2±2.5 mmHg), and diastolic BP (+8.2±1.0 vs. -0.1±1.7 mmHg) was significantly greater after the 4-hr infusion of Intralipid vs. placebo ($p<.001$). The change in BP with Intralipid resulted from an increase in SVR (mean BP/$\dot{Q}$; $p<0.001$) vs. baseline, without a change in $\dot{Q}$. MSNA burst frequency increased during Intralipid infusion vs. baseline (+4.9±1.3 bursts/min; $p<0.05$), and total MSNA (frequency * amplitude) was augmented 65% ($p<0.001$), with no change during placebo infusion. Lipid infusion increased insulin, aldosterone, and F_2-isoprostanes, but not leptin, concentrations. The concomitant increases in BP, MSNA, and SVR suggest that central sympathetic activation contributes to the pressor response to FFA.

The fourth study tested the hypothesis that aging exacerbates the sympathetic and cardiovascular responses to elevated FFA. The objectives of this study were to characterize the cardiovascular, neural, and endocrine response to acute elevation of FFA. Seventeen healthy older volunteers (7M/10F; 69±1 yrs; BMI 24±0 kg/m^2) received a 4-hr iv infusion of Intralipid 20% or placebo (single-blind, randomized, balanced order) on 2 different days separated by at least 2 wks. MSNA, HR, BP (brachial auscultation), $\dot{Q}$ (C_2H_2 rebreathing), leptin, insulin, aldosterone, angiotensin II, and F_2-isoprostanes were measured. The change in HR (+8.8±0.9 vs. +3.0±0.9 beats/min), systolic BP (+13.9±2.2 vs. +6.6±2.4 mmHg), and diastolic BP (+7.4±1.5 vs. +1.3±0.8 mmHg) was significantly greater after the 4-hr infusion of Intralipid vs. placebo ($p<.001$). The increase in BP with Intralipid resulted from variable fluctuations in SVR (mean BP/$\dot{Q}$) and $\dot{Q}$. MSNA burst frequency increased during Intralipid infusion vs. baseline (+6.7±1.6 bursts/min; $p<0.05$), and total MSNA (frequency * amplitude) was augmented 45% ($p<0.001$), with no change

during placebo infusion. Lipid infusion increased insulin (+40%), aldosterone (+50%), and F_2-isoprostanes (+80%), but not leptin, concentrations. Direct vascular mechanisms and central sympathetic activation contribute to the pressor response to FFA; this response is not different compared to previous studies in a younger population.

The results of these studies suggest that nutritional/metabolic factors alter autonomic control of the cardiovascular system. Specifically, moderate caloric/fat restriction diminishes BP control during orthostatic stress and static exercise. Conversely, acute elevation in FFA concentration increases BP by activating the central nervous system, and the response to FFA is not different in older individuals.

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LIST OF ABBREVIATIONS

AgRP	Agouti-Related Peptide
ANOVA	Analysis of Variance
AP	Area Postrema
BMI	Body Mass Index
BMR	Basal Metabolic Rate
BP	Blood Pressure
BR	Bedrest
CART	Cocaine-and Amphetamine-regulated Transcript
CBF	Calf Blood Flow
CCK	Cholecystokinin
CNS	Central Nervous System
CP	Cold Pressor
CR	Caloric Restriction
CSI	Cumulative Stress Index
CVR	Calf Vascular Resistance
DBP	Diastolic Blood Pressure
FFA	Free Fatty Acids
HA	Hypocaloric Ambulatory
HG	Hand Grip
HOMA-IR	Homeostasis Model Assessment

HR	Heart Rate
I/H	Intralipid/Heparin
LBNP	Lower Body Negative Pressure
MAP	Mean Arterial Pressure
MSNA	Muscle Sympathetic Nerve Activity
MVC	Maximal Voluntary Contraction
NA	Normocaloric Ambulatory
NB	Normocaloric Bedrest
NE	Norepinephrine
NPY	Neuropeptide Y
OI	Orthostatic Intolerance
OT	Orthostatic Tolerance
POMC	Proopiomelanocortin
Q	Cardiac Output
QUICKI	Quantitative Insulin Sensitivity Check
S/G	Saline/Glycerol
SBP	Systolic Blood Pressure
SNS	Sympathetic Nervous System
SV	Stroke Volume
SVR	Systemic Vascular Resistance
TG	Triglycerides

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Chapter 1

INTRODUCTION

Background and Significance

It was thirty years ago that Lewis Landsberg first showed changes in sympathetic control following caloric restriction and feeding in rats (Landsberg & Young, 1978). Though enhanced caloric intake alone stimulates central sympathetic activation through multiple signals of energy surfeit which aim to reduce caloric intake and increase diet-induced thermogenesis, evidence suggests that various macronutrients exert specific effects on the sympathetic nervous system (SNS). Modulation of caloric composition revealed the stimulatory effect of fat (Schwartz *et al.*, 1983) and carbohydrates (Young & Landsberg, 1977a), but not protein (Kaufman *et al.*, 1986), on norepinephrine turnover and cardiovascular variables in rats, even while maintaining isocaloric intake. Moreover, research has shown that low-fat vs. high-fat diets are accompanied by reductions in blood pressure and heart rate, as well as reduced responsiveness to sympathetic reflex testing (Straznicky *et al.*, 1993).

Extending the literature on the effects of individual macronutrients, more recent studies have investigated the role of altered plasma fatty acid concentration on cardiovascular control in health and pathological states in humans (Manzella *et al.*, 2001; Paolisso *et al.*, 2000; Stojiljkovic *et al.*, 2001). Importantly, elevated free fatty acids (FFA), which are characteristic of obesity, genetic dyslipidemia, and chronic high-fat

intake, have been linked to conditions associated with metabolic syndrome including hypertension (Fagot-Campagna *et al.*, 1998). However, these studies fail to distinguish between central nervous system (CNS) mechanisms that raise sympathetic activity and peripheral (paracrine) mechanisms that increase systemic vascular resistance (SVR) directly independent of CNS involvement. In contrast, moderate caloric restriction, analogous to conditions associated with spaceflight (Stein *et al.*, 1999), may alter autonomic control of the cardiovascular system contributing to the well-characterized reductions in orthostatic tolerance succeeding return from space (Buckey, Jr. *et al.*, 1996).

Four studies comprising this dissertation were conducted to determine how modification of diet and acute changes in plasma lipids alter autonomic control of the cardiovascular system. Specifically, these studies investigated 1) the sympathetic and hemodynamic response to caloric restriction from reduced fat intake at rest and during an orthostatic stress, 2) the effect of reduced caloric/fat intake on physiological reflex responses to tests of autonomic function, 3) the contribution of the SNS to the pressor response to elevated plasma fatty acids, and 4) whether aging in the absence of overt pathology alters the sympathetic and hemodynamic response to augmented plasma FFA levels.

Specific Aims and Hypotheses

Specific Aim 1: The purpose of the study, "Caloric restriction decreases orthostatic tolerance," was to determine the influence of combinations of normo- and hypocaloric intake coupled with ambulation or bedrest on hemodynamic variables and muscle sympathetic nerve activity during orthostatic stress.

Hypothesis 1: Chronic hypocaloric low-fat energy supply will intensify the development of orthostatic intolerance associated with bedrest.

Specific Aim 2: The purpose of the study, "Hypocaloric intake diminishes the pressor response to static exercise," was to determine effect of hypocaloric intake alone or coupled with bedrest on reflex autonomic and cardiovascular control.

Hypothesis 2: Reduced caloric/fat intake will diminish sympathetic and cardiovascular responses to static handgrip and cold pressor tests.

Specific Aim 3: The purpose of the study, "Free fatty acids increase arterial pressure via central sympathetic activation in humans," was to relate sympathetic activity and hemodynamic responses to plasma FFA concentrations in lean, normotensive young men and women.

Hypothesis 3a: Infusion of Intralipid® will significantly increase muscle sympathetic nerve activity and blood pressure, establishing a role for FFA in central sympathetic activation.

Hypothesis 3b: Responses will be similar between women and men.

Specific Aim 4: The purpose of the study, "Sympathetic and hemodynamic responses to lipids in healthy aging," was to determine whether age-related changes alter the FFA-induced sympathoexcitation response in women and men.

Hypothesis 4a: Augmentation of sympathetic activity and blood pressure following Intralipid® infusion will be greater with aging.

Hypothesis 4b: The greater rise in sympathetic activity and blood pressure with aging will be induced by larger increases in serum insulin and leptin concentrations following Intralipid® infusion.

Chapter 2

REVIEW OF LITERATURE

This review briefly discusses some of the basic findings regarding the role of lipids in modulating cardiovascular regulation. Though most dietary proteins have been shown to elicit no direct effect on sympathetic activation and blood pressure (BP) regulation, and carbohydrates (depending on the structure) may augment cardiovascular responses, their contributions are beyond the scope of this review. The information presented will provide the logic for the following broad hypotheses: 1) plasma free fatty acids (FFA) have a direct relation with BP and increase central sympathetic outflow, 2) elevation of sympathetic activity with FFA is mediated neurally and hormonally, and 3) primary aging may exaggerate the autonomic and hemodynamic responses to increased FFA concentrations.

Dietary Lipid Regulation of Blood Pressure

Dietary lipids are important modulators of BP during both times of excess fat intake and restriction. Rats fed high-fat diets consisting of lard (Tamaya-Mori *et al.*, 2003) or saturated or unsaturated fats (Song *et al.*, 2006) developed elevated BP vs. rats fed a normal chow diet. Stemming from earlier studies examining the effects of caloric restriction (CR) and norepinephrine (NE) turnover (Landsberg & Young, 1978), Schwartz and colleagues (1983) conducted a study demonstrating that a 5-day fat

supplementation of a reduced chow supply augmented NE turnover (~250%) vs. consumption of a reduced amount of chow alone. Since the difference in calories per se of the two groups may have impacted the results, rats were given isocaloric low- and high-fat diets, resulting in the same increase in cardiac and renal NE turnover (Schwartz *et al.*, 1983; Young & Walgren, 1994; Daly *et al.*, 1992). The responses seem to be dependent on postabsorptive processes (i.e. lipid-related signals, metabolism) since administration of cholestyramine, a bile acid binding resin, resulted in suppressed sympathetic activity (Schwartz *et al.*, 1983). In healthy humans, 6 weeks of a low-fat (~25%) vs. high-fat (~40%) diet lowered heart rate (HR), 24-hr mean arterial pressure (MAP), and BP responses to cold pressor testing and NE infusion. However, NE concentration, spillover, and clearance did not change between the two diets (Straznicky *et al.*, 1993). Strengthening these findings, another study similar in design but only 2 weeks in duration reported decreases in BP of 7/3 mmHg and improved lipid profile (Straznicky *et al.*, 1999). Results from these studies point to both peripheral and central mechanisms that modulate the cardiovascular responses to changes in fat intake.

Free Fatty Acids and Cardiovascular Control

Accumulating evidence indicates that FFA may be a strong factor linking obesity, genetic dyslipidemia, and high-fat caloric intake with altered BP regulation (e.g. hypertension) (Egan *et al.*, 1999). Indeed, obese hypertensive patients have roughly twice the measured FFA than lean nomortensive ones (Davda *et al.*, 1995), and insulin's FFA lowering action is substantially impaired in these individuals. Interestingly, only 1% of the U.S. population has combined familial hyperlipidemia, but they comprise 12%

of the hypertensive population (Castro *et al.*, 1993). Moreover, FFA concentration correlated directly with BP measured at rest and over 24 hrs, independently of insulin and insulin-mediated glucose disposal (Egan *et al.*, 1996). Furthermore, in compiling resting data from previous investigations we show a strong and significant correlation (Figure 2-1) between FFA and MAP from multiple data sets.

Figure 2-1

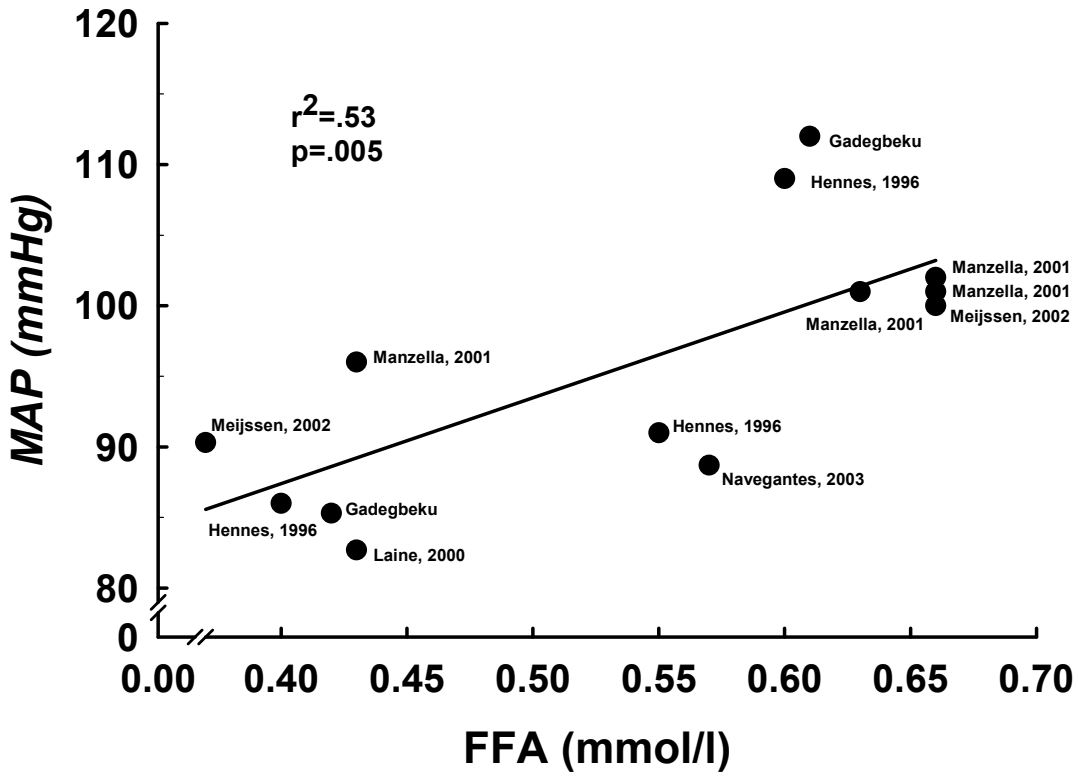

Figure 2-1. Direct linear relation between FFA and MAP using data from previous studies in health and disease.

Though the data show a strong relation, correlation does not prove causality. Therefore, a number of investigations have been performed to determine the acute effects of plasma FFA elevation or reduction on BP regulation. Increasing FFA with an infusion

of Intralipid/heparin increases BP and vascular resistance in most tissue beds in minipigs (Bulow *et al.*, 1990). Likewise, portal and femoral venous infusions of oleate induce a pressor response in normotensive rats mediated by efferent adrenergic activity and/or stimulation of hepatic afferents (Grekin *et al.*, 1995; Grekin *et al.*, 1997).

In human subjects, raising FFA via Intralipid/heparin infusion may induce central sympathetic activation resulting with increases in BP of ~8-15/5-10 mmHg and an elevation of heart rate by 6-10 beats/min on average in most (Lopes *et al.*, 2001; Lopes *et al.*, 2003a; Lopes *et al.*, 2003b; Paolisso *et al.*, 2000; Steinberg *et al.*, 1997; Stojiljkovic *et al.*, 2001), but not all investigations (Polak *et al.*, 2001). Further support for a positive relation between FFA and BP is provided by two studies involving both acute and chronic reductions of FFA. Infusion of nicotinic acid to reduce FFA significantly decreased arterial pressure (Gadegbeku *et al.*, 2003) in hypertensives. Similarly, chronic reduction of FFA in Type 2 diabetic subjects lowered BP and plasma NE (Manzella *et al.*, 2001), but it is not known whether these findings can be extended to healthy individuals.

Peripheral Mechanisms in the Cardiovascular Response to Altered FFA Levels

Do peripheral (vascular) or central factors mediate this acute BP response? The answer appears to be, "yes," and "not yet known." Davda *et al* (1995) showed that FFA impair endothelial cell nitric oxide synthase activity and endothelium-dependent vasodilation *in vitro*. Following lipid infusion in humans to raise FFA endothelium-dependent vasodilation is impaired (Steinberg *et al.*, 1997), and vascular α_1-adrenoceptor-mediated responses are augmented (Steinberg *et al.*, 1997; Stepniakowski *et al.*, 1995;

Stepniakowski *et al.*, 1996). Other mediators of the cardiovascular response to FFA include direct stimulation of protein kinases that cause constriction (Farooqui *et al.*, 1988), inhibition of ATPases and elevation of intracellular calcium in vascular smooth muscle (Ahmed & Thomas, 1971). To date, limited data suggest (Paolisso *et al.*, 2000), but have not definitively proven, a link between the rise in BP and sympathetic activation (Grekin *et al.*, 2005). Considering the pivotal role of the SNS in obesity-related hypertension and the data relating FFA to hypertension, it is critical to determine the contribution of the SNS in this regard. Specific Aims 3 and 4 (Chapters 5 and 6) investigate the role of FFA on central sympathetic outflow by direct measurement of MSNA.

Potential Central Mediators of FFA-induced Sympathetic Activation

This section documents evidence for 5 putative mechanisms by which FFA act to modulate central sympathetic outflow and hemodynamic variables (Figure 2-2). The roles of leptin and insulin will be investigated in Chapters 5 and 6, while the contributions of mechanisms 3-5 are potential topics of study in future investigations.

Figure 2-2

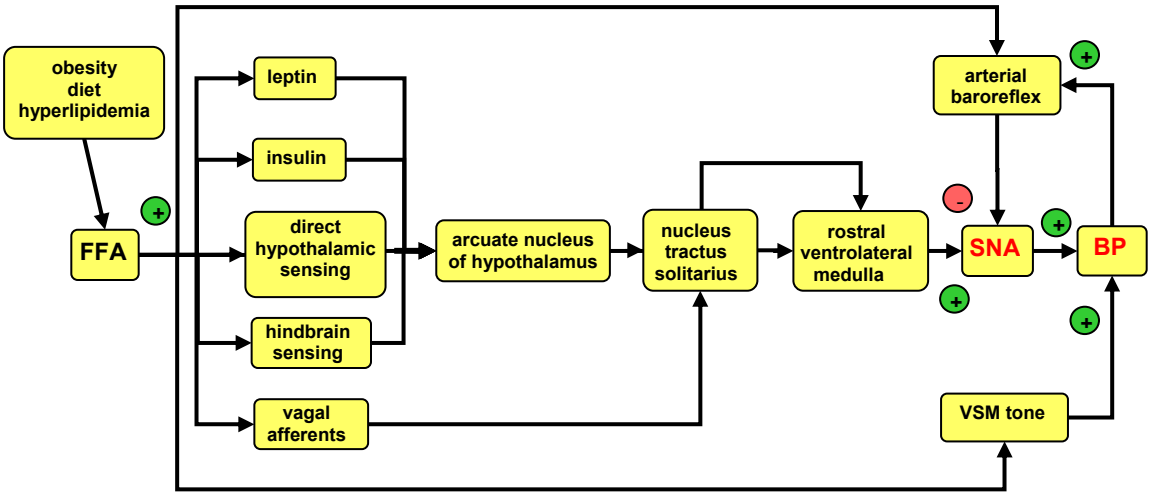

Figure 2-2. Schematic depiction of the potential central mechanisms underlying the increase in MSNA and blood pressure in response to elevated FFA. The roles of leptin and insulin will be considered in this investigation. Note that sympathetic activity is an important discriminating variable, as the direct effects on vascular smooth muscle should disinhibit arterial baroreflexes to reduce sympathetic activity and oppose central activation.

*Leptin***.** Discovered in 1994 (Zhang *et al.*, 1994), leptin (product of *ob* gene) may be considered the hormonal signal bridging peripheral adipose tissue to the central nervous system (CNS) for energy expenditure and control of appetite. The control of leptin secretion from white adipose cells is very complex, following both acute and chronic control. Fasting levels rise in proportion to adiposity (Zhang *et al.*, 1994), though both fasting and feeding modulate leptin levels greatly (Boden *et al.*, 1996; Kolaczynski *et al.*, 1996b; Kolaczynski *et al.*, 1996a), even without a change in body fat. Leptin crosses the blood-brain barrier to the CNS via a saturable transport-mediated endocytosis, and binds to the Ob-R receptor in the arcuate nucleus (Cowley *et al.*, 2001). Subsequent to binding, leptin stimulates POMC/CART (proopiomelanocortin / cocaine- and amphetamine-regulated transcript) neurons (Swart *et al.*, 2002). Concomitantly, leptin

hyperpolarizes the NPY/AgRP (neuropeptide Y / agouti-related peptide) neurons and decreases mRNA expression. Inhibition of NPY/AgRP increases POMC/CART to a greater extent by blocking the tonic GABAergic inhibition of AgRP on POMC. Additionally, decreasing the endogenous melanocortin receptor (i.e., MC-4R) antagonist AgRP further facilitates the action of α-MSH (cleaved from POMC) at MC-4R. The POMC/CART neurons project to the paraventricular nucleus (PVN) and the lateral hypothalamus (LHA), stimulating second-order neurons that synapse in the nucleus tractus solitarius (NTS) and the rostral ventrolateral medulla (RVLM). The leptin-activated arcuate POMC/CART neurons synapse on sympathetic preganglionic neurons to modulate energy expenditure and BP (Elmquist, 2001). Further support is evidenced by the observations that i.v. and i.c.v. administration of leptin increases both sympathetic activity and BP (Dunbar *et al.*, 1997; Matsumura *et al.*, 2000; Shek *et al.*, 1998) in conscious animals.

Whether the increased sympathetic activity and BP observed in obesity and high-fat consumption is mediated by up-regulated gene expression and leptin secretion has been the focus of many investigations, without consistent results. *In vitro*, palmitate and/or 2-bromopalmitate down-regulate leptin secretion from rat adipocytes (Garcia-Lorda *et al.*, 2003), whereas eicosapentanoic and/or arachidonic acids stimulate leptin production in rat adipocytes, with a greater secretion of leptin in the presence of insulin (Perez-Matute *et al.*, 2003; Perez-Matute *et al.*, 2005). Several investigations in both humans and rats have shown that increasing FFA concentrations with lipid infusion produces an increase of both *ob* mRNA levels and plasma leptin (Fabris *et al.*, 2001; Nisoli *et al.*, 1999; Wang *et al.*, 1998). Alternatively, FFA have been shown to have no

effect, or actually decrease, leptin (Chen & Song, 2002; Garcia-Lorda *et al.*, 2003). The reason for the disparate findings *in vivo* is unclear; however, study durations may have been too brief (less than 3 hours) to observe a change in leptin (Wang *et al.*, 1998), and other factors such as infusion rate and concentrations of FFA may play a role. Thus taken together, these data suggest, but are not definitive, that leptin mediates the sympathetic response to FFA.

Insulin. The pancreatic hormone, insulin, was the first hormonal signal to be implicated in the control of body weight by the CNS (Woods *et al.*, 1979) and may play a role in the pathogenesis of obesity-related hypertension (Landsberg, 2001). While insulin circulates in proportion to body fat (Bagdade *et al.*, 1967), acute modulation of energy intake is best known to alter insulin levels (Boden *et al.*, 1996). Insulin enters the brain via a saturable transport mechanism, and binds to its receptor in the arcuate nucleus. Similar to leptin but via a different signaling mechanism, insulin stimulates POMC/CART neurons and inhibits NPY/AgRP neurons to increase sympathetic activity, BP, and energy expenditure (Rahmouni *et al.*, 2004; Schwartz *et al.*, 2000).

Although early studies demonstrated that acute elevations of plasma FFA levels increase serum insulin (Crespin *et al.*, 1969; Crespin *et al.*, 1973), many recent lipid infusion studies (Nisoli *et al.*, 1999; Schwartz *et al.*, 2000; Fugmann *et al.*, 2003; Grekin *et al.*, 1997; Polak *et al.*, 2001) failed to detect a significant change in insulin. A possible explanation for the discrepancy is that by supplying a large amount of FFA, triglyceride synthesis is facilitated by mass action without the need for increased insulin levels. Moreover, insulin clearly augments the leptin response to FFA (Pagano *et al.*, 2004), yet

elevated insulin concentrations are not necessary to evoke a leptin response (Chelikani *et al.*, 2003; Donahoo *et al.*, 1997; Nisoli *et al.*, 1999). Thus the likelihood that insulin is potentially a direct or indirect (via leptin facilitation) mediator of sympathetic activity warrants monitoring of insulin levels during these investigations.

Direct hypothalamic sensing. Long-chain fatty acids, the most abundant fatty acids in Intralipid, cross the blood-brain barrier by simple diffusion in the unbound form in proportion to the circulating plasma FFA concentration (Rapoport, 1996). Within the hypothalamus long-chain fatty acids are esterified to long-chain fatty acyl-CoAs, and are subsequently transported to lipid oxidative and synthetic pathways. The pool of long-chain fatty acyl-CoAs which signals energy surfeit activates the POMC/CART neural pathway resulting in decreased energy intake and activation of the SNS (Lam *et al.*, 2005). Therefore, the possibility that direct sensing within the hypothalamus may be a modulating factor of sympathetic activity in these studies must be acknowledged.

Hindbrain sensing. The area postrema (AP) is a circumventricular organ located in the medulla oblongata, lacking a complete blood-brain barrier. Thus, circulating factors such as FFA as well as afferent fibers can influence AP neurons (Horn *et al.*, 1999) which modulate cardiovascular function. Neurons originating in the AP project to the NTS and RVLM to elicit increases in sympathetic activity. Additionally, signals from the AP synapse on NPY/AgRP and POMC/CART neurons in the hypothalamus (Bishop & Hay, 1993). Therefore, changes in plasma fat content – mainly reductions in oxidizable fatty acids – can elicit sympathetic responses mediated by the AP.

Vagal afferents. FFA may induce a pressor response through vagal afferents. Chemosensitive afferents arising from abdominal viscera respond to a range of endogenous molecules including sugars, lipids, peptide hormones, and cytokines to influence discharge of vasomotor neurons of the RVLM (Verberne et al., 2003) and release of NE in the paraventricular nucleus (Ueta et al., 2000). Moreover, infusion of palmitate, myristate, and oleate in animals increased vagal afferent nerve activity (Orbach & Andrews, 1973;Randich et al., 2004). The contribution of this putative mechanism is beyond the scope of the proposed investigations, but will be the focus of future studies.

Other signals for consideration. Adipocyte hormones such as adiponectin and resistin may be potential candidates for modulating FFA-mediated sympathetic and cardiovascular responses. Adiponectin circulates at high levels, which are reduced with obesity, elevated FFA, and insulin resistance (Arita *et al.*, 1999). In contrast, lipid infusion in rats induces peripheral insulin resistance and increased plasma resistin levels (Yang *et al.*, 2005). However, very limited information is currently available regarding their interaction with the SNS and cardiovascular control (Ahima, 2005). On the other hand, the intestinal peptide cholecystokinin (CCK) stimulates the CNS (Covasa, 2006). However, since CCK is secreted in response to the entrance of fat and protein in the duodenum, it is unlikely that CCK elicits any major effects in response to lipid infusion.

Effect of FFA on Baroreflex Regulation

Currently, there is limited information available addressing modulation of baroreflex function with changes in plasma FFA concentration. Gadegbeku *et al* (2002) concluded that baroreflex sensitivity, as determined by the regression line of R-R interval and systolic BP during phenylephrine infusion, was acutely impaired by augmented FFA concentration in both normotensive and hypertensive participants. A more recent study suggests that the primary mechanism underlying the enhanced α-adrenergic sensitivity from lipid infusion results from reduced baroreflex sensitivity (Gadegbeku *et al.*, 2006). In contrast, cardiovagal and sympathetic baroreflex sensitivity to the modified Oxford technique was not impaired following 2-hr lipid infusion (Monahan *et al.*, 2007). The explanation for disparate results is unclear; however, methodology, subject characteristics, and sample size may play a role.

Does Aging Facilitate the Pressor Response to Fatty Acids?

Aging is associated with a much higher prevalence of hypertension and increased activation of the SNS, which may be driven by progressive accumulation of body fat (Seals & Bell, 2004). Although insulin secretion in response to a glucose challenge or mixed meal is impaired with aging (Muzumdar *et al.*, 2004), the total acute (Basu *et al.*, 2003) and chronic (Fraze *et al.*, 1987; Tamaya-Mori *et al.*, 2003) insulin response is significantly enhanced. Moreover, the age-related defect in insulin secretion appears to be specific to glucose and not FFA (Muzumdar *et al.*, 2004). There are no data concerning a leptin meal response with aging or with lipid infusion in humans; however,

based on the evidence presented previously one could speculate an enhanced leptin response resulting from the increase in insulin. In fact, Tamaya-Mori *et al* (2003) demonstrated an augmented leptin, insulin, and BP response to high-fat dietary intake in aged rats. They hypothesized that the hyperinsulinemia-induced hyperleptinemia accelerated the age-related increase in sympathetic nerve activity, contributing to the significant BP elevation observed in the older rats.

The relation between visceral obesity and factors such as MSNA, insulin resistance, and hypertension in humans is well-documented (Bjorntorp, 1991; Bjorntorp & Rosmond, 2000; Alvarez *et al.*, 2002; Alvarez *et al.*, 2004). Thus, the possibility that the increase in visceral adiposity with age (Wilson & Kannel, 2002) may confound hormonal responses (i.e., insulin, leptin) should be considered. By matching young and older volunteers for the studies in Chapters 5 and 6 for adiposity, BMI, waist-hip ratio, the homeostasis model assessment (HOMA-IR), and quantitative insulin sensitivity check (QUICKI), we are better able to elucidate mechanisms associated with FFA-induced autonomic and hemodynamic responses with primary aging.

It is questionable whether lipid infusion in older vs. young volunteers elicits a greater FFA-induced sympathetic response, contributing to enhanced hemodynamic responses in older volunteers during lipid infusion. Recent studies clearly demonstrate an extra level of sympathetic activation (i.e., MSNA) when comparing older hypertensives to older normotensives (Grassi *et al.*, 1997; Grassi *et al.*, 2000; Yamada *et al.*, 1989). Measurements of plasma NE and NE spillover (e.g., Esler) under these conditions may not be valid because aging may enhance NE reuptake (Supiano *et al.*, 1999). Therefore,

one would anticipate an increase in MSNA in addition to the FFA-associated rise in sympathetic activity, resulting in an enhanced BP response to acute FFA elevation.

To summarize, FFA could contribute to a metabolic link between caloric balance and BP regulation with advancing age or adiposity, mediated by central sympathetic outflow. The results of the investigations comprising this thesis provide, for the first time, a direct measure of sympathetic nerve activity and the hormonal mediators thereof, in response to FFA in women and men in the framework of a carefully-controlled research design. Furthermore, the study in Chapter 6 tests the novel hypothesis that age exacerbates increases in BP and sympathetic activation in response to FFA, (i.e., the population in which hypertension is most prevalent). These studies establish a basis for future mechanistic and interventional studies focusing on the role of diet affecting central nervous system regulation of BP.

Chapter 3

CALORIC RESTRICTION DECREASES ORTHOSTATIC TOLERANCE

Introduction

Orthostatic intolerance (OI), the inability to maintain blood pressure (BP) while standing, affects over 500,000 individuals in the United States (Robertson, 1999), and up to 64% of astronauts upon return from microgravity (Buckey, Jr. *et al.*, 1996). Symptoms include fatigue, headache, nausea, presyncope, and occasionally syncope, resulting from inadequate cerebral perfusion upon standing (Robertson, 1999). Though the etiology of orthostatic hypotension has been extensively researched, its pathophysiology is still poorly understood. One well-known factor predisposing astronauts to orthostatic hypotension is a reduction in blood volume (Fischer *et al.*, 1967), leading to decreased cardiac filling and diminished stroke volume (Blomqvist *et al.*, 1994). Other potential factors contributing to the reduced cardiac filling include altered cardiovascular neurohumoral regulation, diminished carotid-cardiac baroreflex responsiveness (Fritsch *et al.*, 1992), and augmented peripheral pooling (e.g. legs, abdomen) and venous compliance (Buckey *et al.*, 1992).

Despite the effort to simulate the effects of spaceflight employing bedrest (BR), water immersion, and other interventions, the role of diet has been largely unexplored. Astronauts consume ~25% fewer calories than necessary during spaceflight (Heer *et al.*, 2000; Stein *et al.*, 1999). Inadequate food intake can severely impact endocrine,

muscular, and cardiovascular performance. Even moderate caloric restriction (CR) can impact fluid homeostasis resulting in reduced blood volume and cardiovascular function (Shetty, 1999). For example, pilots who fasted during Ramadan had reduced weight (-2.7%), plasma volume (-7%), augmented HR, and diminished pulse pressure during orthostasis after Ramadan (Bigard *et al.*, 1998). Furthermore, CR reduces heart rate (HR), BP, and norepinephrine turnover in rats (Williams *et al.*, 2002; Young & Landsberg, 1977b) and in obese normotensive humans following a reduction in weight (Grassi *et al.*, 1998). However, it is not known how actual or simulated microgravity in conjunction with hypoenergetic intake affect the cardiovascular and sympathetic neural responses to orthostasis in healthy individuals.

Therefore, the objective of the current study was to determine the influence of combinations of normo- and hypocaloric intake coupled with ambulation or BR on hemodynamic variables and muscle sympathetic nerve activity (MSNA) during orthostatic stress. We hypothesized that chronic hypocaloric energy supply would intensify the development of OI.

Methods

Subjects

Nine healthy men (age: 23.8 ± 3.0 years; BMI: 22.8 ± 3.2 kg/m^2) completed a randomized crossover BR and CR study to simulate the effects of spaceflight. This studied conformed with the Declaration of Helsinki, and all subjects signed consent forms approved by the Ethical Committee of the 'Arztekammer Nordrhein', Dusseldorf,

Germany. Subjects were enrolled if they met all of the following inclusion criteria: physical examination, ECG, urinalysis and routine laboratory without clinically relevant findings, total cholesterol ≤200 mg/dL, LDL ≤130 mg/dL, HDL ≥35 mg/dL, and fasting glucose ≤106 mg/dL. Exclusion criteria included hyperlipidemia, arterial hypertension, diabetes, regular medication and/or treatment with drugs within the last 6 wk, acute or chronic illness, smoking within a period of 1-year preceding the study, and drug and/or alcohol abuse.

Study Design

The study was performed in a randomized cross-over design as part of a multi-disciplinary project (Short-term Bedrest – Integrated Physiology: STBR-IP) evaluating the effects of simulated microgravity and hypocaloric nutrition on cardiovascular and sympathetic nervous function. The subjects participated in 4 study phases that were separated by at least 5 months to allow complete recovery of the participants. Each study phase started with a 9-day adaptation period followed by a 14-day intervention period; in each of the 4 intervention periods the participants were exposed to either BR or ambulatory control conditions, while receiving either a tailored normocaloric or hypocaloric diet. Cardiovascular and sympathetic responses to lower body negative pressure (LBNP) were investigated twice in each phase: on the last day of the adaptation period and on day 14 of the intervention period. All four study phases were identical with respect to environmental conditions and study protocol; only the variables posture (BR/ambulation) and energy intake (normocaloric/hypocaloric) were changed.

Ambulatory and Bedrest Conditions

The participants resided in a metabolic ward (Institute of Aerospace Medicine, German Aerospace Center (DLR), Cologne, Germany) during the entire period of the four interventions. Room temperature (24°C) and relative humidity (50%) were kept constant in the metabolic ward and the laboratory. During the BR phases, all activities, including food intake, using the toilet, showering, and weighing, were carried out in the 6° head-down-tilt or horizontal position. 6° head-down-tilt was chosen because it is a validated model for simulation of microgravity (Nixon *et al.*, 1979). Though the induced cardiovascular changes occur more rapidly, their nature and extent is very similar to those observed in supine position. During the ambulatory control phases, the participants maintained upright position during the day and were allowed to walk around in the ward. Though they were not allowed to exercise voluntarily, they followed a light exercise protocol (including bicycle ergometry ~125 W - 15 min twice/day).

Diet

During all adaptation and recovery periods as well as during the normocaloric, ambulatory intervention the participants received a normocaloric standard diet. Energy requirements were calculated for each individual according to the FAO/WHO equations (Lin *et al.*, 2003): participants received a specifically prepared diet containing 1.4 times their basal metabolic rate (BMR). Ten percent of the total calories was added to account for dietary-induced thermogenesis. The average caloric intake was 2722±310 Kcal/day, which consisted of 1 g protein per kg body weight/day, 50 ml water per kg body

weight/day, 2.5 mmol sodium per kg body weight/day, 1000 mg calcium/day, and vitamin D 400 IU/day administered as fixed dose tablets. Dietary protein, fat (saturated and polyunsaturated fatty acids), and carbohydrate intakes were calculated according to dietary reference intake values (Yates *et al.*, 1998) (i.e. 10-15% of energy intake administered as protein, 30% as fat, and 55-60% as carbohydrates). The German recommended dietary intake levels were used for nutrients without experiment-specific requirements. No caffeine, methylxanthine, or alcohol was allowed. Six meals were prepared daily which included three main meals and three snacks. The volunteers received and ate the exact amount of food that was predefined in their individual menu. During the intervention periods, the energy content of the diet was modified as follows: normocaloric, ambulatory: ~2500 Kcal; hypocaloric, ambulatory: about 25% decrease in calories compared to standard diet, ~1875 Kcal; normocaloric, BR: energy content was reduced from the standard diet to adjust to the reduced physical workload; participants received a diet containing 1.1 (instead of 1.4) times their BMR, ~2200 Kcal; hypocaloric, BR: about 25% decrease in calories compared to the respective normocaloric, BR phase, ~1650 Kcal. Reduction in energy intake was mainly achieved by reduction of fat intake to a minimum level of 60g/day in order to keep the recommended level of essential fatty acids. Other than fat, nutrient composition of each experiment day was identical to the normocaloric study periods.

Heart Rate and Arterial Pressure

Heart rate (HR) was derived from a surface electrocardiogram. Beat-to-beat finger arterial pressure was measured by finger photoplethysmography (Portapres,

Amsterdam, The Netherlands), and auscultatory blood pressure (BP) was taken at baseline and at the end of each stage during the protocol.

Muscle Sympathetic Nerve Activity

Peroneal nerve muscle sympathetic activity (MSNA) was recorded as described previously (Wallin & Eckberg, 1982). Briefly, the nerve was located with cutaneous electrical stimulation (Isostim A320, World Precision Instruments). A tungsten reference electrode (FHC, Bowdoinham, ME, USA) was inserted subcutaneously, ~2 cm from the nerve, and a tungsten recording electrode with an uninsulated tip diameter of ~10 μm was inserted through the skin near the nerve. Adjustments of the recording electrode position were made according to auditory signals generated by impaled nerves. Both electrodes were connected in series to a differential preamplifier and an amplifier (NASA, Houston, TX, USA), isolated by two 100 mA current limiters. The nerve signal was amplified (total gain 40,000 to 80,000), band-pass filtered (high pass of .7 kHz and low pass of 2-3 kHz), and then full-wave rectified and smoothed with a resistance-capacitance circuit (time constant, 0.1 s) to produce a recording of "integrated" MSNA. Satisfactory recordings of MSNA were defined by pulse-synchronous bursts that increased during end-expiratory apnoea or Valsalva straining and did not change during tactile or auditory stimulation.

Lower Body Negative Pressure

Suction was applied with subjects supine in lower body chambers sealed at the iliac crests. The chamber, developed by the Deutsche Agentur Raumfahrtagelegenheiten (DARA), was made of collapsible fabric, and had windows to allow leg access for microneurography. After 7 minutes of baseline recording, steady pressure was applied at -15, -30, and -45 mmHg, in fixed order, for 7 minutes each, or until presyncope. Data were analyzed during the third to fifth minute of each 7 minute period, and blood was drawn at the end of each segment. Presyncope was defined as a decrease in systolic blood pressure (SBP) to <80 mmHg; a decrease in SBP to <90 mmHg associated with symptoms of lightheadedness, nausea, or diaphoresis; or progressive symptoms of presyncope accompanied by a request from the subject to discontinue the test.

Data Analysis

Three minutes of data from recordings of MSNA, BP, and HR collected at baseline were each averaged to a single value. Likewise, data for minutes 2-5 of each stage of LBNP were averaged to a single point. Data where subjects became presyncopal are not included in the analysis.

Student's one-tailed paired t-test was utilized to determine differences between resting MSNA values. A repeated measures analysis of variance (ANOVA) was conducted to determine the influence of caloric intake, posture, and time on MSNA and hemodynamic variables. Least squares means with Bonferroni correction were

performed when appropriate to detect where differences between factors occurred. The level of significance was set at α=0.05. Values are presented as means ± SEM.

Results

Subject Characteristics

The subject clinical characteristics at screening are presented in Table 3-1. All subjects were young, healthy, normotensive, and nonobese. Subject weights were not different at baseline for each of the intervention: hypocaloric ambulatory (79.8±3.6 kg), hypocaloric BR (78.7±3.3 kg), normocaloric ambulatory (76.9±3.2 kg), normocaloric BR (78.1±2.8 kg). Figure 3-1 depicts the change in subject weight over the course of each intervention. Weight significantly decreased from baseline for all interventions except for control (normocaloric ambulatory).

Heart Rate and Arterial Pressure

Hemodynamic measurements before and during lower body suction following each intervention are depicted in Figure 3-2. Heart rate was significantly lower at baseline and throughout LBNP following CR, whereas BR was associated with a higher HR. As expected, HR increased during the 3 stages of LBNP ($p<0.001$), and the cardioacceleration was greater following BR, independent of caloric intake ($p<0.001$). Caloric restriction was associated with a lower SBP during LBNP ($p=0.007$), and SBP decreased in all interventions by the last stage of LBNP. No main effects for calorie or

posture were identified for DBP; however, CR abolished, and BR enhanced, the increase in DBP during LBNP. A trend toward reduced MAP following CR alone was present during LBNP (p=0.070).

Muscle Sympathetic Nerve Activity

Microneurography was not attempted during the 1st intervention; therefore, only MSNA data for the hypocaloric and normocaloric ambulatory interventions were examined. Additionally, due to the large attrition of subjects and microneurography electrode shifts with increasing lower body suction, only baseline MSNA data before and after the hypocaloric ambulatory intervention could be analyzed, showing a significant reduction of MSNA (p=0.035) with CR (Figure 3-3).

Tolerance to Lower Body Suction

Data for 8 of the 9 subjects were analyzed for LBNP due to technical problems with the data acquisition system. The number of individuals remaining at a given time of LBNP is presented in Figure 3-4. Seven of 8 individuals completed the entire LBNP protocol following the normocaloric ambulatory intervention, and half the subjects completed following both BR interventions. Interestingly, only 2 of 8 subjects finished after the hypocaloric intervention alone. Survival analysis indicates a significant effect (p=0.03) of CR compared to normocaloric intake to reduce orthostatic tolerance and a tendency (p=0.09) toward reduced orthostatic tolerance with CR independent of BR when compared with the other interventions. Moreover, CR alone reduced the cumulative

stress index (CSI; Σ(LBNP * time) compared to the pre-intervention CSI (587±32 vs. 483±47 mmHg * min; p=0.04).

Discussion

This investigation was conducted to determine the effects of hypoenergetic low-fat intake, BR, and their combination on neural and cardiovascular control in healthy young volunteers at rest and during orthostatic stress.

Effect of Caloric Restriction

The major finding of the present study was that tolerance to lower body suction was reduced following 14 days of a hypocaloric, low-fat diet alone. Additionally, HR and SBP at baseline and throughout LBNP were significantly attenuated following hypocaloric interventions compared with normocaloric interventions. Furthermore, CR with ambulation dramatically affected the BP responses to LBNP at -30 and -45 mmHg, thus contributing to the reduced orthostatic tolerance.

Previous studies have reported reductions in hemodynamic variables following CR in animals and humans. Similar to this investigation, 2 weeks of 60% of normal caloric intake decreased MAP and HR in normotensive, nonobese mice under thermoneutral conditions (Williams *et al.*, 2002), and 2 weeks of low-fat consumption reduced BP by 7/3 mmHg and HR measured over 24 hours (Straznicky *et al.*, 1999; Straznicky *et al.*, 1993). To our knowledge no other studies have examined the effects of reduced caloric/fat intake on hemodynamic responses to LBNP. However, our results are

supported by an investigation that assessed BP and HR responses to 10-min of 80° head-up tilt before and after 1 month of Ramadan fasting in healthy male pilots (Bigard *et al.*, 1998). In concord with our study, fasting reduced SBP at baseline and during tilt; however, cardioacceleration was enhanced during tilt, in opposition to the attenuated HR response observed in the present investigation.

Why Does Caloric Restriction Reduce Blood Pressure During LBNP?

Several mechanisms may have contributed to the reduced cardiovascular responsiveness and orthostatic tolerance with caloric/fat restriction including: (1) reduced blood volume, (2) modulation of autonomic function, (3) altered baroreflex function, and (4) altered vascular smooth muscle tone and responsiveness.

Reduced blood volume. Consistent with the loss of weight in this study, short-term CR produces a 7-8% fall in plasma volume in lean (Bigard *et al.*, 1998) and obese individuals (Messaoudi *et al.*, 1998). Thus, reduced caloric intake may contribute to the 10-15% fall in blood volume during spaceflight (Buckey *et al.*, 1992). In fact, it has been shown that nutritional energy supply during missions is associated with chronic total body mass (presumably fluid) loss or gain during spaceflight (Drummer *et al.*, 2000). Therefore, the reduced orthostatic tolerance observed in this investigation may be explained, at least in part, by reduced blood volume (Pawelczyk *et al.*, 2001).

Autonomic nervous system modulation. Caloric/fat restriction and fasting suppress sympathetic activity in animals (Young & Landsberg, 1977b; Young & Landsberg, 1982) and overweight or obese individuals (Gohler *et al.*, 2000; Grassi *et al.*, 1998; Kushiro *et al.*, 1991). Diminished sympathetic outflow may be mediated by a

reduction in leptin and/or insulin levels, which are altered acutely by caloric intake independent of changes in body weight (Coleman & Herrmann, 1999). Additionally, free fatty acids modulate leptin (Fabris *et al.*, 2001; Heptulla *et al.*, 2001; Nisoli *et al.*, 1999; Wang *et al.*, 1998) and insulin (Paolisso *et al.*, 2000; Steinberg *et al.*, 1997; Vollenweider *et al.*, 1995), and directly activate the sympathetic nervous system (Lam *et al.*, 2005). Thus, a reduction in FFA (as may occur in moderate but not severe CR) may directly or indirectly reduce central sympathetic outflow. The trend toward reduced sympathetic activity at rest in the present study supports this idea. Moreover, it is possible that the sympathetic response during LBNP is also diminished (Straznicky *et al.*, 1993), contributing to the decreased BP and LBNP tolerance.

Baroreflex function. Though carotid-cardiac baroreflex responsiveness may be reduced after spaceflight (Fritsch *et al.*, 1992), previous studies have suggested that CR alone enhances baroreflex function (Alvarez *et al.*, 2005; Grassi *et al.*, 1998). Therefore, we would have expected a greater tachycardic response since BP decreased during LBNP. Interestingly, a blunted HR response was observed following CR in this study.

Altered vascular function. Reduced fat intake and plasma fatty acids may decrease vascular tone through a variety of mechanisms including increased insulin sensitivity (Roberts & Schoeller, 2007), nitric oxide synthase and nitric oxide production (Davda *et al.*, 1995), enhanced endothelium-dependent and –independent vasodilation (Hesse *et al.*, 2005), and reduced α-adrenergic sensitivity (Stepniakowski *et al.*, 1996). Additionally, CR reduces oxidative stress (Hesse *et al.*, 2005) which has been linked to hypertension (Minuz *et al.*, 2002).

Effect of Bedrest

Consistent with other studies of simulated or actual microgravity, half the participants in this experiment were unable to finish the protocol during orthostatic stress. We cannot determine the exact underlying mechanism(s) associated with the altered hemodynamic response and reduced orthostatic tolerance from our results. Moreover, MSNA could not be analyzed due to experimental limitations for the normocaloric BR intervention. However, our findings are in accord with a BR study of similar duration (Pawelczyk *et al.*, 2001). Following 18 days of 6° head-down BR, subjects had reduced LBNP tolerance through a combination of cardiac atrophy (Levine *et al.*, 1997) and hypovolemia, coupled with augmented sympathetic activation (Pawelczyk *et al.*, 2001). The elevated basal HR and enhanced cardioacceleration during LBNP in the present study may be indicative of a hypovolemic, hyperadrenergic form of orthostatic intolerance (Pawelczyk *et al.*, 2001) instead of a form resulting from downregulated baroreflex control (Fritsch *et al.*, 1992).

Combined Effect of Bedrest and Caloric Restriction

When hypocaloric intake was combined with BR, tolerance to lower body suction mirrored the response observed during BR alone. Thus, our hypothesis that CR would intensify the development of OI during BR is not supported. Diastolic BP at rest and during LBNP was also similar to BR alone, whereas HR was in between that of the normocaloric BR and hypocaloric ambulatory interventions, and SBP was lower throughout. These data may, therefore, indicate that caloric/fat restriction may

antagonize the enhanced sympathetic activation and endothelial dysfunction (Hesse *et al.*, 2005) of BR and/or enhance the hypovelemia resulting from BR or CR alone.

Limitations

Several factors limit our interpretation of the results. First, the study was relatively underpowered; though ten subjects were recruited, only nine completed the study. Further, data for one subject were not analyzed due to technical problems with data acquisition during one of the trials. Consequently, strong trends were identified for certain variables (e.g. MAP) following the hypocaloric ambulatory intervention alone which may have achieved significance with data from more subjects. Similarly, microneurography was not attempted during the 1st phase, limiting our analysis of MSNA data for all phases. Second, blood volume and cardiac output were not measured; therefore, we can only speculate the contribution of these factors from other related studies. Finally, data for catecholamines, aldosterone, angiotensin II, leptin, insulin, and free fatty acids were either not measured or are not available for the current investigation. These variables would be extremely valuable in determining possible mechanisms to explain our findings.

Conclusion

This study provides the first evidence that 14 days of caloric/fat restriction in healthy men decreases orthostatic tolerance. This condition may result from blunted neurogenic and cardiovascular responses to orthostatic stress. Hypocaloric intake

combined with BR may offset the adaptation to simulated microgravity alone. Future mechanistic studies examining possible neural and hormonal mediators of these responses are warranted.

Table 3-1. Subject characteristics

Age (years)	24±1
Height (cm)	182±2
Weight (kg)	76±2
BMI (kg/m^2)	23±3
Total cholesterol (mg/dl)	161±7
HDL (mg/dl)	51±3
LDL (mg/dl)	97±5
SBP (mmHg)	123±2
DBP (mmHg)	78±3

Values are mean±SEM. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Figure 3-1

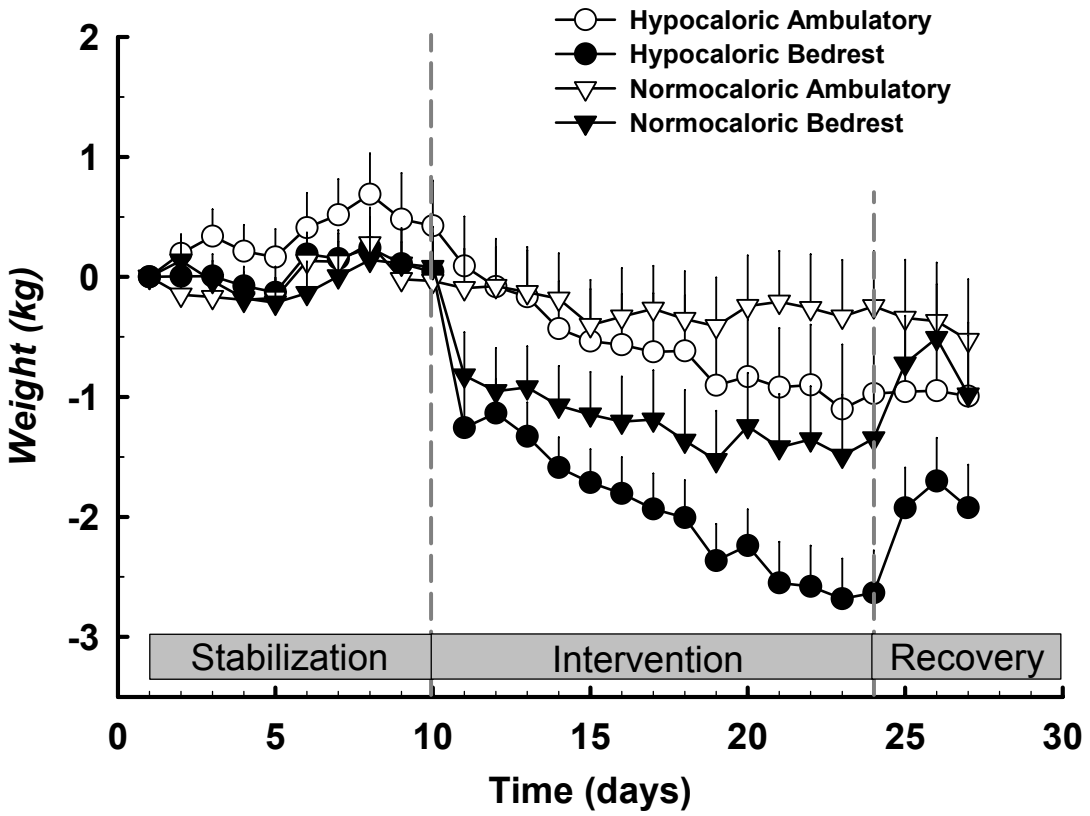

Figure 3-1. Change in subject weight before, during, and after each intervention. Data are presented as mean ± SEM. Weight was significantly reduced vs. baseline and control following all experimental interventions.

Figure 3-2

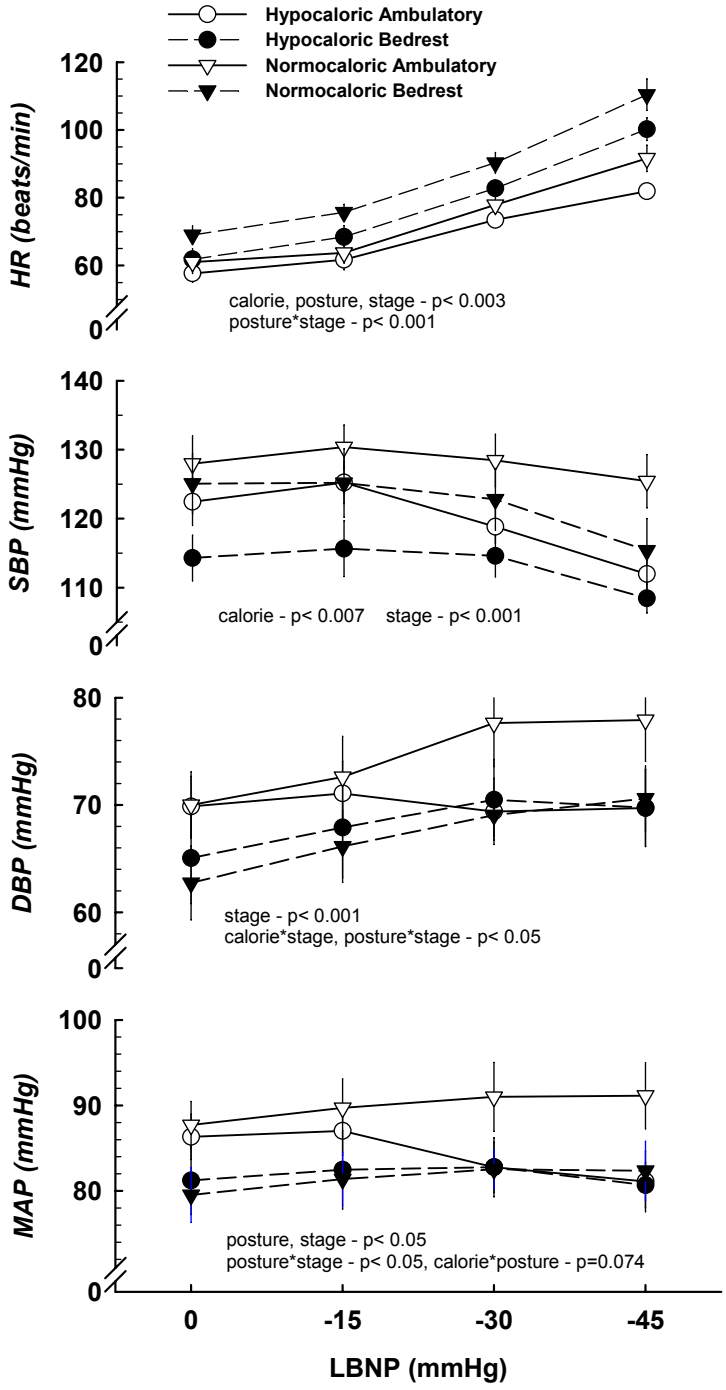

Figure 3-2. Systemic hemodynamic responses to 3 stages of LBNP. Data are presented as mean ± SEM. The main effects calorie, posture, and stage are indicated below each respective graph. Following BR, HR at rest and during LBNP is augmented (posture * time interaction); however, tachycardia was attenuated with CR. Both CR and BR are associated with lower SBP during LBNP. MAP tended to decrease (p=0.074) during LBNP following CR alone.

Figure 3-3

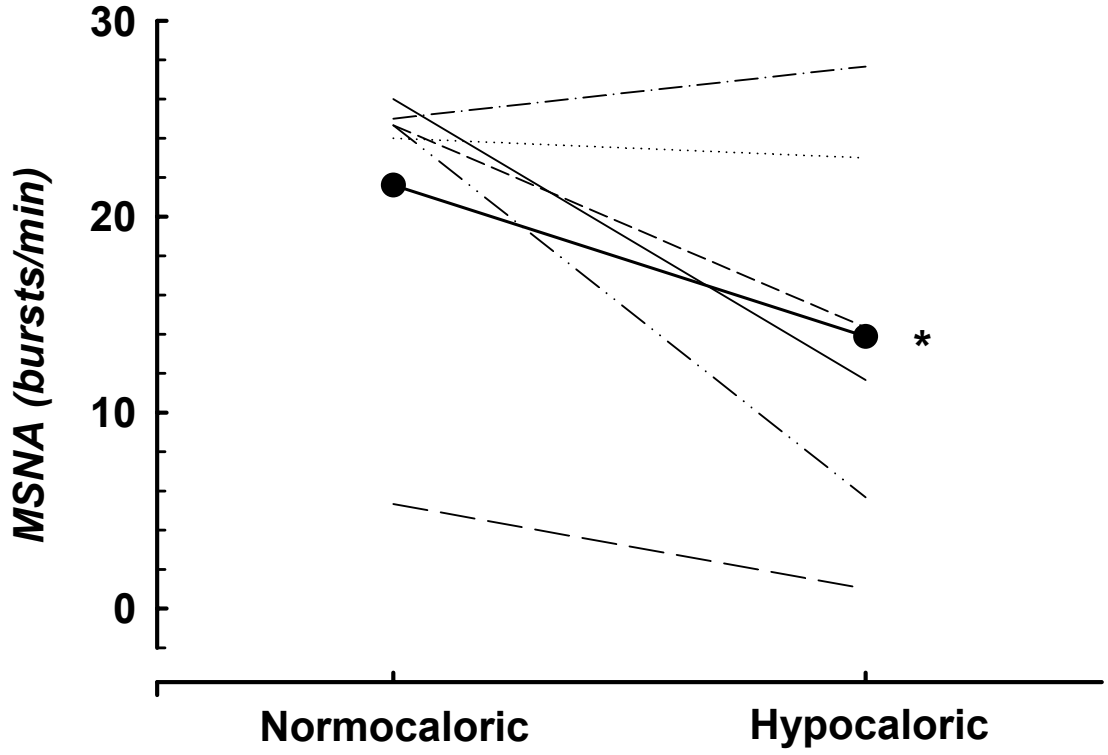

Figure 3-3. Baseline MSNA values before and after the hypocaloric ambulatory trial for 6 subjects. MSNA significantly declined (p=0.035) following CR.

Figure 3-4

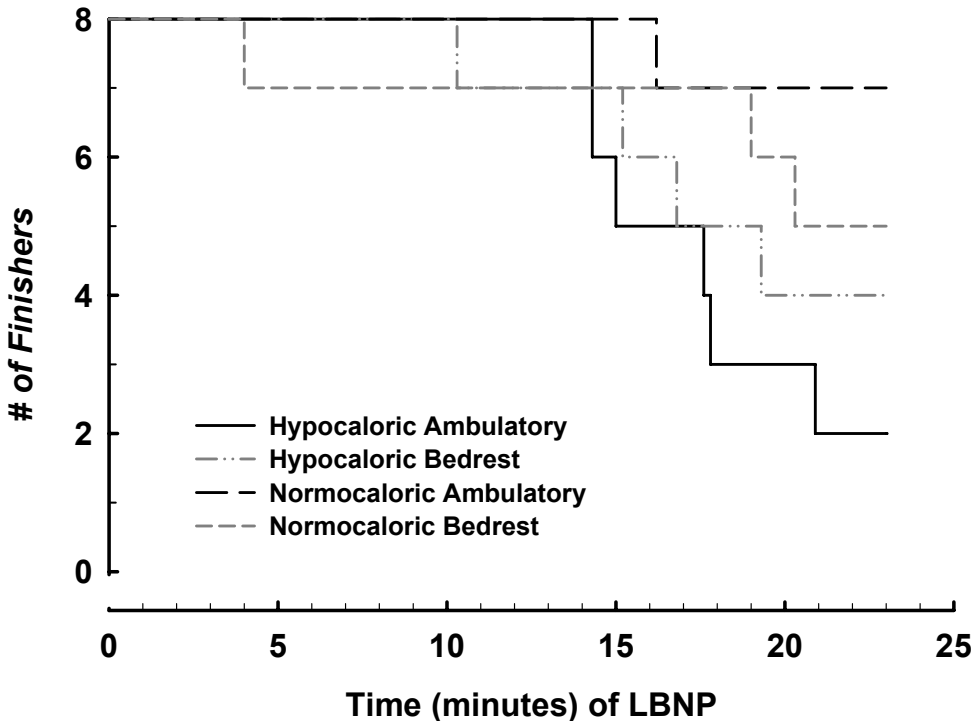

Figure 3-4. Survival analysis comparing the number of finishers and time of failure for non-finishers during LBNP. Caloric restriction vs. normocaloric intake reduces orthostatic tolerance (χ^2, p=0.03), and CR alone shows a tendency (χ^2, p=0.09) toward reduced orthostatic tolerance independent of bedrest.

Chapter 4

HYPOCALORIC INTAKE DIMINISHES THE PRESSOR RESPONSE TO STATIC EXERCISE

Introduction

Exposure to actual (Spaak *et al.*, 2001; Levine *et al.*, 1996; Trappe *et al.*, 2006) or simulated (Kamiya *et al.*, 2000; Kamiya *et al.*, 2004; Pagani *et al.*, 2001; Shykoff *et al.*, 1996; Spaak *et al.*, 2001) microgravity reduces tolerance for physical exertion and alters cardiovascular responses to exercise in humans. Most (Convertino *et al.*, 1998; Kamiya *et al.*, 2000; Kamiya *et al.*, 2004; Pagani *et al.*, 2001; Spaak *et al.*, 2001), but not all (Fu *et al.*, 2002) studies have shown impaired reflex responses to static exercise or cold pressor tests. However, the extent to which spaceflight or bedrest (BR) influences these reflexes and the underlying cause(s) remain unclear.

By employing various stressors, it is possible to characterize afferent and efferent reflex pathways and determine how environmental adaptations (i.e. spaceflight, BR) modulate neural and cardiovascular responses. Static handgrip to fatigue elicits increases in blood pressure (BP), heart rate (HR), and muscle sympathetic nerve activity (MSNA) (Seals & Victor, 1991). Two primary mechanisms are responsible for neural and cardiovascular responses: feedforward control (central command) by activation of the cardiovascular center from descending central neural pathways, and a feedback control

mechanism (exercise pressor reflex) emanating from mechano- and metaboreceptors in skeletal muscles (Rowell & O'Leary, 1990; Seals & Victor, 1991). Reflex pathways originating from cold nociceptors in the skin and involving central vasomotor centers can be assessed by sympathetic and pressure responses to the cold pressor test (Fu *et al.*, 2002; Yamamoto *et al.*, 1992).

A recent study (Stein *et al.*, 1999) reported that astronauts were in negative energy balance (~30%) during a 17-day shuttle mission. Hypocaloric intake reduces HR, BP, and sympathetic activity (Grassi *et al.*, 1998; Williams *et al.*, 2002; Young & Landsberg, 1977b), and we have documented reduced orthostatic tolerance following caloric restriction (Florian *et al.*, 2004). However, the impact of reduced caloric intake alone and in conjunction with microgravity adaptation on neural control of the cardiovascular system during static exercise or cold pressor is not known. Accordingly, the purpose of this study was to test the hypothesis that hypoenergetic intake reduces the responses to cold pressor and static handgrip exercise, and to a greater extent when combined with BR.

Methods

Subjects

Nine healthy men (age: 23.8 ± 3.0 years; BMI: 22.8 ± 3.2 kg/m^2) completed a randomized crossover BR and caloric restriction (CR) study to simulate the effects of spaceflight. This studied conformed with the Declaration of Helsinki, and all subjects signed consent forms approved by the Ethical Committee of the ‘Arztekammer

Nordrhein', Dusseldorf, Germany. Subjects were enrolled if they met all of the following inclusion criteria: physical examination, ECG, urinalysis and routine laboratory without clinically relevant findings, total cholesterol ≤200 mg/dL, LDL ≤130 mg/dL, HDL ≥35 mg/dL, and fasting glucose ≤106 mg/dL. Exclusion criteria included hyperlipidemia, arterial hypertension, diabetes, regular medication and/or treatment with drugs within the last 6 wk, acute or chronic illness, smoking within a period of 1-year preceding the study, and drug and/or alcohol abuse.

Study Design

The study was performed in a randomized cross-over design as part of a multi-disciplinary project (Short-term Bedrest – Integrated Physiology: STBR-IP) evaluating the effects of simulated microgravity and hypocaloric nutrition on cardiovascular and sympathetic nervous function. The subjects participated in 4 study phases that were separated by at least 5 months to allow complete recovery of the participants. Each study phase started with a 9-day adaptation period followed by a 14-day intervention period; in each of the 4 intervention periods the participants were exposed to either BR or ambulatory control conditions, while receiving either a tailored normocaloric or hypocaloric diet. Cardiovascular and sympathetic responses to static handgrip exercise and the cold pressor test were investigated twice in each phase: on the last day of the adaptation period and on day 14 of the intervention period. All four study phases were identical with respect to environmental conditions and study protocol; only the variables posture (BR/ambulation) and energy intake (normocaloric/hypocaloric) were changed.

Ambulatory and Bedrest Conditions

The participants resided in a metabolic ward (Institute of Aerospace Medicine, German Aerospace Center (DLR), Cologne, Germany) during the entire period of the four interventions. Room temperature (24°C) and relative humidity (50%) were kept constant in the metabolic ward and the laboratory. During the BR phases, all activities, including food intake, using the toilet, showering, and weighing, were carried out in the 6° head-down-tilt or horizontal position. 6° head-down-tilt was chosen because it is a validated model for simulation of microgravity (Nixon *et al.*, 1979). Though the induced cardiovascular changes occur more rapidly, their nature and extent is very similar to those observed in supine position. During the ambulatory control phases, the participants maintained upright position during the day and were allowed to walk around in the ward. Though they were not allowed to exercise voluntarily, they followed a light exercise protocol (including bicycle ergometry ~125 W - 15 min twice/day).

Diet

During all adaptation and recovery periods as well as during the normocaloric, ambulatory intervention the participants received a normocaloric standard diet. Energy requirements were calculated for each individual according to the FAO/WHO equations (Lin *et al.*, 2003): participants received a specifically prepared diet containing 1.4 times their basal metabolic rate (BMR). Ten percent of the total calories was added to account for dietary-induced thermogenesis. The average caloric intake was 2722±310 Kcal/day, which consisted of 1 g protein per kg body weight/day, 50 ml water per kg body

weight/day, 2.5 mmol sodium per kg body weight/day, 1000 mg calcium/day, and vitamin D 400 IU/day administered as fixed dose tablets. Dietary protein, fat (saturated and polyunsaturated fatty acids), and carbohydrate intakes were calculated according to dietary reference intake values (Yates *et al.*, 1998) (i.e. 10-15% of energy intake administered as protein, 30% as fat, and 55-60% as carbohydrates). The German recommended dietary intake levels were used for nutrients without experiment-specific requirements. No caffeine, methylxanthine, or alcohol was allowed. Six meals were prepared daily which included three main meals and three snacks. The volunteers received and ate the exact amount of food that was predefined in their individual menu. During the intervention periods, the energy content of the diet was modified as follows: normocaloric, ambulatory: ~2500 Kcal; hypocaloric, ambulatory: about 25% decrease in calories compared to standard diet, ~1875 Kcal; normocaloric, BR: energy content was reduced from the standard diet to adjust to the reduced physical workload; participants received a diet containing 1.1 (instead of 1.4) times their BMR, ~2200 Kcal; hypocaloric, BR: about 25% decrease in calories compared to the respective normocaloric, BR phase, ~1650 Kcal. Reduction in energy intake was mainly achieved by reduction of fat intake to a minimum level of 60g/day in order to keep the recommended level of essential fatty acids. Other than fat, nutrient composition of each experiment day was identical to the normocaloric study periods.

Heart Rate and Arterial Pressure

Heart rate (HR) was derived from a surface electrocardiogram. Beat-to-beat finger arterial pressure was measured by finger photoplethysmography (Portapres,

Amsterdam, The Netherlands), and auscultatory blood pressure (BP) was taken at baseline and at the end of each stage during the protocol.

Muscle Sympathetic Nerve Activity

Peroneal nerve muscle sympathetic activity (MSNA) was recorded as described previously (Wallin & Eckberg, 1982). Briefly, the nerve was located with cutaneous electrical stimulation (Isostim A320, World Precision Instruments). A tungsten reference electrode (FHC, Bowdoinham, ME, USA) was inserted subcutaneously, ~2 cm from the nerve, and a tungsten recording electrode with an uninsulated tip diameter of ~10 μm was inserted through the skin near the nerve. Adjustments of the recording electrode position were made according to auditory signals generated by impaled nerves. Both electrodes were connected in series to a differential preamplifier and an amplifier (NASA, Houston, TX, USA), isolated by two 100 mA current limiters. The nerve signal was amplified (total gain 40,000 to 80,000), band-pass filtered (high pass of .7 kHz and low pass of 2-3 kHz), and then full-wave rectified and smoothed with a resistance-capacitance circuit (time constant, 0.1 s) to produce a recording of "integrated" MSNA. Satisfactory recordings of MSNA were defined by pulse-synchronous bursts that increased during end-expiratory apnoea or Valsalva straining and did not change during tactile or auditory stimulation.

Protocol

Experiments were carried out immediately before and after the 14-day intervention. Data for tests conducted following all four interventions are presented in this report. Each subject was studied while lying supine, with his lower body enclosed in a chamber made of collapsible fabric, which had zippers to access the leg for microneurography. The chamber was used for another study in the STBR-IP autonomic investigations, and was open to air during the cold pressor and static handgrip tests. Each subject performed 3 brief (~3 sec) maximal contractions to determine his maximal voluntary contraction (MVC) by using a handgrip dynamometer subsequent to microneurography electrode placement. The average of the 3 values was used as the MVC.

Cold pressor test. The cold pressor test was carried out after controlled-frequency breathing and Valsalva maneuvers, results of which are not included in this report. Baseline measurements were recorded for 1 minute, and then the subject placed his right hand in a 0-1°C mixture of ice and water for 2 min while maintaining a steady, relaxed breathing pattern. Immediately following the test the subject's hand was removed from the ice water and warmed in a towel while recovery data were recorded for 2 min.

Static handgrip to fatigue. After a sufficient recovery period to allow all signals to return to baseline values following the cold pressor test, baseline HR, arterial pressure, and MSNA were recorded for 1 min. Static handgrip was then performed with the dominant hand at 40% of MVC until fatigue, followed by 2 min of post-handgrip forearm circulatory arrest with an upper arm cuff inflated to 250 mmHg and 2 min of recovery. When the achieved force declined to $< 80\%$ of the target for ≥ 5 sec, the cuff was

inflated. During exercise, the subjects were instructed to avoid the Valsalva maneuver, as well as leg or abdominal muscle tension.

Data Analysis

Each minute of data was analyzed for cold pressor test, and the 2 min of recovery for cold pressor and static handgrip were each averaged to a single value. Because the duration of handgrip was not constant between subjects and interventions, and since sympathetic and hemodyamic responses to static handgrip are dependent on fatigue and not actual duration, data are expressed as a percentage of total time and divided into four equal sections. Since the units for burst frequency are bursts/min, MSNA values for each stage of handgrip are normalized to 1 min.

A repeated measures analysis of variance (ANOVA) was conducted to determine the influence of caloric intake, posture, and time on MSNA and hemodynamic variables. Least squares means with Bonferroni correction were performed when appropriate to detect where differences between factors occurred. The level of significance was set at α=0.05. Values are presented as means ± SEM.

Results

The subject clinical characteristics at screening are presented in Table 4-1. All subjects were young, healthy, normotensive, and nonobese. Subject weights, which were similar at baseline for each intervention, significantly declined following all interventions except control (hypocaloric ambulatory: 79.8±3.6 vs. 78.4±3.7 kg; hypocaloric BR:

78.7±3.3 vs. 76.0±3.3 kg; normocaloric BR: 78.1±2.8 vs. 76.7±2.8 kg; normocaloric ambulatory: 76.9±3.2 vs. 76.7±3.3 kg).

Cardiovascular Response to Handgrip

The time to fatigue during static handgrip was similar following all 4 interventions. Hemodynamic measurements before, during, and after handgrip and post-exercise circulatory arrest are presented in Figure 4-1. Heart rate was significantly lower at baseline and throughout the protocol following CR, whereas BR was associated with a higher HR. At the same relative forces, HR gradually increased during static handgrip, reached its peak at fatigue, and immediately returned to baseline values during post-exercise circulatory arrest following each intervention. The contraction-induced increases in HR were diminished with CR (calorie * time interaction $p<0.001$). Systolic BP and DBP increased progressively during static handgrip, peaked at fatigue, and decreased but remained elevated compared to baseline during post-handgrip circulatory arrest. The increase in DBP (Figure 4-1) and SBP (Figures 4-1 and 4-2) during handgrip were greatly attenuated with CR independent of BR. Responses were well-maintained during post-exercise ischemia.

Sympathetic Neural Response to Handgrip

The MSNA responses to static handgrip and post-handgrip circulatory arrest are depicted in Figure 4-1 (bottom panel). Data for only hypocaloric and normocaloric ambulatory interventions were analyzed because MSNA was not recorded during the 1st

intervention. Baseline MSNA was similar; however, the response during static exercise was significantly attenuated with CR (calorie * time interaction p=0.04). Burst frequency remained elevated during post-exercise ischemia in both interventions.

Cardiovascular and Sympathetic Neural Responses to Cold Pressor

Figure 4-3 shows the hemodynamic and neural responses to the cold pressor test. Heart rate at baseline and during cold pressor was increased with BR. As expected, the cold pressor test increased SBP, DBP, and MSNA, and levels returned to baseline following recovery. Heart rate increased during the 1st minute of cold pressor followed by a decline during the 2nd minute and recovery. No differences in sympathetic and pressure responses were identified for between any of the interventions.

Discussion

Previous studies examining reflex neural control of the cardiovascular system following spaceflight or BR have reported impaired (Convertino *et al.*, 1998; Kamiya *et al.*, 2000; Kamiya *et al.*, 2004; Pagani *et al.*, 2001; Spaak *et al.*, 2001) or intact (Fu *et al.*, 2002; Haruna *et al.*, 1994) functional responses to cold pressor and/or static handgrip exercise. Therefore, the current study was conducted to determine the effects of hypocaloric intake, similar to that during spaceflight (Stein *et al.*, 1999), on neural and cardiovascular control. We hypothesized that caloric/fat restriction alone would alter physiological responses, and that these changes would be exacerbated when CR was combined with BR; however, our findings support only the first part of our hypothesis.

The major findings of this investigation are fivefold: (1) HR at rest and throughout the static handgrip protocol was elevated from BR and reduced with CR; (2) HR and BP responses to handgrip exercise were significantly attenuated following CR trials, independent of BR; (3) MSNA exhibited a blunted response to exercise, but not to post-exercise circulatory arrest (metaboreceptor stimulation), following hypoenergetic intake; (4) HR and BP responses after the normocaloric ambulatory and BR interventions were identical; and (5) HR following both BR trials was significantly greater at rest and throughout cold pressor; however, responses for all variables were well-maintained.

Cold Pressor

Though the overall HR main effect was dependent on posture, we found that the sympathetic and cardiovascular responses to cold pressor were well-maintained following CR, BR, and the combination of the two. This is in agreement with most (Fu *et al.*, 2002; Haruna *et al.*, 1994) but not all (Convertino *et al.*, 1998; Straznicky *et al.*, 1993) studies. The cold pressor test augments central sympathetic activation independent of the baroreflex and so can be utilized to test the efferent limb of the sympathetic loop (Victor *et al.*, 1987). Therefore, maintenance of the neural and cardiovascular responses may confirm that central reflex activation of MSNA and the corresponding vasomotor response is intact following BR and CR.

Why Does Caloric Restriction Severely Attenuate Responses During Static Handgrip?

To our knowledge this is the first experiment to examine the effects of reduced caloric intake on autonomic control during isometric handgrip exercise and cold pressor. Our findings, though mediated by different mechanisms, are synchronous with a previous report (Florian *et al.*, 2004) that CR reduces reflex control of the circulation during orthostatic stress. In certain circumstances the schedule may not have allowed sufficient time for return to the basal state before cold pressor or static handgrip tests, so baseline values should be interpreted with caution. Overall, however, the reduced HR and BP responses are consistent with physiological adaptation to reduced caloric intake. For example, reduced caloric or fat intake lowered HR and BP in rats (Williams *et al.*, 2002; Young & Landsberg, 1977b; Mager *et al.*, 2006) and humans (Straznicky *et al.*, 1993; Bigard *et al.*, 1998).

From this investigation it is not apparently clear why the response is drastically reduced following CR. During static exercise, the increase in HR is controlled primarily by central command and the mechanoreflex, whereas BP is regulated by central command along with mechano- and metaboreflexes, and MSNA predominantly by the metaboreflex (Rowell & O'Leary, 1990). Alterations can occur at a number of points along the muscle mechano- and metaboreflex arcs (e.g. afferent response, central integration, efferent signal) and central command in addition to changes in stimuli and end-organ responses. From our results, it seems most likely that central command and/or the mechanoreflex are attenuated.

Central Command. Immediately at the onset of exercise, central command modulates the level of parasympathetic and sympathetic efferent activity to the heart and

vasculature (Rowell & O'Leary, 1990). The magnitude of control is largely influenced by the individual's perceived effort during actual or attempted exercise, independent of absolute workload or force production. For example, increasing or decreasing central command at a given muscle tension during static exercise results in a corresponding increase or decrease in cardiovascular responses (Goodwin *et al.*, 1972). Although the exact location of integration of these signals is unknown, it appears to include regions of the insular and anterior cingulate cortexes that interact with thalamic and brainstem structures of cardiovascular integration (Williamson *et al.*, 2006).

Nutrient signaling within the hypothalamus and dorsal vagal complex that controls appetite and sympathetic outflow may modulate the influence of central command. A reduction in free fatty acids or other oxidizable fuels with moderate CR influences central neural pathways (Lam *et al.*, 2005; Wade & Jones, 2004) shared by that of central command (Dampney *et al.*, 2002). The blunted neural and cardiovascular responses observed during handgrip are consistent with this concept, though the exact contribution of central command cannot be determined in the present study.

Muscle Mechanoreflex. Mechanosensitive afferents primarily consisting of group III and some group IV fibers respond to stimuli such as stretch, contraction, and pressure (McCloskey & Mitchell, 1972). The mechanoreflex increases HR mainly through cardiac vagal inhibition (Gladwell & Coote, 2002) and may also augment sympathetic activation (McClain *et al.*, 1993; McClain *et al.*, 1994). That the HR and MSNA responses during static handgrip were reduced whereas MSNA continued to increase comparable to the normocaloric intervention during muscle ischemia collectively suggest that the mechanoreflex may be impaired.

We suggest several possibilities for the impaired reflex. First, the sensitivity of muscle afferents are directly proportional to interstitial fluid (Fisher & White, 2004; McClain *et al.*, 1993). Therefore, a reduction in plasma volume and interstitial fluid that may occur with CR (Bigard *et al.*, 1998), and even more so with BR (Pawelczyk *et al.*, 2001), may desensitize the mechanoreceptors. However, this seems unlikely since the cardiovascular responses to handgrip following the normocaloric BR intervention were similar to control. Second, CR may modulate central integration of the mechanoreflex, similar to that of central command. Third, reduced caloric/fat intake may decrease adrenergic sensitivity (Straznicky *et al.*, 1993) while increasing endothelium-dependent and –independent vasodilation (Hesse *et al.*, 2005). This may be consistent with the attenuated BP response; however, it would not explain the diminished sympathetic outflow compared to normocaloric conditions.

Muscle Metaboreflex. Stimulation of the metaboreceptors situated in the interstitial space of muscle elicits increases in MSNA and arterial pressure (Rowell & O'Leary, 1990). The metaboreflex (as well as mechanoreflex and central command) is activated during static handgrip due to buildup of metabolites from mechanical occlusion of blood vessels by the contracting muscle; however, it can be isolated during post-exercise circulatory arrest when mechanical stimulation and central command influences are absent. Since the neural and hemodynamic responses during muscle occlusion were similar following all four interventions, caloric modulation of the metaboreflex is unlikely to contribute to the reduced exercise responses.

Limitations

Several limitations may be associated with the present study. First, microneurography was not attempted during the first phase, limiting the MSNA analysis. Second, sympathetic outflow included only efferent outflow to skeletal muscle, so these findings may not represent sympathetic outflow to other vascular beds. Third, cardiac output and thus systemic vascular resistance was not measured, limiting our ability to interpret the results. Finally, the experiment was performed after controlled breathing and Valsalva maneuvers, which may have affected the neural and cardiovascular responses to exercise and cold pressor. Additionally, due to time constraints during a few experiments, recovery time between cold pressor and static handgrip may have been less than optimal, possibly obscuring baseline results.

Conclusion

In summary, 14-day caloric/fat restriction attenuated MSNA and pressor responses during isometric exercise to fatigue but not to post-exercise muscle ischemia. This indicates that the integrity of the metaboreflex is maintained whereas the influence of the mechanoreflex and/or central command may be reduced. Similar increases in MSNA, HR, and BP during the cold pressor test were recorded following each intervention indicating that central reflex activation may remain intact. We conclude that hypoenergetic low-fat intake reduces the pressor response to static exercise. Further research will be required to 1) more clearly elucidate which portion(s) of the reflexes are

impaired following CR and 2) determine the underlying metabolic/hormonal signals associated with the observed neural and cardiovascular changes.

Table 4-1. Subject characteristics

Age (years)	24±1
Height (cm)	182±2
Weight (kg)	76±2
BMI (kg/m^2)	23±3
Total cholesterol (mg/dl)	161±7
HDL (mg/dl)	51±3
LDL (mg/dl)	97±5
SBP (mmHg)	123±2
DBP (mmHg)	78±3

Values are mean±SEM. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Figure 4-1.

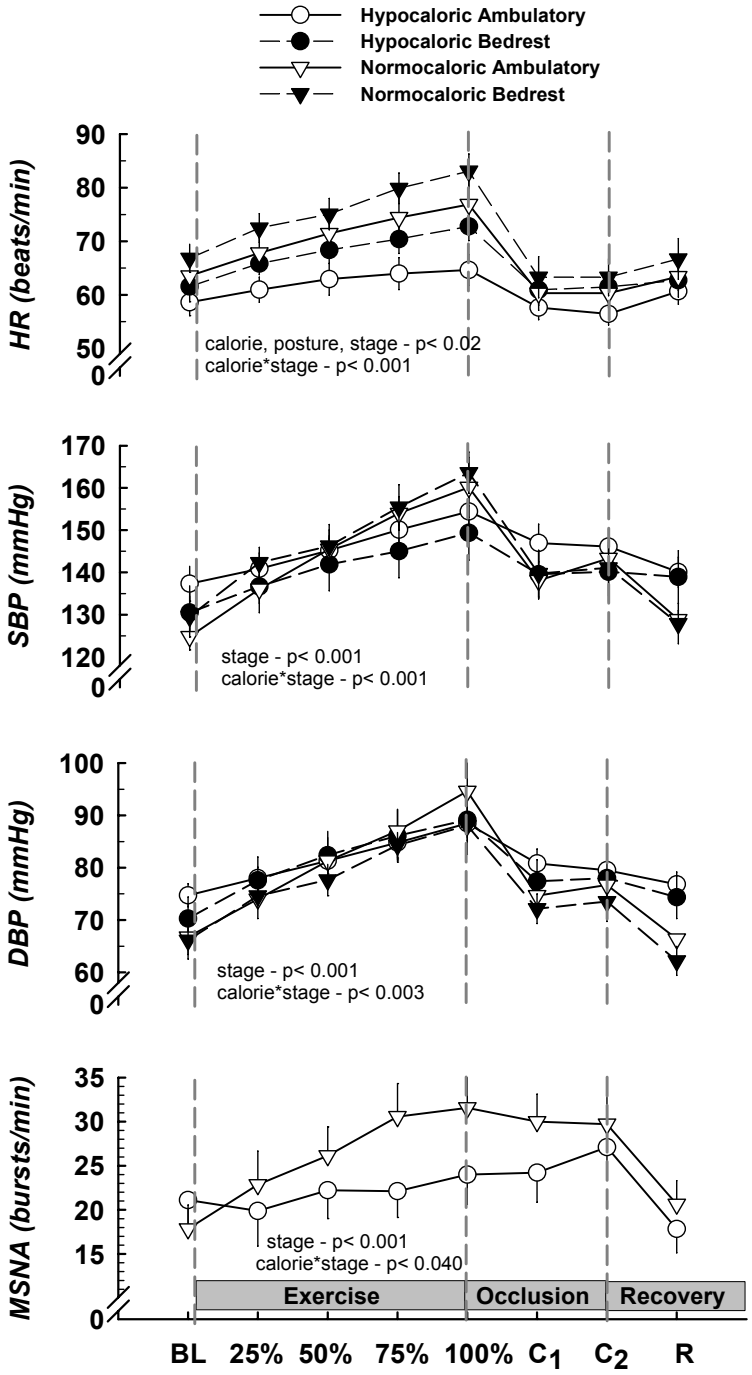

Figure 4-1. Systemic neural and hemodynamic responses to static handgrip and post-exercise muscle ischemia. Data are presented as mean ± SEM. The x-axis during exercise corresponds to the % of time to fatigue; MSNA is adjusted to minute values and expressed as bursts/min. The main effects calorie, posture, and time are significantly different for HR. Following CR, the responses of all variables during exercise are attenuated (calorie * time interaction). Values during 2-min of occlusion are similar.

Figure 4-2

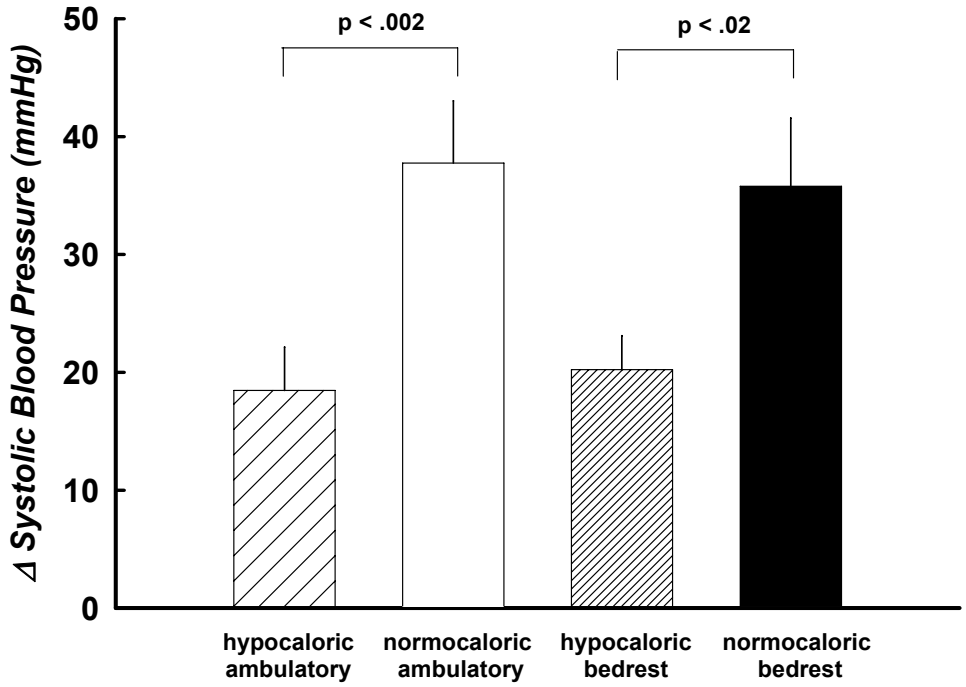

Figure 4-2. The change in SBP at the point of maximum fatigue. Data are presented as mean ± SEM. The SBP response to static handgrip to fatigue was significantly attenuated following CR, independent of BR.

Figure 4-3

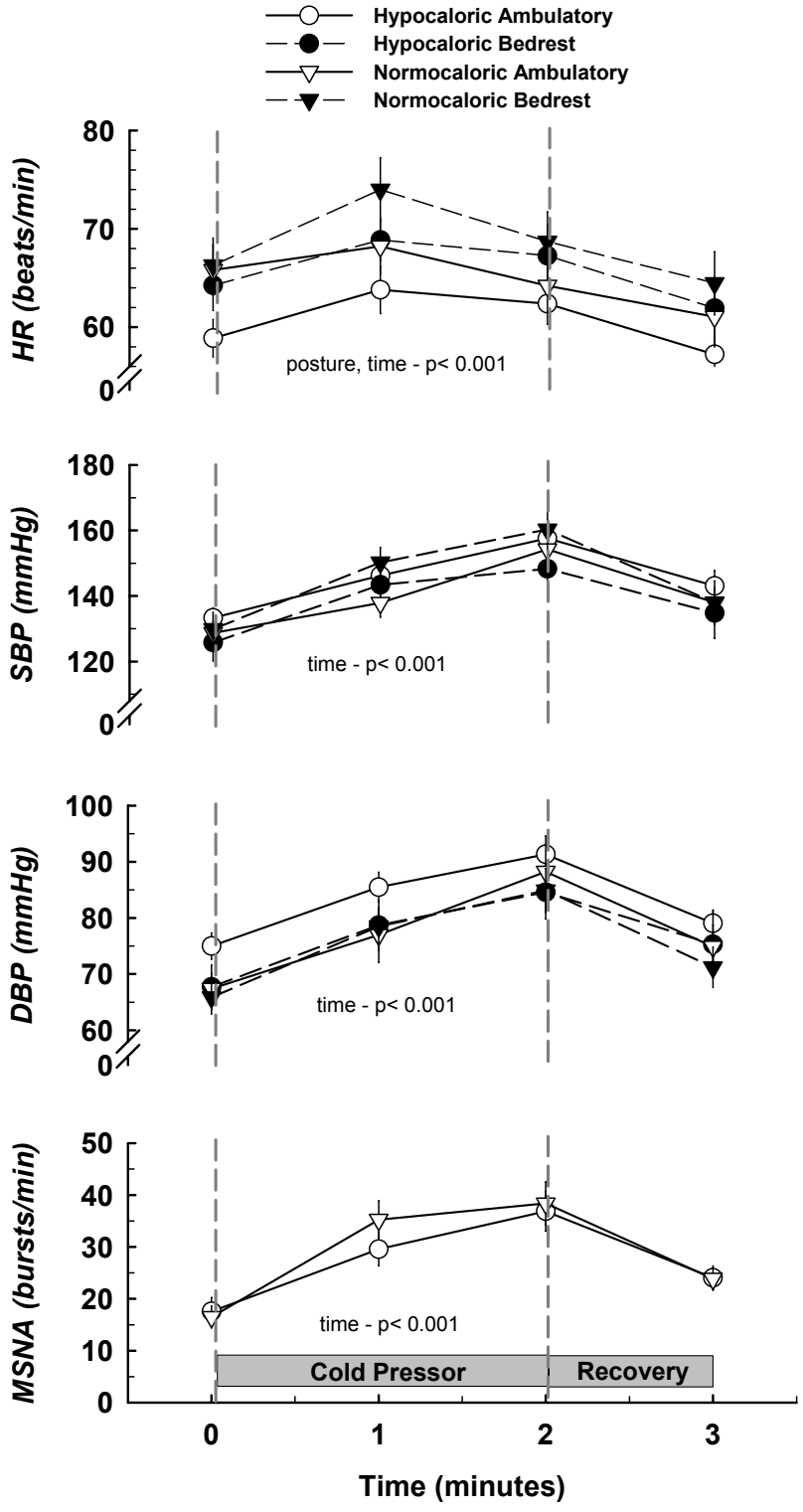

Figure 4-3. Systemic neural and hemodynamic responses to cold pressor test. Data are presented as mean ± SEM. The main effects of posture and time are significantly different for HR, whereas time is significant for BP as well as MSNA.

Chapter 5

FREE FATTY ACIDS INCREASE ARTERIAL PRESSURE VIA CENTRAL SYMPATHETIC ACTIVATION IN HUMANS

Introduction

Accumulating evidence links elevated plasma free fatty acids (FFA) with insulin resistance and hypertension, two components of the metabolic syndrome. Epidemiological research indicates that elevated fasting FFA concentration is a strong independent predictor for the development of hypertension (Fagot-Campagna *et al.*, 1998). Levels of FFA in obese hypertensive patients are approximately twice that measured in lean normotensive individuals (Davda *et al.*, 1995), and insulin's ability to lower FFA is severely impaired in these patients. Moreover, FFA concentration correlates directly with blood pressure (BP) measured at rest and over 24 hours, independently of insulin and insulin-mediated glucose disposal (Egan *et al.*, 1996).

Studies over the past decade have examined whether acutely elevating FFA increases BP, and the underlying mechanisms associated with the pressor response to FFA. For example, Intralipid/heparin (I/H) infusion in minipigs significantly augmented BP and vascular resistance in most tissue beds (Bulow *et al.*, 1990). Likewise, portal and femoral venous infusions of oleate induce a pressor response in rats (Grekin *et al.*, 1995; Grekin *et al.*, 1997), and I/H infusion in humans increased BP and resistance in most

(Stojiljkovic *et al.*, 2001; Paolisso *et al.*, 2000; Lopes *et al.*, 2003a), but not all investigations (Polak *et al.*, 2001).

Despite the emerging literature linking FFA to metabolic abnormalities and hypertension, there is a paucity of data regarding the role of the sympathetic nervous system (SNS) in the pressor response to FFA. This is surprising given the evidence that some forms of hypertension with obesity and high-fat diets may be neurogenically mediated (Hall, 2003; Straznicky *et al.*, 1993). In normotensive rats, the pressor response induced by oleate infusion was inhibited by the α_1-adrenoceptor antagonist prazosin, suggesting sympathetic activation. Paolisso *et al* (2000) reported an increase in plasma norepinephrine (NE) with I/H infusion in humans, whereas another study (Grekin *et al.*, 2005) showed a small but significant decline in NE and a trend toward decreased renal venous NE spillover. It is difficult to reconcile these disparate findings, particularly in the absence of a control group and hemodynamic data in the latter study. More recently, a study assessing baroreflex function did not show a change in sympathetic activation during a 2-hr lipid infusion (Monahan *et al.*, 2007); however, the limited duration of the study may have precluded a complete sympathetic response to elevated FFA. Collectively, these data suggest (Paolisso *et al.*, 2000; Grekin *et al.*, 1997), but do not definitively prove (Grekin *et al.*, 2005; Monahan *et al.*, 2007), a role for the SNS in the pressor response to FFA.

Therefore, the main goal of this study was to investigate the role of FFA on central sympathetic activation by direct measurement of muscle sympathetic nerve activity (MSNA) for a sufficient duration to establish a complete temporal response (i.e. direct sympathetic activation or indirect stimulation by hormonal mediators). A

secondary goal was to determine whether leptin and/or insulin are plausible mediators of the sympathetic response to FFA. Finally, we sought to fill the gaps of previous studies by measuring various hormonal (e.g. aldosterone, angiotensin II) and hemodynamic variables, together with MSNA, in the framework of a randomized, blinded, placebo-controlled research design. We hypothesized that FFA would augment MSNA and BP, and that this response would be associated with increases in insulin and leptin.

Methods

Subjects

Seventeen young (18-31yrs; 10 men, 7 women) subjects participated in the present study. All women were tested during days 2-7 of the follicular phase of their menstrual cycle to minimize the potential influence of hormonal variations on physiological responses (Minson *et al.*, 2000). Each subject underwent a complete medical screening, including CBC, CHEM-24, PT-PTT, lipid profile evaluation, physical examination, DXA, and determination of maximal oxygen uptake ($\dot{V}O_{2max}$). All subjects were healthy, active but not athletically trained, normotensive, non-diabetic, non-obese nonsmokers who were not taking any medications including oral contraceptives. Subjects abstained from alcohol for 2 days and caffeine for 1 day prior to the experiment, and refrained from taking aspirin 1 week prior to the experiment. Approval was obtained from the Institutional Review Board of the Pennsylvania State University. Each subject

gave verbal and written informed consent prior to participation in the study, and all procedures conformed to the standards of the Declaration of Helsinki.

Instrumentation

The study utilized a crossover design involving 2 identical experimental days (differing only in the substance infused), separated by a period of at least 2 weeks. The infusion of 20% I/H or 2.25% glycerol/saline (S/G) was randomized, balanced, and single-blinded. All subjects consumed a standardized meal of 13 kcal/kg, consisting of 55% carbohydrate, 30% fat, and 15% protein between 6 and 8 PM the night prior to the experiment. After an overnight fast, subjects reported to the General Clinical Research Center (GCRC) ~ 7 AM on 2 separate occasions to complete the study. A short 20 gauge polyethylene catheter was inserted into one antecubital vein for infusion of all test substances, while an 18 gauge catheter was placed retrograde into the contralateral dorsal hand vein for blood sampling. This arm was kept in a heating pad throughout the experiment to arterialize venous blood. Patency was maintained in the sampling line by infusion of saline (15 ml/hr). Following catheter insertion, subjects moved to a thermoneutral room and lay supine in a comfortable bed with their arms placed at heart level. Subjects were then instrumented to measure heart rate (HR), BP, calf blood flow (CBF), cardiac output ($\dot{Q}$), and MSNA.

Measurements

Heart rate and arterial pressure. Heart rate (3-lead ECG) was measured continuously and recorded using a customized beat-to-beat data collection software program. Blood pressure was measured by the automated brachial auscultation function of the Colin 7000 after a 30-minute period of rest (2 times at 1-minute interval) and every ten minutes throughout infusion. Mean arterial pressure (MAP) was estimated as diastolic pressure + 1/3 pulse pressure.

Calf blood flow. Calf blood flow (CBF) was determined by venous occlusion plethysmography on the left calf. Changes in limb volume were transduced by using a dual-strand mercury-in-Silastic strain gauge (Hokanson *et al.*, 1975) and amplified (Hokanson EC-4). Strain gauges were sized to approximate the largest circumference of the calf. Blood flow to the foot was excluded from the measurement, with an ankle arresting cuff inflated to 250 mmHg just before flow measurements and deflated following the measurements. A venous occlusion cuff wrapped around the thigh was rapidly inflated to 50 mmHg every 20 s (8 s inflate, 12 s release) 4 times in succession. The lower leg was elevated 15 cm above the subjects' midaxillary line to facilitate venous drainage between determinations.

Blood flows were calculated from the slope of the ascending, linear portion of the plethysmographic response curve by using the line-fitting option in the Hokanson analysis software package (NIVP3) and manual adjustment (to exclude cuff inflation artifact). Calf vascular resistance (CVR) was estimated as corresponding brachial MAP/CBF.

Cardiac output. Cardiac output was measured using the acetylene rebreathing technique first described by Triebwasser *et al* (1977). A mass spectrometer (Perkin-Elmer MGA1100) was used to analyze gas concentration during a 20 second rebreathing period. The accuracy and precision of this method has been validated against thermodilution and direct Fick. Systemic vascular resistance (SVR) was calculated as $\dot{Q}$ /MAP. Stroke volume (SV) was calculated as $\dot{Q}$/HR.

Muscle sympathetic nerve activity. Peroneal MSNA was recorded as described previously (Wallin & Eckberg, 1982). Briefly, the nerve was located with cutaneous electrical stimulation (Isostim A320, World Precision Instruments). A tungsten reference electrode (FHC, Bowdoinham, ME, USA) was inserted subcutaneously, ~2 cm from the nerve, and a tungsten recording electrode with an uninsulated tip diameter of ~10 μm was inserted through the skin near the nerve. Adjustments of the recording electrode position were made according to auditory signals generated by impaled nerves. Both electrodes were connected in series to a differential preamplifier and an amplifier (U. Iowa, Dept. of Bioengineering). The nerve signal was amplified (total gain 40,000 to 80,000), band-pass filtered (high pass of .7 kHz and low pass of 2-3 kHz), and then full-wave rectified and smoothed with a resistance-capacitance circuit (time constant, 0.1 s) to produce a recording of "integrated" MSNA. Satisfactory recordings of MSNA were defined by pulse-synchronous bursts with signal:noise >2 that increased during end-expiratory apnoea or Valsalva straining and did not change during tactile or auditory stimulation. Nerve traffic was expressed both as bursts/min, and as bursts/min * mean burst amplitude, an index of total MSNA.

Protocol

Following instrumentation and a 30 minute adaptation period, baseline hemodynamic and sympathetic data were obtained, and blood was sampled for metabolic and hormonal assays. Subjects then received a 4-hr infusion of either 20% Intralipid (0.8 $ml \cdot m^{-2} \cdot min^{-1}$) (Stojiljkovic *et al.*, 2001) and heparin (200 U bolus, followed by 0.3 $U \cdot kg^{-1} \cdot min^{-1}$) or sham infusion (2.25% glycerol/saline; New England Compounding, Framingham, MA, infused at the same rate and volume as Intralipid/heparin). Heparin was infused with Intralipid to activate lipoprotein lipase and to accelerate the hydrolysis of fatty acids from triglycerides (TG) (Stepniakowski *et al.*, 1995). Glycerol was infused with saline to match for the glycerol contained in 20% Intralipid. During the infusion, $\dot{Q}$ and CBF were measured in duplicate every 30 minutes; MSNA and HR were recorded and averaged over a 5-min period every 30 minutes; and BP was taken every 10 min. Arterialized blood was sampled at baseline and for each hour of infusion thereafter for FFA, TG, glycerol, glucose, insulin, leptin, and catecholamines. Additional blood was sampled at baseline and at the end of infusion for aldosterone, angiotensin II (AT-II), and F_2-isoprostanes. F_2-isoprostanes are a biomarker of oxidative stress that may affect endothelial function.

Blood Samples

Glucose was determined by the oxidase method (Ascencia® ELITE®) immediately after blood collection. All other samples were collected on ice, centrifuged at 4°C, and the plasma was stored at -80°C until assay. Blood for FFA, TG, and glycerol

was drawn into prechilled test tubes containing EDTA and 0.275 g paraoxon/L (Sigma, St. Louis, MO) to inhibit lipoprotein lipase and prevent hydrolysis of FFA from TG *in vitro* (Zambon *et al.*, 1993). Samples for catecholamines were collected in prechilled tubes containing EGTA and glutathione.

Serum insulin (OEM Concepts, Saco, ME) and leptin (R&D Systems, Minneapolis, MN) concentrations were determined by ELISA. Plasma FFA and TG concentrations were determined by a colorimetric method. Glycerol was measured enzymatically (Sigma, St. Louis, MO), catecholamines by HPLC, and aldosterone (Siemens Medical Solutions Diagnostics, Los Angeles, CA) and AT-II (ALPCO Diagnostics, Salem, NH) by RIA. The analytical sensitivity of the aldosterone assay was 0.07 nmol/L. Intraassay and interassay coefficients of variation averaged 2 and 3%, respectively. The analytical sensitivity of the AT-II assay was 0.97 pmol/L. Intraassay and interassay coefficients of variation averaged 9 and 11%, respectively. F_2-isoprostanes were determined by immunoassay (Assay Designs, Ann Arbor, MI). The analytical sensitivity of the F_2-isoprostanes assay was 0.04 ng/ml, and the intraassay and interassay coefficients of variation were 5 and 6%, respectively.

Data Analysis

Five minutes of data from recordings of MSNA and HR were collected at baseline and every 30 minutes during infusion; each 5-minute period was averaged to a single value. Calf blood flow (CBF) was measured 8 times and $\dot{Q}$ was measured in duplicate at

baseline and every 30 minutes during infusion; likewise, data from each period were averaged to a single value.

Statistical Analysis

A repeated measures ANOVA was conducted to detect treatment effects on neural, hormonal, and hemodynamic variables. Least squares means with Bonferroni correction were performed when appropriate to identify where differences between treatments occurred. The level of significance was set at α=0.05. Values are presented as mean±SEM.

Results

Anthropometric, Metabolic, and Hormonal Measurements

All subjects were young, healthy, normotensive, nonobese, and were not insulin resistant (Table 5-1). No sex differences in any responses were detected, so data from men and women were pooled for analysis. Infusions of I/H were well-tolerated by all the subjects.

Plasma FFA, TG, glycerol, and glucose concentrations during the infusions of S/G and I/H are shown in Table 5-2. Infusion of I/H was associated with a significant rise in plasma FFA and TG vs. baseline and S/G, whereas no changes occurred with S/G. Plasma glycerol increased with S/G infusion and to a greater extent during I/H infusion. No significant changes in plasma glucose concentrations occurred with either day.

Serum leptin and insulin, and plasma epinephrine concentrations are reported in Figure 5-1. Plasma aldosterone, AT-II, and F_2-isoprostane concentrations are presented in Table 5-3. Baseline hormone concentrations were similar in both experimental conditions. Leptin concentration decreased during both infusions; however, the treatment * time interaction ($p=0.053$) suggests a tendency for FFA to maintain leptin levels compared to the decline in leptin with placebo. Insulin concentration rose significantly by hours 3 and 4 of I/H infusion, but did not increase in response to S/G. Infusion of I/H significantly lowered plasma NE concentration by hours 3 and 4, whereas NE increased by the final hour of S/G infusion. When expressed as a change from baseline (Figure 5-2), NE concentration was unchanged with fat; however, a significant treatment interaction ($p=0.001$) was still present. Both F_2-isoprostanes and aldosterone increased during I/H infusion, and aldosterone significantly decreased during S/G administration. No changes in AT-II and epinephrine concentrations were noted with either I/H or placebo.

Hemodynamic Measurements

As shown in Table 5-3, I/H infusion was associated with a significant increase systolic BP (+14.0±1.6 vs. +3.2±2.5 mmHg), diastolic BP (+8.2±1.0 vs. -0.1±1.7 mmHg), and HR (+8.2±1.0 vs. +2.4±1.2 beats min^{-1}) vs. S/G infusion ($p<0.001$), especially during the last 2 hours of infusion. Despite the increase in HR with I/H, $\dot{Q}$ (Figure 3) remained unchanged due to a decrease in SV, which was not different compared to the S/G day. Paralleling the increase in MSNA, with I/H infusion SVR was

significantly elevated vs. baseline ($p<0.001$) at hour 4, but did not change during S/G infusion. Whereas CBF (Figure 2) increased significantly by hour 2 and remained elevated for the duration of I/H, the increase during S/G infusion was not significant until the final time point. The change in CBF was greater with I/H vs. S/G over the last 2 hours of infusion.

MSNA and Vascular Resistance

Changes in MSNA burst frequency and total MSNA in response to I/H and S/G infusion, along with CVR, are displayed in Figure 5-2. MSNA recordings could not be obtained for 4 subjects because of shifts in electrode position or failure to meet signal-to-noise criteria. Furthermore, data for total MSNA were excluded if there was a shift in electrode position at any point during the experiment, leaving data for 8 subjects for the total MSNA quantification. Due to the challenges in maintaining the microneurography recording over a period of 4-5 hrs, we recruited more subjects than necessary to attain sufficient statistical power to identify differences in MSNA in the case of missing data. Thus, the more limited size of the MSNA sample should be regarded as adequate and representative of the overall sample.

Baseline burst frequency was not different between the two days, and Bland-Altman analysis revealed that 85% of the baseline MSNA values for both days were repeatable within 25%. This compares favorably to previous studies (Kimmerly *et al.*, 2004). Both the change in burst frequency (+29.9% vs. +0.0%) and total MSNA (+65.1% vs. +12.3%) in comparison to baseline and S/G control were augmented after

the 2nd hour with I/H and continued to increase until the end of infusion. CVR tended to decrease over the 4-hr I/H vs. S/G infusion (p=0.08). Finally, the change in SVR during lipid infusion was directly related to the change in burst frequency (Figure 4).

Discussion

This investigation was conducted to determine the role of the SNS in the pressor responses to FFA and to examine possible mediators of the sympathetic and cardiovascular response to high FFA levels. The primary new findings of the present study were that (1) acute elevation of plasma FFA with infusion of I/H increases central sympathetic activation measured from sympathetic postganglionic neurons, (2) the augmentation of MSNA was associated with a rise in insulin but not leptin concentrations, and (3) FFA increased aldosterone and F_2-isoprostanes, but not AT-II, to contribute to the hemodynamic response.

Our results confirm earlier reports that increased FFA and/or TG concentrations, as might be seen in insulin resistant individuals following a meal, elevate BP and HR (Lopes *et al.*, 2003a; Paolisso *et al.*, 2000; Stojiljkovic *et al.*, 2001). Our findings are similar to one study which reported a rise in SBP/DBP and HR by 13.5/8.0 ± 2.1/1.5 mmHg and 9.4 ± 1.4 beats/min following 4-hr infusion of I/H, resulting from both an increase in SVR and $\dot{Q}$ (Stojiljkovic *et al.*, 2001). However, the increase in BP in the present study resulted from augmented SVR, despite a rise in CBF and no change in $\dot{Q}$. The observation that SVR was elevated while $\dot{Q}$ was unaltered and CBF was increased with lipid infusion suggests a redistribution of blood flow and vasoconstriction in

vascular beds other than muscle (Bulow *et al.*, 1990). Most studies are in agreement that FFA promote insulin resistance and decrease endothelium-dependent vasodilation (de Kreutzenberg *et al.*, 2000; Steinberg *et al.*, 1997; Steinberg *et al.*, 2000); however, the endothelial dysfunction documented in these investigations is typically associated with a paradoxical increase in basal limb blood flow. Although few studies have examined the underlying mechanisms of increased basal limb blood flow from elevated FFA (Kearney *et al.*, 2002; Stepniakowski *et al.*, 1997), possible explanations include insulin-induced NO release or vasodilating prostaglandin production (Stepniakowski *et al.*, 1997).

To date, limited data suggest (Grekin *et al.*, 1997; Paolisso *et al.*, 2000), but have not definitively proven (Grekin *et al.*, 2005; Monahan *et al.*, 2007), a link between the rise in BP and sympathetic activation. The increase in burst frequency (~30%) and total MSNA (~65%) recorded in this study clearly support a role for central sympathetic activation in the pressor response to FFA. Our results are not inconsistent with a recent study that failed to show an increase in MSNA with just two hours of hyperlipidemia (Monahan *et al.*, 2007) since MSNA did not begin to increase in the current study until ~2.5 hours of lipid infusion. An additional finding in this investigation is that the increase in SVR was directly associated with central sympathetic activation. Further studies blocking FFA-induced sympathetic activation are necessary to determine a causal relation.

Notably, NE decreased modestly during I/H infusion despite an increase in MSNA. At rest, MSNA is representative of sympathetic activity to other vascular beds and is positively related to plasma concentration of NE. However, the change in NE concentration may reflect as little as 20% of the change in MSNA (Wallin, 1988),

demonstrating that measurement of NE alone in some cases may be an inadequate representation of sympathetic outflow. Moreover, dissociation of the MSNA-plasma NE relation has been documented with modulation of blood flow, neuronal reuptake, NE degradation, or clearance (Esler *et al.*, 1990; Floras, 2003; Khan *et al.*, 2002; Thompson *et al.*, 1995; Leuenberger *et al.*, 1991). Prior studies have shown either an increase (Paolisso *et al.*, 2000) or a slight decrease in NE concentration with lipid infusion (Grekin *et al.*, 2005), paralleled by an increase in clearance of NE in the latter study. Thus, increased clearance may explain, at least in part, the decline in NE in this study.

Possible Mechanisms of Central Sympathetic Activation

We considered 4 potential mechanisms by which FFA increased MSNA: (1) activation of vagal afferents, (2) direct sensing in the hypothalamus, (3) mediation by nutrient-sensing hormones such as leptin and/or insulin, and (4) modulation of the baroreflex. First, chemosensitive afferents arising from the abdominal viscera respond to lipids to influence discharge of vasomotor neurons of the rostral ventrolateral medulla (Ueta *et al.*, 2000). Second, FFA cross the blood-brain barrier in proportion to circulating plasma FFA concentrations and activate the POMC/CART neural pathway resulting in decreased energy intake and activation of the SNS (Lam *et al.*, 2005). Though we cannot rule out the contribution of either of these mechanisms, the latter two can be examined in greater detail.

Hormonal mediation. Leptin and insulin emerged as possible mediators of central sympathetic activation from FFA due to their characteristic responses to changes in

adiposity and food intake. Both leptin and insulin stimulate POMC/CART neurons in the hypothalamus, which project to other brain centers and ultimately stimulate sympathetic preganglionic neurons to modulate energy expenditure and BP (Elmquist, 2001). Further support is evidenced by the observations that i.v. and i.c.v. administration of leptin increase both sympathetic activity and BP in conscious animals (Dunbar *et al.*, 1997).

Our results support a modest role for insulin as a mediator of sympathetic activation; however, leptin does not seem to be regulated extensively by elevated FFA, at least in the acute setting. The roughly 30% increase in plasma insulin reported in the present study is in agreement with some (Paolisso *et al.*, 2000; Steinberg *et al.*, 2000; Vollenweider *et al.*, 1995), but not all previous studies (Fugmann *et al.*, 2003; Polak *et al.*, 2001).

Previous studies have reported divergent findings regarding a leptin response to changes in FFA. Peak leptin is positively correlated with a nocturnal peak in FFA levels (Heptulla *et al.*, 2001). Studies in rats (Wang *et al.*, 1998) and humans (Nisoli *et al.*, 2000) reported elevated leptin concentrations and leptin mRNA, respectively, following lipid infusion. In contrast, leptin did not change following 150 min lipid infusion (Chen & Song, 2002) and actually decreased in another study (Garcia-Lorda *et al.*, 2003). Since the nadir for leptin levels occurs in the early afternoon, it is possible that the decline in leptin on the control day simply reflected the diurnal rhythm, whereas elevated FFA supported maintenance of leptin concentration. Though serum leptin levels did not change substantially, the influence of leptin on the sympathetic response cannot be ruled out completely. Recent studies have shown rapid exocrine leptin secretion by the gastric mucosa in response to a meal which can act in concert with cholecystokinin to activate

vagal afferents (Cammisotto *et al.*, 2005). It is not known, however, whether systemic lipid infusion initiates this form of leptin secretion. Nevertheless, it is unlikely that endocrine leptin in the acute setting is a mediator of FFA-induced sympathoexcitation.

Baroreflex modulation. Two recent studies have presented conflicting evidence regarding acute baroreflex resetting and reduced baroreflex sensitivity in response to 1-hr lipid infusion (Gadegbeku *et al.*, 2006; Monahan *et al.*, 2007). Although our study was not designed to assess baroreflex function, the concomitant increase in BP, HR, and MSNA supports the concept of resetting of the arterial baroreflexes to defend higher pressures. Alternatively, increases in MSNA may be a reflex response to muscle vasodilation (rather than direct or indirect central activation from elevated FFA). Two observations suggest that muscle vasodilation is not the mechanism for increased MSNA during lipid infusion. First, the BP response to I/H far exceeds expected baroreflex compensation although CBF increased 20% within the first hour and 60% by the fourth hour of I/H infusion. By estimating the total skeletal muscle mass and volume of each subject and assuming that the increased CBF occurred in all the skeletal muscle vasculature, the expected final MAP if $\dot{Q}$ and resistance in non-skeletal muscle beds remained at control levels would be 72.8 mmHg vs. the basal MAP of 76.2 mmHg, considerably less than the measured BP after I/H infusion. Second, since MSNA did not increase until after the second hour of infusion with I/H, whereas CBF and BP increased by the first hour, it is unlikely that MSNA augmentation resulted from a pure baroreflex response.

Could Peripheral Mechanisms Contribute to the Pressor Response?

While central sympathetic activation contributes to the pressor response to FFA, data from this study also suggest other peripheral mechanisms are involved. As in previous studies (Lopes *et al.*, 2003a), F_2-isoprostanes, a biomarker of oxidative stress that has been linked to hypertension, doubled during I/H infusion in the present study, supporting its role in the BP response to FFA. Although no changes were observed in AT-II concentrations on either experimental day, aldosterone increased by ~38% by the end of I/H infusion, in contrast to the ~44% drop on the control day. The increase in aldosterone is consistent with a recent study which demonstrated the adrenal stimulating effects of oxidized linoleic acid derivatives (Goodfriend *et al.*, 2004). The reason for the decline in aldosterone on the control day is unclear; however, it is likely that the postural shift in blood volume and central venous pressure is a controlling factor (Gauer, 1975). Other mediators of a pressor response to FFA not measured in this study include direct stimulation of protein kinases that cause constriction and increased sensitization of vessels to α-adrenergic stimulation (Stepniakowski *et al.*, 1995). From our data it seems likely that both peripheral and central mechanisms play a role in the hemodynamic response to elevated FFA. Interestingly, in agreement with a previous study of similar duration (Stojiljkovic *et al.*, 2001), a biphasic response was observed such that BP increased within the first 30 min and remained relatively stable, followed by an even greater increase during the final 2 hr of lipid infusion. Additionally, in our study HR and SVR did not begin to rise until the final 2 hr, coinciding with the increase in MSNA.

These observations may reflect an initial direct vascular effect of fat followed by central activation (after ~2 hrs of infusion) and the corresponding hemodynamic responses.

Limitations

Data from previous studies suggested that an infusion duration of at least 3-4 hrs was necessary to evoke the hypothesized hormonal, neural, and hemodynamic responses (Paolisso *et al.*, 2000; Wang *et al.*, 1998). Due to the length of the study two issues were of concern. First, any leg movement by the subject could alter the nerve recording or disrupt the signal altogether. Consequently, data could not be analyzed for burst frequency in ~20% and for total MSNA in ~50% of subjects. Despite the reduction in MSNA data, the statistical power was large enough to identify differences for lipid infusion compared to control. Second, it is possible that stress or bladder distension contributed to the sympathetic and hemodynamic response (Fagius & Karhuvaara, 1989) over the roughly 5 hr basal and infusion period. To mitigate bladder distension subjects voided using a condom catheter or urinal while remaining supine and stationary. Notably, MSNA did not rise during the placebo infusion, suggesting that the autonomic responses were indeed not altered by a time-dependent confounding effect such as bladder distension or stress. Additionally, from this study we cannot determine the relative contributions of central vs. peripheral mechanisms underlying the BP response to fat. Future studies that inhibit sympathetic activation from FFA may provide further clarification. Finally, the data result from an acute increase in FFA; any conclusion about

the long-term effects of chronic augmentation of FFA concentration on BP or cardiovascular risk should be considered speculative.

Conclusion

This study provides direct evidence that in humans the pressor response to FFA is mediated, at least in part, by sympathetic activation. Moreover, insulin, F_2-isoprostanes and aldosterone, but not leptin, may contribute to the hemodynamic response during lipid infusion. The neurogenic response documented in this investigation implicates central sympathetic activation as a possible mechanism linking conditions characterized by high FFA (e.g. obesity, dyslipidemia) with hypertension. Further studies examining the effect of more chronic elevation of FFA on autonomic control in humans and whether reducing FFA concentration results in a corresponding decrease in sympathetic activity are necessary to more precisely elucidate the relation between FFA and sympathetic activation in some forms of hypertension.

Table 5-1. Subject characteristics

Sex (M, F)	10, 7
Age (years)	22±1
Height (cm)	173±0
Weight (kg)	69±3
BMI (kg/m^2)	23±1
Body fat (%)	20±2
Waist-to-hip ratio	0.88±0.01
$\dot{V}O_{2\,max}$ (ml/kg/min)	45±2
Total cholesterol (mg/dl)	155±6
HDL (mg/dl)	53±3
LDL (mg/dl)	86±5
HOMA-IR	0.73±0.11
QUICKI	0.43±0.01

Values are mean±SEM. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check.

Table 5-2. **Time course of plasma FFA, TG, glucose, and glycerol concentrations during infusion**

Time (hours)		0	1	2	3	4
FFA (μmol/L)	I/H	502±52	1419±51*†	2104±142*†	2301±93*†	2266±110*†
	S/G	502±52	518±50	556±50	617±36	657±38
TG (mmol/L)	I/H	0.87±0.07	3.03±0.13*†	4.17±0.30*†	4.99±0.43*†	5.76±0.54*†
	S/G	0.79±0.07	0.97±0.07	0.94±0.06	0.97±0.07	1.00±0.07
Glycerol (μmol/L)	I/H	72±9	774±44*†	1072±61*†	1239±81*†	1288±101*†
	S/G	79±7	239±10*	244±12*	243±16*	262±16*
Glucose (mmol/L)	I/H	4.45±0.10	4.69±0.07	4.50±0.09	4.35±0.09	4.46±0.08
	S/G	4.52±0.10	4.52±0.12	4.38±0.13	4.57±0.13	4.55±0.12

Values are mean±SEM of 17 subjects.
*Significant vs. baseline ($p<0.001$). †Significant vs. S/G control day ($p<0.001$).

Table 5-3. Baseline and final values for neural, cardiovascular, and hormonal variables during infusion

	Intralipid/heparin		Saline/glycerol		
	0 hrs	4 hrs	0 hrs	4 hrs	Interaction
Heart Rate (beats/min)	58.7±2.4	69.0±3.1*†	60.9±2.3	63.3±2.5	<0.001
Systolic BP (mmHg)	109.2±2.1	123.2±2.7*†	112.4±2.5	116.0±3.3	<0.001
Diastolic BP (mmHg)	59.7±1.9	68.5±1.8*†	62.1±1.4	61.7±2.4	<0.001
MAP (mmHg)	76.2±1.7	86.7±2.0*†	78.8±1.6	79.8±2.5	<0.001
Cardiac Output (L/min)	6.5±0.4	6.4±0.4	6.5±0.4	6.4±0.4	0.983
Stroke Volume (ml)	86.7±6.1	79.1±6.9	85.3±5.6	79.8±6.4	0.904
SVR (dyne · s · cm^{-5})	991±80	1151±80*	1007±56	1063±64	0.070
MSNA (bursts/min)	16.3±1.7	21.2±1.8*	20.5±2.2	20.8±2.3	0.005
Total MSNA (% baseline)	100±0	165±24*†	100±0	112±16	0.002
CBF (ml/100 ml/min)	1.65±0.23	2.60±0.29*†	1.95±0.14	2.36±0.17*	0.032
CVR (units)	54.4±4.6	38.7±3.2	45.1±4.5	37.2±3.5	0.080
Norepinephrine (nmol/L)	0.93±0.06	0.74±0.06*†	0.76±0.07	0.99±0.08*	0.001
Leptin (pg/ml)	5.43±1.24	5.11±1.16*	5.37±1.19	4.55±1.05*	0.053
Insulin (pmol/L)	23.5±4.2	30.0±4.3*†	23.3±2.3	18.8±2.0	0.004
Epinephrine (pmol/L)	480±34	492±58	461±35	456±29	0.937
Aldosterone (nmol/L)	0.08±0.01	0.11±0.01*†	0.09±0.01	0.05±0.01*	<0.001
Angiotensin II (pmol/L)	3.84±0.58	3.02±0.51	4.0±0.62	3.5±0.54	0.624
F_2-isoprostanes (pg/ml)	12.4±1.2	24.2±1.1*†	12.9±1.1	14.8±0.7	0.015

Values are mean±SEM. MAP, mean arterial pressure; SVR, systemic vascular resistance; CBF, calf blood flow; CVR, calf vascular resistance.
*Significant vs. baseline ($p<0.05$). †Significant vs. S/G control day ($p<0.05$).
Interaction indicates p-value of 2-way (treatment * time) repeated measures ANOVA interaction.

Figure 5-1

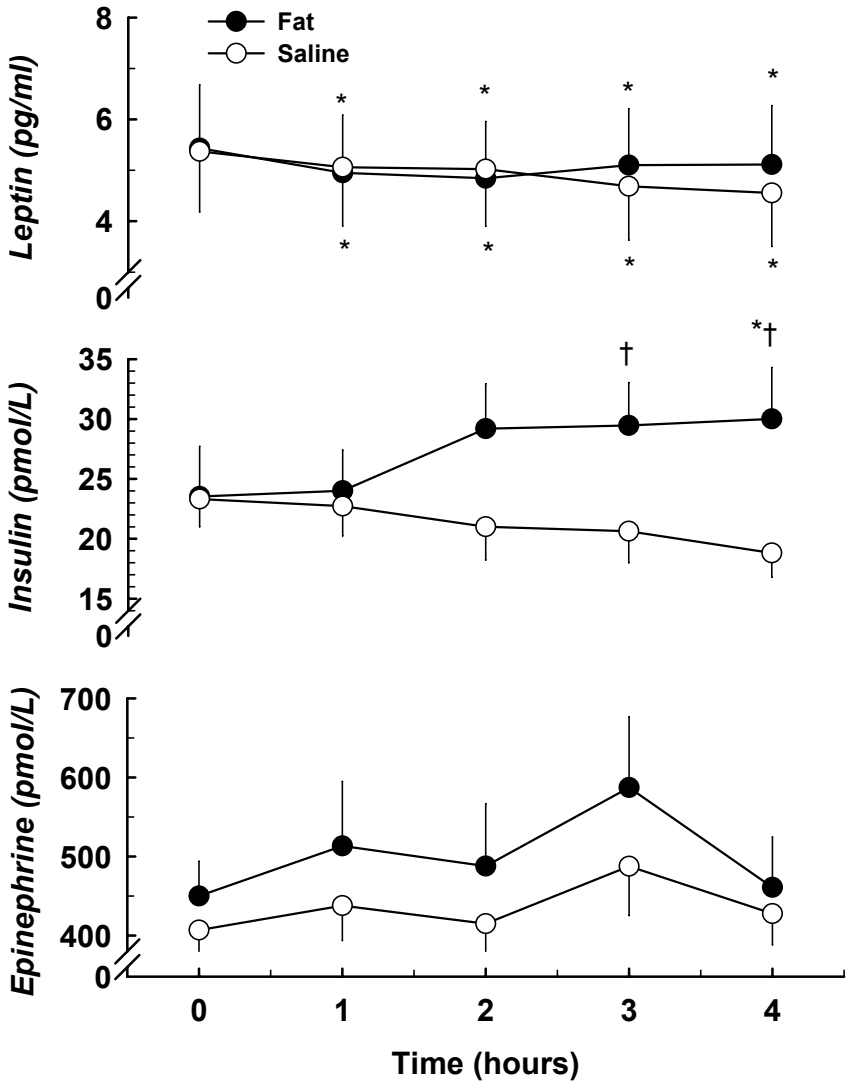

Figure 5-1. Hormonal responses to infusion of Intralipid/heparin (fat ●) and saline/glycerol (saline ○). Values are mean±SEM of 17 subjects. *Significant vs. baseline ($p<0.05$). †Significant vs. saline control day ($p<0.05$).

Figure 5-2

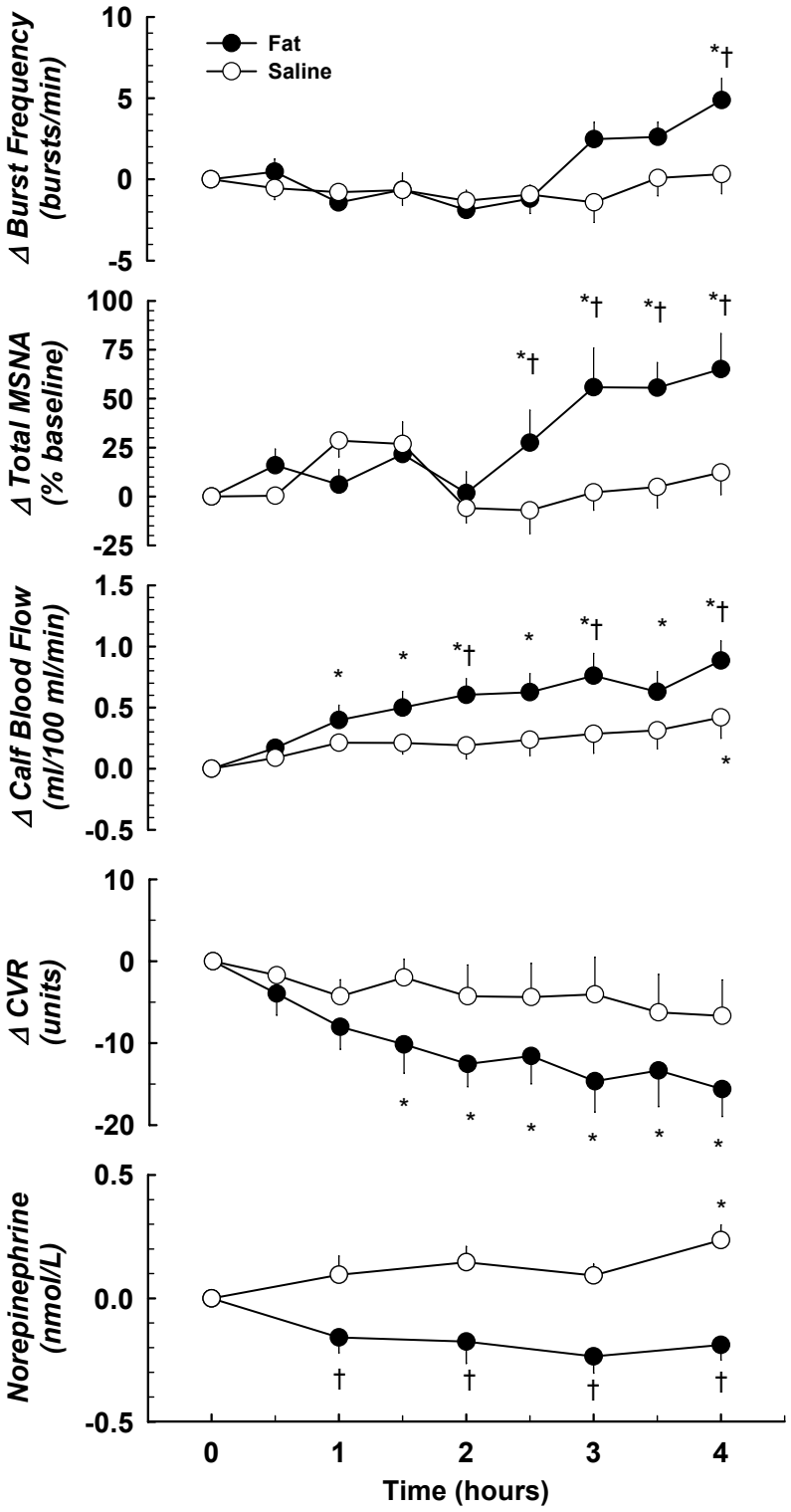

Figure 5-2. Responses to infusion of Intralipid/heparin (fat ●) and saline/glycerol (saline ○). Values are mean±SEM. CVR, calf vascular resistance. *Significant vs. baseline ($p<0.05$). †Significant vs. saline control day ($p<0.05$).

Figure 5-3

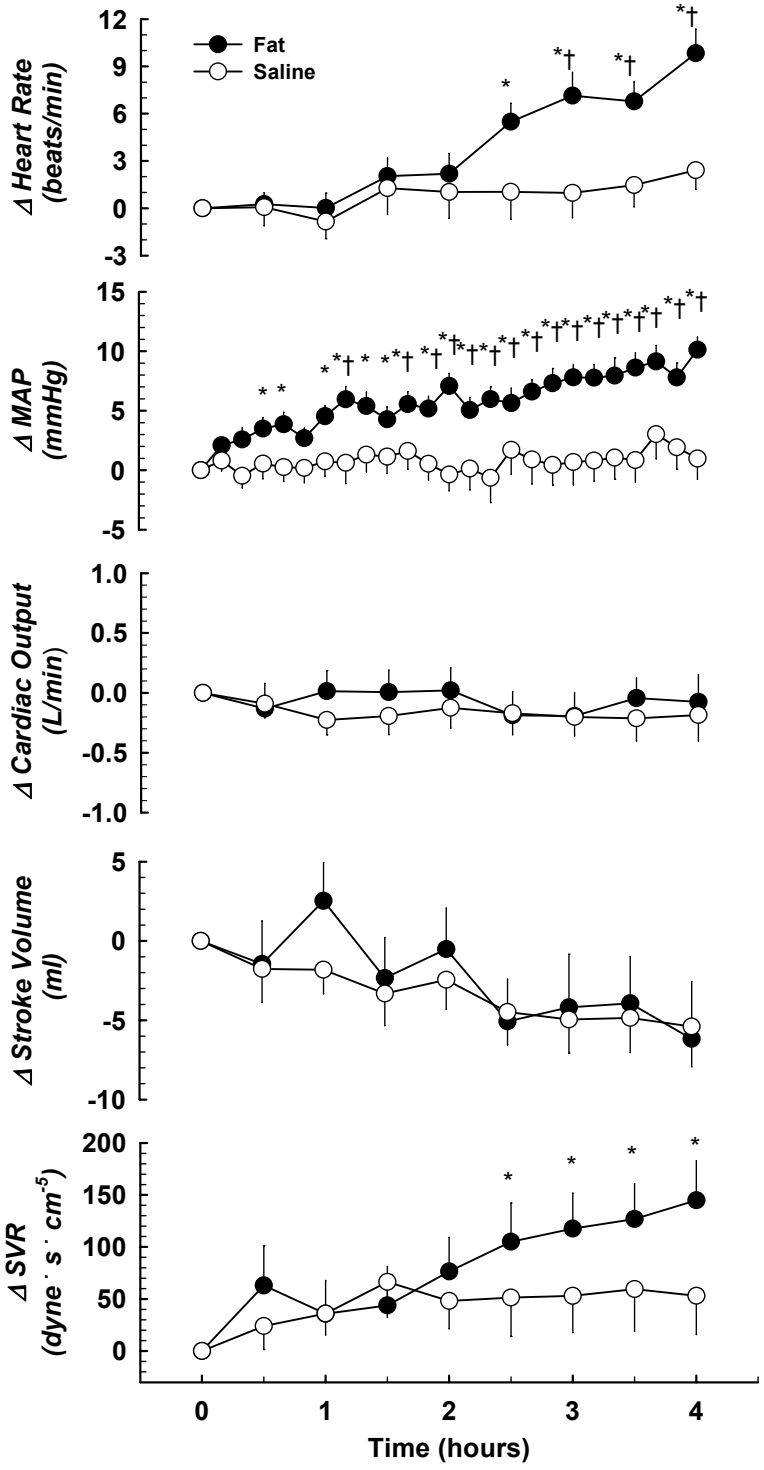

Figure 5-3. Systemic hemodynamic responses to infusion of Intralipid/heparin (fat ●) and saline/glycerol (saline ○). Values are mean±SEM of 17 subjects. MAP, mean arterial pressure; SVR, systemic vascular resistance. *Significant vs. baseline ($p<0.05$). †Significant vs. saline control day ($p<0.05$).

Figure 5-4

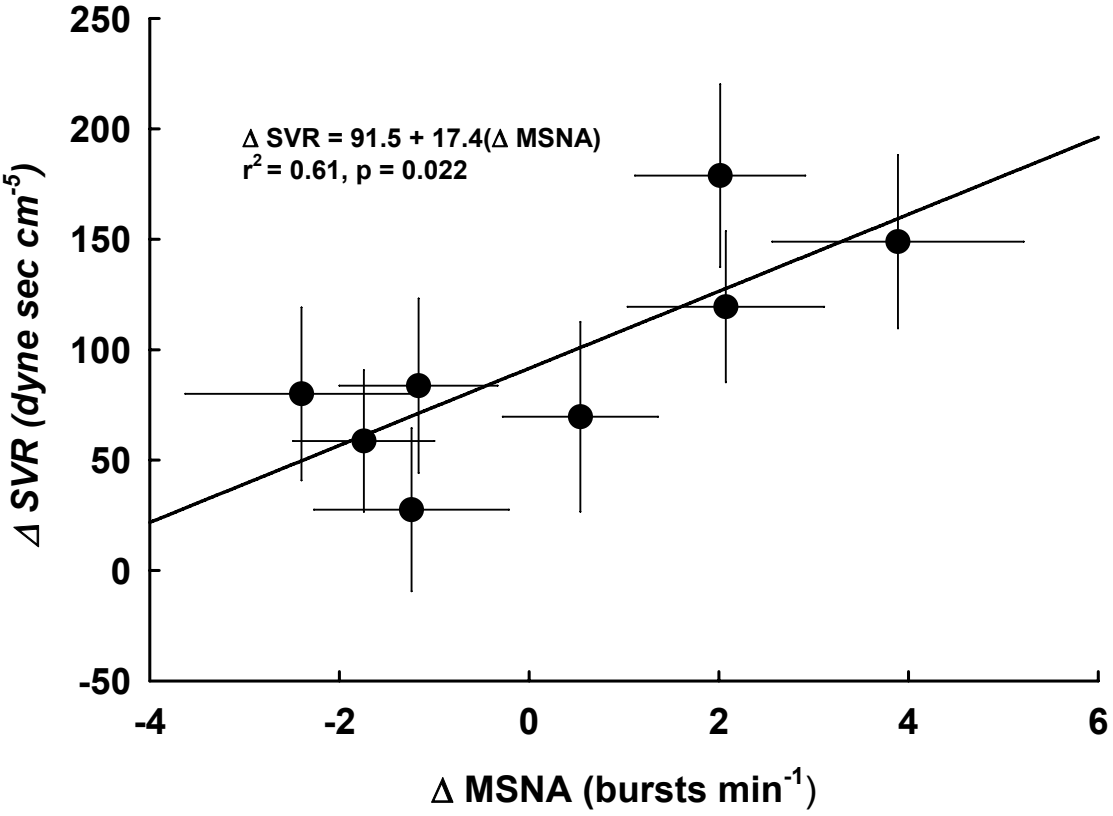

Figure 5-4. Linear least squares fit regression reveals a direct relation between the change in muscle sympathetic nerve activity (MSNA) and the change in systemic vascular resistance (SVR) during lipid infusion.

Chapter 6

SYMPATHETIC AND HEMODYNAMIC RESPONSES TO LIPIDS IN HEALTHY AGING

Introduction

Aging is associated with a much higher prevalence of hypertension and increased activation of the sympathetic nervous system (SNS), which may be driven by progressive accumulation of body fat and metabolic abnormalities (Seals & Bell, 2004). Muscle sympathetic nerve activity (MSNA) is augmented with age in both men and women (Ng *et al.*, 1993; Tanaka *et al.*, 1999; Narkiewicz *et al.*, 2005), and plasma norepinephrine (NE) concentrations increase ~10-15% per decade, both by an increase in spillover and decreased clearance due to lower cardiac output and flow (Seals and Dinenno, 2004). Also accompanying aging is increased arterial stiffening due to vascular smooth muscle proliferation and arterial remodeling, partly resulting from tonic increases in MSNA (Dinenno *et al.*, 2000); increased collagen crosslinking; calcification; and decreased elastin (Lakatta & Sollott, 2002). Together, with reduced baroreflex buffering (Jones *et al.*, 2003) and vasodilator capacity (Proctor *et al.*, 2005; Ridout *et al.*, 2005), these factors may contribute to age-related increases in systemic vascular resistance (SVR) and blood pressure (BP).

Changes in cardiovascular regulation coupled with age-associated increases in free fatty acids (FFA) (Aberg *et al.*, 2006; Toth *et al.*, 1996; Al Jaouni *et al.*, 2002) could have important implications for autonomic and cardiovascular regulation with aging since elevated FFA levels are associated with insulin resistance (Boden, 2001) and hypertension (Fagot-Campagna *et al.*, 1998), and FFA concentration correlates directly with BP (Egan *et al.*, 1996). Studies have shown that acute elevation of FFA levels in minipigs with infusion of a lipid emulsion increases BP and vascular resistance in most tissue beds (Bulow *et al.*, 1990), and portal and femoral oleate infusion induces a pressor response in rats (Grekin *et al.*, 1995; Grekin *et al.*, 1997). Similarly, infusion of a lipid emulsion and heparin to hydrolyze the triglycerides reduced arterial distensibility (Stojiljkovic *et al.*, 2001) and increased vascular resistance and arterial pressure in most (Fugmann *et al.*, 2003; Lopes *et al.*, 2001; Lopes *et al.*, 2003a; Lopes *et al.*, 2003b; Paolisso *et al.*, 2000; Stojiljkovic *et al.*, 2001) but not all (Amery *et al.*, 2000; Polak *et al.*, 2001) investigations.

The hemodynamic response to FFA in young individuals results from 1) paracrine mechanisms that act directly on the smooth muscle vasculature to increase resistance and/or 2) central sympathetic activation which increases vasomotor tone (Florian & Pawelczyk, 2007). On one hand, FFA impair endothelial cell nitric oxide synthase activity (Davda *et al.*, 1995) and endothelium-dependent vasodilation (Steinberg *et al.*, 1997), and increase vascular α-adrenergic sensitivity (Haastrup *et al.*, 1998; Stepniakowski *et al.*, 1995; Stepniakowski *et al.*, 1996) and oxidative stress (Lopes *et al.*, 2003a). Additionally, reduced baroreflex sensitivity and resetting has been shown in some (Gadegbeku *et al.*, 2002; Gadegbeku *et al.*, 2006) but not all (Monahan *et al.*, 2007)

studies. On the other hand, FFA increase NE (Paolisso *et al.*, 2000), and more recently we have directly shown that a 4-hr lipid infusion in healthy young men and women increases MSNA which augments SVR and BP (Florian & Pawelczyk, 2007). Moreover, the increase in sympathetic activation was preceded by a rise in insulin concentration, suggesting that insulin may have mediated a portion of the sympathetic response.

Although insulin secretion in response to a glucose challenge or mixed meal is impaired with aging (Muzumdar *et al.*, 2004), the total acute (Basu *et al.*, 2003) and chronic (Fraze *et al.*, 1987; Tamaya-Mori *et al.*, 2003) insulin response is significantly enhanced. Moreover, the age-related defect in insulin secretion appears to be specific to glucose and not FFA (Muzumdar *et al.*, 2004). In fact, Tamaya-Mori *et al* (2003) demonstrated an augmented leptin, insulin, and BP response to high-fat dietary intake in aged rats. They hypothesized that the hyperinsulinemia-induced hyperleptinemia accelerated the age-related increase in sympathetic nerve activity, contributing to the significant BP elevation observed in the older rats.

Research focusing on the physiological responses to i.v. lipid infusion and elevated FFA (Aberg *et al.*, 2006) or parenteral nutrition (Al Jaouni *et al.*, 2002; McCowen *et al.*, 2002) in older individuals is extremely scarce, limited to studies examining metabolic function and hormonal responses in healthy (Aberg *et al.*, 2006) and diseased (Al Jaouni *et al.*, 2002; McCowen *et al.*, 2002) populations. Given the age-related autonomic and cardiovascular changes and the detrimental responses to elevated FFA, an important question arises: Are the neural and cardiovascular responses to elevated FFA augmented with aging, mediated by greater increases in insulin and other hormonal mediators and a reduced ability to buffer the increase in pressure? Therefore,

the aim of this study was to test the hypothesis that aging exacerbates the pressor response to FFA and to determine possible hormonal mediators of the response. Employing a randomized placebo-controlled crossover design, we directly measured sympathetic nerve activity and hemodynamic/hormonal variables. Our results demonstrate that the pressor response to FFA is maintained in healthy aging.

Methods

Subjects

Seventeen older (60-77 yrs; 7 men, 10 women) subjects participated in the present study. All women were postmenopausal and not taking any form of hormone replacement therapy for at least the last 12 months. Each subject underwent a complete medical screening, including blood chemistry, blood clotting characteristics, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantilly, VA, USA), physical examination, DXA, and an assessment of maximal oxygen uptake ($\dot{V}O_{2max}$) (SensorMedics Corporation, Yorba Linda, CA, USA). All subjects were healthy, active, normotensive, non-diabetic, non-obese nonsmokers who were currently not taking any medications that would affect neural or cardiovascular function. Subjects abstained from alcohol for 2 days and caffeine for 1 day prior to the experiment, and refrained from taking any aspirin for 1 week prior to the experiment. Approval was obtained from the Institutional Review Board of the Pennsylvania State University. Each subject gave

verbal and written informed consent prior to participation in the study, and all procedures conformed to the standards of the Declaration of Helsinki.

Instrumentation

The study utilized a crossover design involving 2 identical experimental days (differing only in the substance infused), separated by a period of at least 2 weeks. The infusion of 20% Intralipid/heparin (I/H) or 2.25% glycerol/saline (S/G) was randomized, balanced, and single-blinded. All subjects consumed a standardized meal of 13 kcal/kg, consisting of 55% carbohydrate, 30% fat, and 15% protein between 6 and 8 PM the night prior to the experiment. After an overnight fast, subjects reported to the laboratory ~ 7 AM on 2 separate occasions to complete the study. Nude weight was measured, and a short polyethylene catheter was inserted into one antecubital vein for infusion of all test substances, while another catheter was placed retrograde into the contralateral dorsal hand vein for blood sampling. This arm was kept in a heating pad throughout the experiment to arterialize venous blood. Patency was maintained in the sampling line by infusion of saline (15 ml/hr). Following catheter insertion, subjects moved to a thermoneutral room and lay supine in a comfortable bed with their arms placed at heart level and both legs elevated 15 cm. Subjects were then instrumented to measure heart rate (ECG), arterial pressure (brachial auscultation), calf blood flow (venous occlusion plethysmography), cardiac output (C_2H_2 rebreathing), and MSNA (peroneal nerve microneurography).

Measurements

Heart rate and arterial pressure. Heart rate (3-lead ECG) was measured continuously and recorded using a customized beat-to-beat data collection software program. Blood pressure was measured by automated brachial auscultation (Colin 7000) after a 30-minute period of rest (2 times at 1-minute interval) and every ten minutes throughout infusion. Mean arterial pressure (MAP) was estimated as diastolic pressure + 1/3 pulse pressure.

Calf blood flow. Calf blood flow (CBF) was determined by venous occlusion plethysmography on the left calf. Changes in limb volume were transduced by using a dual-strand mercury-in-Silastic strain gauge (Hokanson *et al.*, 1975) and amplified (Hokanson EC-4). Strain gauges were sized to approximate the largest circumference of the calf. Blood flow to the foot was excluded from the measurement, with an ankle arresting cuff inflated to 250 mmHg just before flow measurements and deflated following the measurement. A Venous occlusion cuff wrapped around the thight was rapidly inflated to 50 mmHg every 20 s (8 s inflate, 12 s release). The lower leg was elevated 15 cm above the subjects' midaxillary line to facilitate venous drainage between determinations.

Blood flows were calculated from the slope of the ascending, linear portion of the plethysmographic response curve by using the line-fitting option in the Hokanson analysis software package (NIVP3) and manual adjustment (to exclude cuff inflation artifact). Systolic and diastolic blood pressures were measured by auscultation of the

brachial artery of the ipsilateral arm. Calf vascular resistance (CVR) was estimated as corresponding brachial MAP / CBF.

Cardiac output. Cardiac output ($\dot{Q}$) was measured using the acetylene rebreathing technique first described by Triebwasser *et al* (1977) . A mass spectrometer (Perkin-Elmer MGA1100) was used to analyze gas concentration during a 20 second rebreathing period. The accuracy and precision of this method has been validated against thermodilution and direct Fick. Systemic vascular resistance (SVR) was calculated as $\dot{Q}$ / MAP. Stroke volume (SV) was calculated as $\dot{Q}$ / HR.

Muscle sympathetic nerve activity. Peroneal MSNA was recorded as described previously (Wallin & Eckberg, 1982). Briefly, the nerve was located with cutaneous electrical stimulation (Isostim A320, World Precision Instruments). A tungsten reference electrode (FHC, Bowdoinham, ME, USA) was inserted subcutaneously, ~2 cm from the nerve, and a tungsten recording electrode with an uninsulated tip diameter of ~10 μm was inserted through the skin near the nerve. Adjustments of the recording electrode position were made according to auditory signals generated by impaled nerves. Both electrodes were connected in series to a differential preamplifier and an amplifier (UIowa Bioengineering, Iowa City, Iowa, USA). The nerve signal was amplified (total gain 40,000 to 80,000), band-pass filtered (high pass of .7 kHz and low pass of 2-3 kHz), and then full-wave rectified and smoothed with a resistance-capacitance circuit (time constant, 0.1 s) to produce a recording of "integrated" MSNA. Satisfactory recordings of MSNA were defined by pulse-synchronous bursts that increased during end-expiratory apnoea or Valsalva straining and did not change during tactile or auditory stimulation. Nerve traffic was expressed both as bursts/minute, an index of the frequency of the

activity, and as bursts/min times the mean burst amplitude, an index of integrated (total MSNA) activity.

Protocol

Following instrumentation and a 30 minute adaptation period, baseline hemodynamic and sympathetic data were obtained, and blood was sampled for metabolic and hormonal assays. Subjects then received a 4-h infusion of either 20% Intralipid at 0.8 ml $\cdot$ $m^{-2} \cdot min^{-1}$ and heparin (200 U bolus, followed by 0.3 U $\cdot$ $kg^{-1} \cdot min^{-1}$) or 2.25% glycerol/saline (New England Compounding, Framingham, MA) infused at the same rate and volume as Intralipid/heparin. Heparin was infused with Intralipid to activate lipoprotein lipase and to accelerate the hydrolysis of fatty acids from triglycerides (TG) (Stepniakowski *et al.*, 1996). Glycerol was infused with saline to control for the glycerol contained in 20% Intralipid. During the infusion, $\dot{Q}$ and CBF were measured every 30 minutes; MSNA and HR were recorded and averaged over a 5-min period every 30 minutes; and BP was taken every 10 min. Arterialized blood was sampled at baseline and for each hour of infusion thereafter for FFA, TG, glycerol, glucose, insulin, leptin, and catecholamines. Additional blood was sampled at baseline and at the end of infusion for aldosterone, angiotensin II, and F_2-isoprostanes. F_2-isoprostanes are a biomarker of oxidative stress that may affect endothelial function.

Blood Samples

Glucose was determined by the glucose oxidase method with an autoanalyzer (Ascencia® ELITE®) immediately after blood collection. All other samples were collected on ice, centrifuged at 4°C, and stored at -80°C until assay. Blood for FFA, TG, and glycerol was drawn into prechilled test tubes containing EDTA and 0.275 g paraoxon/L (Sigma, St. Louis, MO) to inhibit lipoprotein lipase and prevent hydrolysis of FFA from TG *in vitro* (Zambon *et al.*, 1993). Samples for catecholamines were collected in prechilled tubes containing EGTA and glutathione.

Serum insulin (OEM Concepts, Saco, ME) and leptin (R&D Systems, Minneapolis, MN) concentrations were determined by ELISA. Plasma FFA and TG concentrations were determined by a colorimetric method. Glycerol was measured enzymatically (Sigma, St. Louis, MO), catecholamines by HPLC, and aldosterone (Siemens Medical Solutions Diagnostics, Los Angeles, CA) and AT-II (ALPCO Diagnostics, Salem, NH) by RIA. The analytical sensitivity of the aldosterone assay was 0.07 nmol/L. Intraassay and interassay coefficients of variation averaged 2 and 3%, respectively. The analytical sensitivity of the AT-II assay was 0.97 pmol/L. Intraassay and interassay coefficients of variation averaged 9 and 11%, respectively. F_2-isoprostanes were determined by immunoassay (Assay Designs, Ann Arbor, MI). The analytical sensitivity of the F_2-isoprostanes assay was 0.04 ng/ml, and the intraassay and interassay coefficients of variation were 5 and 6%, respectively.

Data Analysis

The 5 minutes of data from recordings of MSNA and HR collected at baseline and every 30 minutes during infusion were averaged to a single value. CBF was measured 8 times and $\dot{Q}$ was measured twice at baseline and every 30 minutes during infusion; data for each variable was also averaged to a single value.

A repeated measures analysis of variance (ANOVA) was conducted to detect treatment and time effects on MSNA, hemodynamic, and hormonal variables. Least squares means with Bonferroni correction were performed when appropriate to detect where differences between treatments occurred. All statistical analyses were performed using SAS 9.1. The level of significance was set at $\alpha=0.05$. Values are presented as means ± SEM.

Results

Anthropometric, Metabolic, and Hormonal Measurements

The physical characteristics of the subjects are presented in Table 6-1. All subjects were healthy, normotensive, nonobese, and were not insulin-resistant (HOMA-IR and QUICKI) (Katz *et al.*, 2000). Data for men and women were pooled for most variables, except for insulin where sex differences in the response to infusion were identified. Infusions of I/H were well-tolerated by all the subjects.

Plasma FFA, TG, glycerol, and glucose concentrations during the experimental and control days are shown in Table 6-2. Lipid infusion significantly increased plasma

FFA and TG vs. baseline and control, whereas no changes occurred on the control day. Plasma glycerol significantly increased on both days, but to a greater extent during lipid infusion. Glucose levels were diminished after 2 hrs of lipid infusion vs. baseline and were significantly lower vs. placebo by the last hour.

Figure 6-1 illustrates the leptin, insulin, and epinephrine concentrations, and Table 6-3 shows plasma aldosterone, AT-II, and F_2-isoprostane levels. Baseline hormone concentrations were similar in both experimental conditions. Leptin concentration decreased during both infusions, and epinephrine and NE levels were unaffected by infusion of I/H or S/G. Because some of the samples were below the detectable range for the insulin assay, values for 13 and 11 subjects for the experimental and control days, respectively, are presented. Insulin concentration rose significantly by hours 3 and 4 in the experimental group, but did not increase in the control group; men displayed a greater insulin response (Figure 6-2) to lipid infusion compared to women by the 3rd hour (+15.0±4.0 vs. +4.0±3.1 pmol/L; p=0.011). Both F_2-isoprostane and aldosterone concentrations increased during I/H infusion vs. baseline and S/G infusion, whereas no change in AT-II concentration was noted for both experimental days.

Hemodynamic Measurements

As shown in Table 6-3, lipid infusion significantly increased systolic BP (+13.88±1.2.24 vs. +6.57±2.43 mmHg), diastolic BP (+7.41±1.45 vs. +1.29±0.81 mmHg), and HR (+8.88±0.88 vs. +3.02±0.91 beats/min) vs. placebo infusion; systolic BP and HR were significantly elevated vs. baseline by the end of infusion. Stroke Volume

and $\dot{Q}$ (Figure 6-3) tended to increase transiently over the 3rd hour. The change in SVR with I/H infusion was inconsistent throughout I/H infusion; however, it was significantly elevated vs. baseline by the end of infusion on both days. CBF (Figure 6-4) increased significantly by hour 2 and remained elevated for the duration of I/H infusion; however, the increase on the experimental day was not different from the control day.

MSNA and Vascular Resistance

Changes in MSNA burst frequency and total MSNA in response to I/H and S/G infusion, along with CVR, are displayed in Figure 6-4. Data for burst frequency (11 subjects) and total MSNA (8 subjects) were reduced from the goal of 17 subjects due to shifts in microneurography electrode position or failure to meet signal-to-noise criteria. However, the statistical power remained sufficient to identify differences between the two days. Both the change in burst frequency (+7.03 vs. -1.19 bursts/min) and total MSNA (+44.9% vs. -5.52%) in comparison to baseline and the control day were augmented after the 2.5 hrs on the experimental day and continued to increase until the end of infusion. Finally, though CVR decreased by the 4th hour of lipid infusion, it was not different when compared to the placebo day.

Discussion

The principal new findings of this rigorous and carefully-controlled investigation in healthy older individuals are as follows. First, acute hyperlipidemia induces a pressor response by elevating MSNA, BP, and HR. Second, the increase in MSNA was

associated with a rise in insulin but not leptin concentrations. Third, nonneural mechanisms such as sodium and fluid retention (aldosterone) and oxidative stress (F_2-isoprostanes) may have contributed to the hemodynamic response to FFA. Finally, though unexpected, hyperlipidemia caused directionally opposite responses for insulin (↑) and CVR (↓) in men, whereas insulin and CVR responses were severely blunted and nonexistent in women, respectively.

To the best of our knowledge, this is the first investigation to examine simultaneously the neural, cardiovascular, and endocrine responses to elevated FFA in healthy older humans. Importantly, high FFA concentration, as may be seen in individuals with obesity (Davda *et al.*, 1995) or dyslipidemia (Steinberg *et al.*, 1997), has been linked to hypertension (Fagot-Campagna *et al.*, 1998). Likewise, it is possible that age-associated increases in FFA (Toth *et al.*, 1996), possibly related to increased adiposity and altered endocrine function (Wilson & Kannel, 2002), may contribute to increasing BP with age.

Previous studies have demonstrated a pressor response to acute FFA elevation in young participants, mediated by direct vascular effects (Haastrup *et al.*, 1998; Lopes *et al.*, 2003a; Stepniakowski *et al.*, 1995; Stepniakowski *et al.*, 1996; Stojiljkovic *et al.*, 2001) and sympathoexcitation (Paolisso *et al.*, 2000; Florian & Pawelczyk, 2007). However, due to neural, vascular, and endocrine changes that occur with healthy aging, it was not apparent whether the response to FFA and the associated mechanisms would differ in older vs. young humans, particularly since MSNA exhibits a stronger relation with BP throughout the lifespan (Narkiewicz *et al.*, 2005).

Cardiovascular Response to Elevated FFA

The findings in the present investigation do not support our original hypothesis that aging exacerbates the pressor response to FFA. The peak pressor response was similar to that shown in previous studies with young participants (Lopes *et al.*, 2003a; Paolisso *et al.*, 2000; Stojiljkovic *et al.*, 2001; Florian & Pawelczyk, 2007), though BP was more variable throughout the infusions, resulting from inconsistent changes in BP determinants (i.e. $\dot{Q}$, SVR). Whether the pressor response would be greater if basal MAP in this study was similar to that in a previous study (~ +12 mmHg in the current study) cannot be determined. Subjects rested quietly for 30 min after instrumentation to establish a stable baseline; however, the true response would be underestimated if basal levels were not achieved. Since cardiovascular variables did not decrease during S/G infusion, it is likely that a true baseline was established on both days.

During lipid infusion, SVR significantly increased by the first hour followed by a subsequent return to baseline when $\dot{Q}$ tended to increase after the second hour; however, $\dot{Q}$ dropped to the starting value by the end of the study. In contrast, Stojiljkovic *et al* (2001) reported a significant increase in $\dot{Q}$ by the end of a 4-hr I/H infusion at a similar rate, though it is possible our results differ due to changes with aging. The augmented BP and HR response to S/G toward the end of the study may reflect stress and/or bladder distention (Fagius & Karhuvaara, 1989); however, this was not associated with an increase in MSNA. Further, the physiological responses to I/H infusion were significantly greater compared to S/G revealing a true effect of elevated FFA.

Interestingly, unlike the vasodilation observed in young men and women in a previous study (Florian & Pawelczyk, 2007) and older men in the current investigation, women did not exhibit reduced CVR (Figure 6-2B). This finding, though in response to a different stimulus, is consonant with two recent studies which documented impaired vasodilator responses to shear stress in older women (Parker *et al.*, 2006) but not men (Wray *et al.*, 2005). Possible explanations include increased arterial stiffening, reduced smooth muscle responsiveness to endothelium-derived vasodilators, or altered balance of dilator/constrictor release (Parker *et al.*, 2006). The sex difference for CVR may also be related to the blunted insulin response in women (Anderson *et al.*, 1991), though there are not enough data to identify any relation between CVR and insulin in the present study.

Neural Modulation of the Cardiovascular Response to Elevated FFA

Basal sympathetic burst frequency recorded in this study is consistent with previous values obtained in older individuals (Narkiewicz *et al.*, 2005; Yamada *et al.*, 1989), although no sex differences were noted. This contrasts with an earlier study showing greater activation in men vs. women (Ng *et al.*, 1993); however, the inclusion of women taking hormone replacement therapy may have obscured the results in that study. A more recent study utilizing a larger sample size and stricter inclusion criteria supports our results (Narkiewicz *et al.*, 2005).

The increase in burst frequency and total MSNA was similar to our previous study in young subjects (Florian & Pawelczyk, 2007). Interestingly, the sympathoexcitation did not translate to a noticeable increase in circulating NE, possibly

due to the low contribution of muscle to NE spillover at rest or from changes in neuronal reuptake or clearance.

Since the stimulus (i.e. FFA concentration) and level of sympathoexcitation was similar for both men and women, this may suggest concordant mechanisms of sensing and activation. However, contrasting with a previous study in our laboratory, the insulin response, a potential key mediator between FFA and sympathetic activation (Schwartz *et al.*, 2000; Florian & Pawelczyk, 2007), was unexpectedly blunted in women (Figure 6-2A). The reason for these findings is not apparently clear. Though insulin secretion in response to a glucose challenge or mixed meal is impaired with aging (Muzumdar *et al.*, 2004), the total acute (Basu *et al.*, 2003) and chronic (Fraze *et al.*, 1987; Tamaya-Mori *et al.*, 2003) insulin response is significantly enhanced. Moreover, the age-related defect in insulin secretion appears to be specific to glucose and not FFA (Muzumdar *et al.*, 2004). However, Muzumdar et al examined the insulin response to lipid infusion in male rats only, providing no insight to the sex differences noted in the current study. A recent comprehensive study which determined the effects of age and sex on insulin regulation following a mixed meal revealed that insulin secretion and clearance are similar in older men and women (Basu *et al.*, 2006). More studies which examine nutrient-specific insulin responses with age and sex are necessary to provide further clarification.

Leptin, a hormonal signal bridging peripheral adipose tissue and nutrient intake with the central nervous system, correlates with peak nocturnal FFA (Heptulla *et al.*, 2001) levels and activates the SNS (Schwartz *et al.*, 2000). Previous studies have reported inconsistent findings regarding the leptin response to FFA modulation. For example, two studies in rats (Fabris *et al.*, 2001; Wang *et al.*, 1998) and one in humans

(Nisoli *et al.*, 2000) reported elevated leptin concentrations and leptin mRNA, respectively, following lipid infusion. In contrast, leptin did not change following 150 min lipid infusion (Chen & Song, 2002) and actually decreased in another study (Garcia-Lorda *et al.*, 2003). Results from our 4-hr lipid infusion do not support a role for leptin in the acute response to FFA.

Two additional potential mechanisms mediating the sympathetic response cannot be ruled out in the current investigation. First, chemosensitive afferents arising from abdominal viscera respond to a range of endogenous molecules including sugars, lipids, peptide hormones, and cytokines to influence discharge of vasomotor neurons of the rostral ventrolateral medulla (Verberne *et al.*, 2003) and release of NE in the paraventricular nucleus (Ueta *et al.*, 2000). Moreover, infusion of palmitate, myristate, and oleate in animals increased vagal afferent nerve activity (Orbach & Andrews, 1973; Randich *et al.*, 2004).

Second, long-chain fatty acids, the most abundant fatty acids in Intralipid, cross the blood-brain barrier by simple diffusion in the unbound form in proportion to the circulating plasma FFA concentration (Rapoport, 1996). Within the hypothalamus long-chain fatty acids are esterified to long-chain fatty acyl-CoAs, and are subsequently transported to lipid oxidative and synthetic pathways. The pool of long-chain fatty acyl-CoAs which signals energy surfeit activates the POMC/CART neural pathway resulting in decreased energy intake and activation of the SNS (Lam *et al.*, 2005). Therefore, the possibility that activation of vagal afferents and direct sensing within the hypothalamus may be modulating factors of sympathetic activity in this study must be considered.

Peripheral (Nonneural) Modulation of the Cardiovascular Response to Elevated FFA

Several variables measured in the current study support nonneural mechanisms that contribute to the pressor response. No change was noted in AT-II excluding a role for this hormone in the acute pressor response to FFA. The increase in aldosterone with I/H infusion is consistent with two previous studies (Goodfriend *et al.*, 2004; Florian & Pawelczyk, 2007) and could have important implications in long-term BP regulation through a shift in the renal function curve (Guyton, 1990); however, whether aldosterone would remain elevated with chronically elevated FFA levels is unknown. Interestingly, aldosterone decreased on the control day, most likely reflecting a postural shift in central venous pressure and blood volume (Gauer, 1975). Increases in concentrations of F_2-isoprostanes, a stable biomarker of oxidative stress linked to hypertension (Minuz *et al.*, 2002), have been shown in previous studies with young participants (Lopes *et al.*, 2003a; Lopes *et al.*, 2003b; Florian & Pawelczyk, 2007). Our results show a similar change in oxidative stress to high levels of FFA with aging.

Experimental Considerations

Several factors must be considered when interpreting the results of this study. First, due to the length of the study and the difficulty of maintaining the microneurography recording, data could not be analyzed for burst frequency in ~25% and for total MSNA in ~ 50% of the subjects. Therefore, 17 subjects were recruited to maintain the statistical power to identify a difference in sympathetic outflow should a recording be disrupted. Second, since bladder distention increases sympathetic activation

and BP (Fagius & Karhuvaara, 1989), subjects voided using a condom catheter or urinal when necessary while remaining supine and stationary. However, it is possible that stress or other factors contributed to the response. Thus, the control group was essential to discriminate between the response to elevated FFA and other time-dependent confounding factors such as stress or bladder distention. Third, the design of this study does not prove cause and effect between variables such as MSNA and SVR or insulin and MSNA; we can only show associations in the time course of these variables. Fourth, as sympathetic outflow is regionally dependent, we cannot extrapolate our MSNA findings to other vascular beds. Finally, the data correspond to an acute increase in FFA, preventing any explicit conclusion on the long-term effects of chronic FFA elevation.

Conclusion

The results of the present study suggest that the pressor response to FFA is maintained (vs. exaggerated) with healthy aging, and that both neural and nonneural mechanisms contribute to this response. Additionally, though men and women respond similarly in most aspects, women exhibit an attenuated insulin and CBF response to FFA. Further studies are necessary to 1) determine whether blocking sympathetic activation attenuates the pressor response to FFA, 2) examine the effect or chronic FFA elevation on autonomic control of the cardiovascular system, and 3) elucidate the underlying mechanisms of sex-differences in the insulin response to FFA.

Table 6-1. Subject characteristics

Sex (M, F)	7, 10
Age (years)	69±1
Height (cm)	171±0
Weight (kg)	69±2
BMI (kg/m^2)	24±0
Body fat (%)	27±2
Waist-to-hip ratio	0.88±0.01
$\dot{V}O_{2\,max}$ (ml/kg/min)	28±1
Total cholesterol (mg/dl)	186±6
HDL (mg/dl)	57±4
LDL (mg/dl)	112±5
HOMA-IR	0.98±0.19
QUICKI	0.41±0.02

Values are mean±SEM. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check.

Table 6-2. **Time course of plasma FFA, TG, glucose, and glycerol concentrations during infusion**

Time (hours)		0	1	2	3	4
FFA (μmol/L)	I/H	581±30	1660±61*†	2548±94*†	2665±130*†	2586±136*†
	S/G	661±35	631±30	677±33	609±34	684±40
TG (mmol/L)	I/H	1.03±0.06	3.34±0.18*†	4.27±0.27*†	4.97±0.47*†	5.24±0.37*†
	S/G	0.99±0.08	1.15±0.07	1.17±0.07	1.16±0.07	1.18±0.06
Glycerol (μmol/L)	I/H	53±3	866±48*†	1077±70*†	1175±88*†	1277±94*†
	S/G	60±6	238±8*	267±11*	249±7*	255±8*
Glucose (mmol/L)	I/H	5.11±0.10	5.04±0.13	4.86±0.11*	4.80±0.10*†	4.64±0.08*†
	S/G	4.98±0.10	4.95±0.08	4.98±0.08	4.94±0.07	4.96±0.09

Values are mean±SEM of 17 subjects.
*Significant vs. baseline ($p<0.001$). †Significant vs. S/G control day ($p<0.001$).

Table 6-3. Baseline and final values for neural, cardiovascular, and hormonal variables during infusion

	Intralipid/heparin		Saline/glycerol		
	0 hrs	4 hrs	0 hrs	4 hrs	Interaction
Heart Rate (beats/min)	56.8±2.2	65.5±2.5*†	58.6±2.3	62.1±2.7*	<0.001
Systolic BP (mmHg)	125.9±4.0	139.8±3.6*†	128.8±4.0	135.2±4.0*	0.008
Diastolic BP (mmHg)	70.5±1.8	77.9±1.8*†	72.9±1.5	73.6±1.6	0.002
MAP (mmHg)	88.0±2.1	98.5±2.2*†	91.5±2.0	94.1±1.9	<0.001
Cardiac Output (L/min)	4.6±0.3	4.6±0.3	4.3±0.1	4.1±0.2	0.026
Stroke Volume (ml)	75.6±4.3	70.9±4.5	71.5±3.8	65.9±3.9	0.211
SVR (dyne · s · cm^{-5})	1622±88	1782±88*	1710±56	1910±64*	0.011
MSNA (bursts/min)	39.7±2.5	46.8±3.2*†	38.9±3.3	37.7±4.2	<0.001
Total MSNA (% baseline)	100.0±0.0	144.9±9.5*†	100.0±0.0	94.5±8.4	<0.001
CBF (ml/100 ml/min)	1.59±0.13	2.25±0.24*	1.62±0.17	1.64±0.14	0.007
CVR (units)	62.0±5.6	50.7±4.7*	64.6±5.3	65.9±6.7	0.270
Norepinephrine (nmol/L)	1.53±0.19	1.32±0.14	1.76±0.34	1.56±0.16	0.991
Leptin (pg/ml)	6.01±1.20	5.45±0.95*	6.24±1.19	4.97±0.92*	0.689
Insulin (pmol/L)	28.1±6.8	36.6±6.8*†	29.2±6.7	28.3±6.7	<0.001
Epinephrine (pmol/L)	691±82	639±39	757±194	619±38	0.994
Aldosterone (nmol/L)	0.04±0.01	0.06±0.01*†	0.03±0.01	0.02±0.00	0.024
Angiotensin II (pmol/L)	3.01±0.36	2.89±0.35	3.44±0.43	3.20±0.34	0.832
F_2-isoprostanes (pg/ml)	13.5±0.6	24.2±2.7*†	14.3±0.8	13.0±0.8	0.010

Values are mean±SEM. MAP, mean arterial pressure; SVR, systemic vascular resistance; CBF, calf blood flow; CVR, calf vascular resistance.
*Significant vs. baseline ($p<0.05$). †Significant vs. S/G control day ($p<0.05$).
Interaction indicates p-value of 2-way (treatment * time) repeated measures ANOVA interaction.

Figure 6-1

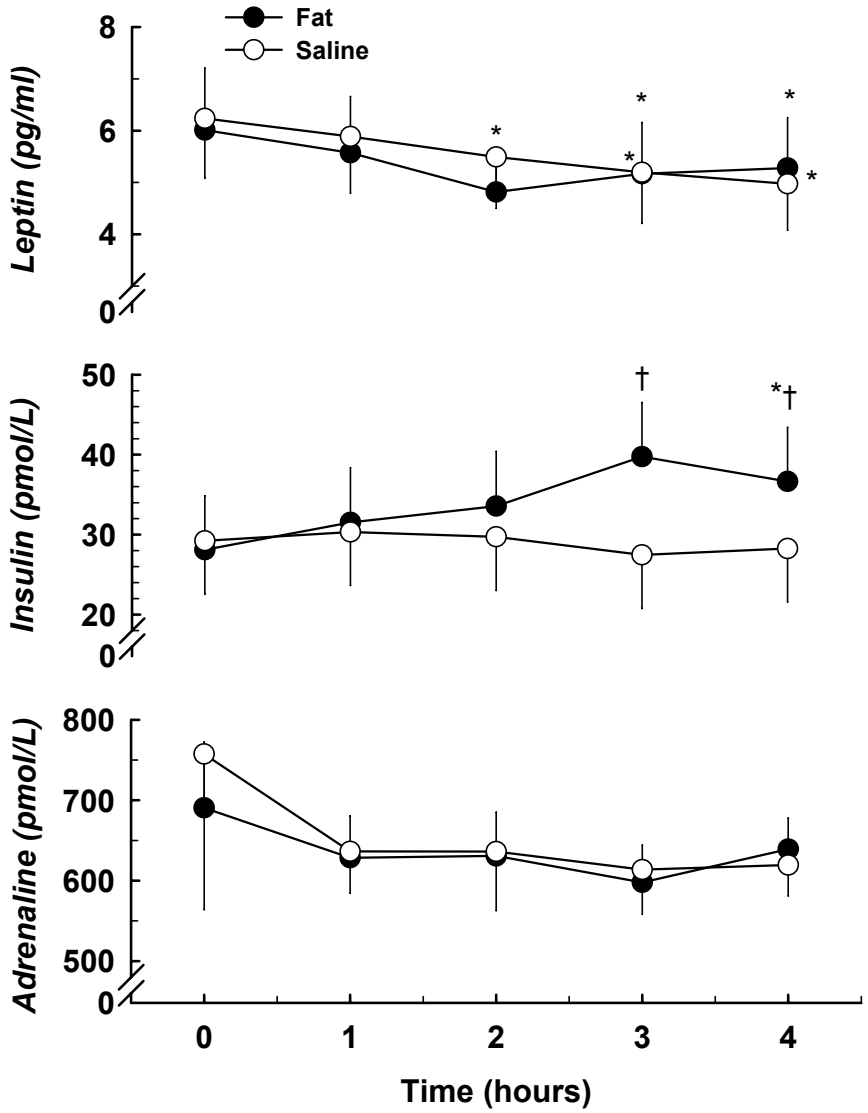

Figure 6-1. Hormonal responses to infusion of Intralipid/heparin (fat ●) and saline/glycerol (saline ○). Values are mean±SEM of 17 subjects. *Significant vs. baseline ($p<0.05$). †Significant vs. saline control day ($p<0.05$).

Figure 6-2

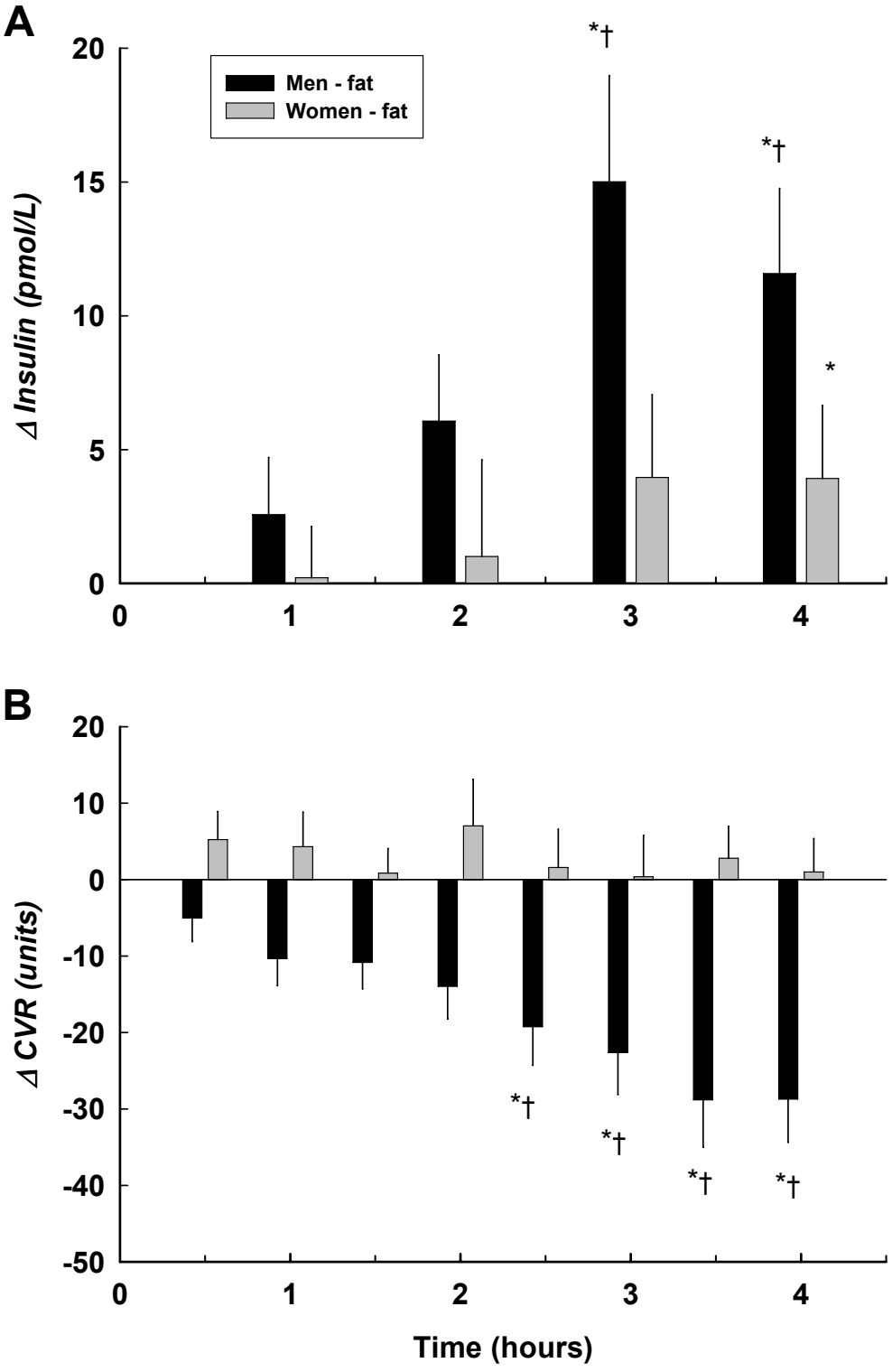

Figure 6-2. Insulin (A) and CVR (B) responses to infusion of Intralipid/heparin in 5 women and 8 men. Values are mean±SEM. *Significant vs. baseline ($p<0.05$). †Significant vs. female response ($p<0.05$).

Figure 6-3

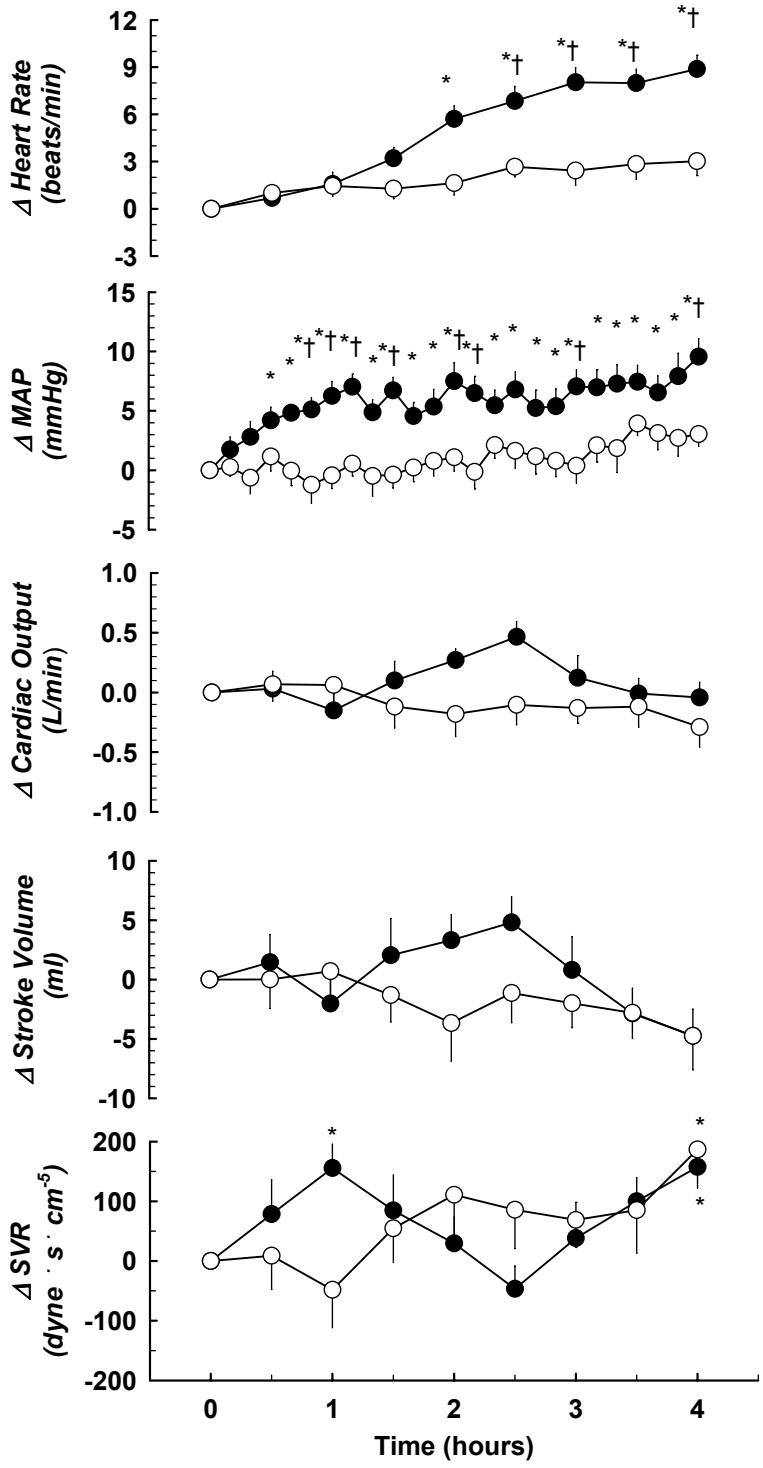

Figure 6-3. Systemic hemodynamic responses to infusion of Intralipid/heparin (fat ●) and saline/glycerol (saline ○). Values are mean±SEM of 17 subjects. MAP, mean arterial pressure; SVR, systemic vascular resistance. *Significant vs. baseline ($p<0.05$). †Significant vs. saline control day ($p<0.05$).

Figure 6-4

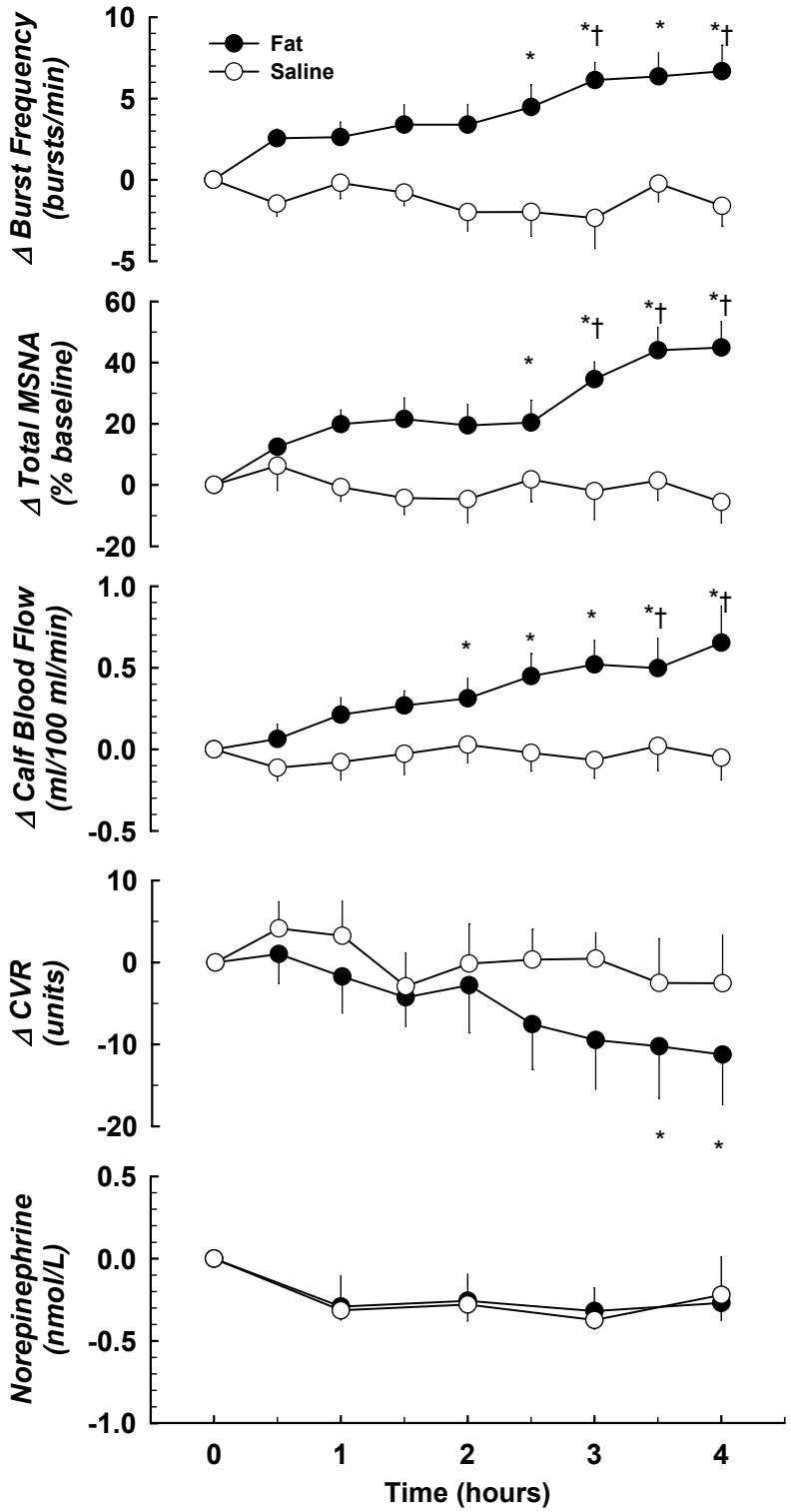

Figure 6-4. Responses to infusion of Intralipid/heparin (fat ●) and saline/glycerol (saline ○). Values are mean±SEM. CVR, calf vascular resistance. *Significant vs. baseline ($p<0.05$). †Significant vs. saline control day ($p<0.05$).

Chapter 7

CONCLUSIONS

The four studies comprising this dissertation were designed to explore the impact of hypoenergetic low-fat intake on autonomic control of the cardiovascular system and to determine the underlying neural mechanisms and hormonal mediators of the pressor response to elevated plasma FFA in young and older individuals. This chapter is intended to summarize the results of these studies and to place them into a broader physiological context. Additionally, future avenues of research that may further clarify mechanistic changes from caloric restriction and altered FFA levels will be identified.

Caloric Restriction and Orthostatic Tolerance

The primary finding of this study (Chapter 3) was that tolerance to lower body suction was reduced following 14 days of a hypocaloric, low-fat diet alone. In addition to a tendency toward reduced MSNA, HR and BP at baseline and throughout LBNP were significantly attenuated following hypocaloric interventions compared with normocaloric interventions. The orthostatic intolerance resulted from a lower hemodynamic starting point compounded by an impaired response to orthostatic stress.

Caloric Restriction and Static Exercise

The primary finding of this study (Chapter 4) was that 14-day caloric/fat restriction attenuated MSNA and pressor responses during isometric exercise to fatigue but not to post-exercise muscle ischemia. This indicates that the integrity of the

metaboreflex is maintained whereas the influence of the mechanoreflex and/or central command may be reduced. Though responses to CP were maintained, these results complement those of the orthostatic tolerance study, suggesting that CR alters autonomic function in response to various stressors via a central mechanism.

Free Fatty Acids and Sympathetic Activation

The primary new findings (Figure 7-1) from the FFA study (Chapter 5) were that 1) acute elevation of plasma FFA with infusion of I/H increases central sympathetic activation measured from sympathetic postganglionic neurons, 2) the augmentation of MSNA was associated with a rise in insulin but not leptin concentrations, and 3) FFA increased aldosterone and F_2-isoprostanes, but not AT-II, to contribute to the hemodynamic response.

Figure 7-1

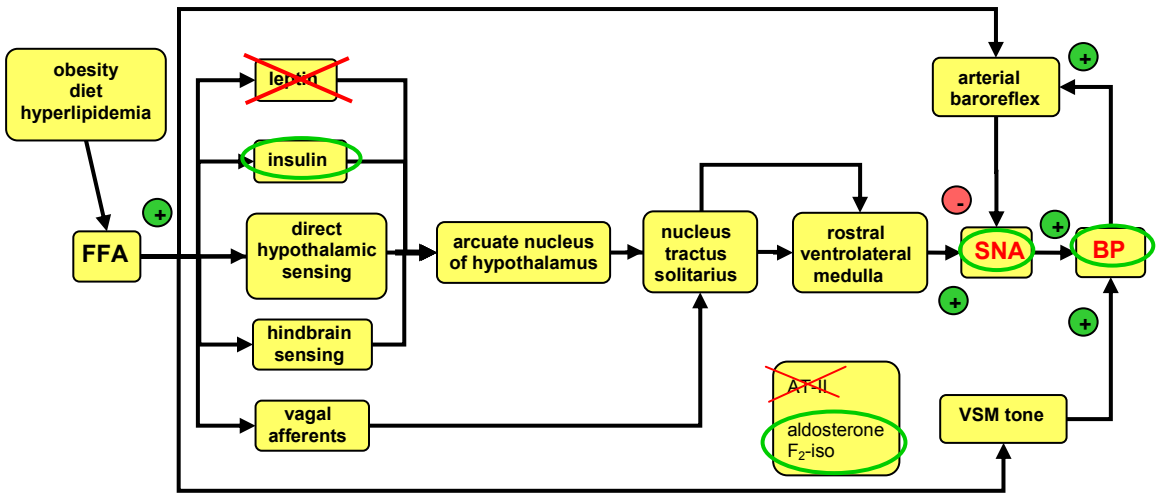

Figure 7-1. Primary findings of the FFA studies. Acute hyperlipidemia raises BP by central sympathetic activation and peripheral mechanisms that directly increase vascular smooth muscle tone, and possibly plasma volume. Sympathetic augmentation was associated with an increase in insulin but not leptin levels.

Free Fatty Acids and Aging

The principal finding of the FFA and aging study (Chapter 6) is that contrary to our hypothesis that age would exacerbate the pressor response to FFA, acute hyperlipidemia induces a pressor response similar to that in young adults by elevating MSNA, BP, and HR. The hormonal responses were also comparable except that hyperlipidemia caused directionally opposite responses for insulin (↑) and CVR (↓) in men, whereas insulin and CVR responses were severely blunted and nonexistent in women, respectively.

Summary

Hypothesis 1: Chronic hypocaloric low-fat energy supply will intensify the development of orthostatic intolerance associated with bedrest.

Conclusion 1: Data from the current study support the hypothesis that caloric restriction decreases orthostatic tolerance; however, orthostatic intolerance was not intensified when caloric restriction was combined with bedrest.

Hypothesis 2: Reduced caloric/fat intake will diminish sympathetic and cardiovascular responses to static handgrip and cold pressor tests.

Conclusion 2: Data from the current study support the hypothesis that reduced caloric/fat intake diminishes sympathetic and cardiovascular responses to static handgrip and cold pressor tests.

Hypothesis 3a: Infusion of Intralipid® will significantly increase muscle sympathetic nerve activity and blood pressure, establishing a role for FFA in central sympathetic activation.

Conclusion 3a: Data from the current study support the hypothesis that elevation of FFA with Intralipid® infusion increases muscle sympathetic nerve activity and blood pressure, thus establishing a role for FFA in central sympathetic activation.

Hypothesis 3b: Responses will be similar between women and men.

Conclusion 3b: Data from the current study support the hypothesis that responses to lipid infusion are similar in men and women (during the low hormone phase of the cycle).

Hypothesis 4a: Augmentation of sympathetic activity and blood pressure following infusion will be greater aging.

Conclusion 4a: Data from the current study refute the hypothesis that sympathetic activation and blood pressure are greater following acute elevation of FFA with Intralipid® infusion.

Hypothesis 4b: The greater rise in sympathetic activity and blood pressure with aging will be induced by larger increases in serum insulin and leptin concentrations following Intralipid® infusion.

Conclusion 4b: Data from the current study refute the hypothesis that aging augments the hormonal response to an increase in FFA.

Conclusion

The results from these studies collectively demonstrate that reduced caloric/fat intake (which may decrease FFA) impacts autonomic function and ultimately translates to reduced orthostatic tolerance and impaired pressor responses to exercise. Plasma FFA have emerged recently as an important factor involved in cardiovascular dysfunction. The studies in chapters 5 and 6 provide direct evidence that in young and older humans the pressor response to FFA is mediated, at least in part, by sympathetic activation. The neurogenic response documented in these investigations implicates central sympathetic activation as a possible mechanism linking conditions characterized by high FFA (e.g. obesity, dyslipidemia) with hypertension.

Future Research Directions

The studies presented in this dissertation have provided insight into the basic neural mechanisms underlying changes in BP regulation from modulation of caloric/fat intake and plasma FFA concentration. However, these studies have also generated many important physiologically significant questions that should be addressed in future investigations. Most notably, future investigations will need to determine:

1. the mechanisms underlying decreased OT in healthy individuals following caloric/fat restriction, including changes in plasma volume, endocrine function, baroreflex responses, and sympathetic regulation.

2. what factor(s) is responsible for the reduced exercise pressor response following hypocaloric intake, including changes along the reflex arc (i.e. afferent, central integration, efferent pathways), plasma volume, etc.
3. the underlying metabolic/hormonal signals associated with the observed neural and cardiovascular changes from caloric restriction.
4. the effect of chronic elevation of FFA on autonomic control in humans and whether reducing FFA concentration results in a corresponding decrease in sympathetic activity.
5. the relative contributions of central vs. peripheral vascular mechanisms underlying the BP response to fat by inhibiting sympathetic activation from FFA.
6. the contributions of putative mediators such as vagal afferents, hindbrain sensing, and direct hypothalamic sensing in FFA-induced sympathoexcitation.
7. whether inhibiting insulin secretion alters the sympathoexcitation from FFA.
8. the effect of individual fatty acids on sympathetic activation and cardiovascular control.
9. mechanisms underlying the divergent insulin responses to FFA in older men and women.

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Appendix

Informed consent form for FFA and sympathetic activation studies (Chapters 5-6):

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: **Influence of Free Fatty Acids on Blood Pressure Regulation: Autonomic Mechanisms**

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This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. James A. Pawelczyk.

1. **Purpose of the study:**
Past research has shown that the blood pressure in animals and humans and nerve activity in animals increase with fat intake and elevated fat levels in the blood. Additionally, older people may have a greater blood pressure response to fat intake than do younger people. The purpose of this research is to learn how fat intake affects nerve-signals to your muscle, as well as blood pressure regulation, and to see if aging changes these signals. We will do this by recording electrical signals from nerve fibers that cause your blood vessels to narrow in response to stress. To complete this study, you will be asked to visit Noll Laboratory and/or the General Clinical Research Center (GCRC) on 6 days.

2. Procedures to be followed: *You will participate on the circled days. Please read the descriptions of the circled days. Then write your initials by the circled days. You may request personnel of the same gender to perform procedures.*

_____ initial **Day 1 (Screening 1):** You will not eat or drink after midnight during the night before your exam. During your first visit, the staff will give you a tour of the Noll laboratory. You will go to the General Clinical Research Center (GCRC) for part one of your screening. The

nurses will draw 15 ml (1 Tbsp) of blood from a vein in your arm with a needle to check your state of wellness. After the blood draw, we will give you a breakfast bar and juice, if you wish. They also measure your blood pressure, height, weight, and resting electrocardiogram (ECG). If you are allergic to shellfish, eggs, or latex please inform the staff. If you are allergic to eggs or latex you will not be able to help with the study.

_____ initial **Day 2 (Screening 2):** Avoid using any type of stimulant (including cold medications and chocolate), drinking caffeine (coffee, tea, cola), and drinking alcohol after 9 PM the night before you come to the lab. You should eat a light breakfast before coming to the lab. Bring shorts, t-shirt, and comfortable shoes in which you can walk or run. If you are a woman, bring a sports bra, too. We can provide a sports bra if you do not have one. You will have an examination by the GCRC medical staff that includes a medical history and check-up. Women of childbearing-age will submit a urine sample for a pregnancy test. If you are pregnant, you will not be able to participate in the project. Following the check-up you will have a Dual-Energy X-Ray Absorptiometry (DXA) scan to determine your body composition and fat distribution. A nurse will also measure the circumference around your waist and hip (middle of buttocks) to determine your waist/hip ratio. The measurements will be taken underneath your outer clothing. You will have a graded exercise test (GXT) to measure your fitness level and test your cardiovascular system. We will measure blood pressure, heart rate, and, the electrical activity of your heart. During the test, you will wear a nose clip and breathe into a tube. We will measure the oxygen and carbon dioxide you breathe out. You will rate how hard you are working by using a numbered scale (rating of perceived exertion or RPE scale). As you walk or run on a treadmill, the grade of the treadmill will increase a little every 2 minutes. The exercise will become harder. If you do your best to exercise for as long as you can, the test will be most accurate. However, you can stop at any time. The test is 10-20 minutes long.

Pregnancy Test (younger female participants): An over the counter urine pregnancy test will be performed before each experimental day. If the test result is positive you will be excused from participating in the study.

_____ initial **Days 3 and 5 (Experiment days):** For two days before the experiment, you will drink normal amounts of fluid (8 glasses water, juice, or sports drinks per day) and will avoid using any type of stimulant (including cold medications and chocolate), drinking caffeine (coffee, tea, cola), and drinking alcohol after 24 hours prior to the experiment. Please bring or wear a t-shirt, socks and shorts, and if you are a woman, a sports bra. We can provide a sports bra if you do not have one.

You will report to the GCRC pick up a meal by 6 p.m. the night prior to the study; the meal must be eaten by 8 p.m. Please notify us if you have any special food requirements. You will then report to the GCRC in the morning following an overnight fast (nothing to eat or drink, except water, following the GCRC meal). GCRC staff will apply topical anesthetic and follow with catheter insertion in one arm vein for infusion and another vein in the other hand or arm for blood sampling. During each study day, small samples of blood will be drawn every hour. The total amount of blood drawn on each study day is about ¼ pint. We will attach our test equipment (including microneurography) while you are lying on a bed. We will then infuse either lipid/heparin (on one day) or saline/glycerol (the other day) for four hours and measure:

- heart rate from sticky patches placed on the chest (3-lead ECG),
- blood pressure from the arm and a sensor on the wrist,
- the electrical activity generated by the nerves leading to muscle in the lower leg,

- blood flow from cuffs attached to the ankle and upper leg,
- your heart's pumping ability by breathing in and out of a bag containing a small amount of gas called acetylene.

You will not know which substance we infuse (i.e., lipid/heparin or saline/glycerol) on each of the study days.

_____ initial **Days 4 and 6 (Post-study days):** You will return to the GCRC on a scheduled day between 7 and 10 days following each experimental day. During this 30 minute visit, a 4 mL (less than 1 tablespoon) sample of blood will be drawn for the purpose of measuring platelet levels. You will also fill out a questionnaire regarding symptoms associated with HIT.

Procedures to be used: Please initial the descriptions to show that you have read and understand them.

____ Blood draw for screening. Skilled GCRC staff will remove blood from your arm using a needle. The blood will be tested for blood cell counts, fat, and liver and kidney function. If you are taking thyroid medication, we will also test your blood to examine thyroid function. The staff uses standard safety measures and sterile techniques that are used in hospitals.

____ Heart Rate. 10 ECG electrodes (sticky patches) are attached to the chest on Days 1 and 2 to measure the heart's electrical activity and rate. On Days 3 and 4, 3 ECG electrodes will be applied to the chest to measure heart rate.

____ Body Composition Test**.** Your body composition (% fat, muscle, bone) will be estimated during the second screening day using a DXA test. This whole body scan requires that you lie flat on a padded table without moving for approximately 10 minutes while an X-ray scanner moves over your body.

____ Blood Pressure. At screenings, a cuff is inflated on the upper arm. The air in the cuff is slowly released while a technician or clinician listens to the area at the inside of the elbow with a stethoscope. During the experiment, a miniature cuff will be placed around a finger on your right hand and a cuff will be placed on your left upper arm. Your finger may turn slightly blue or tingle from the finger cuff, and some people find the cuff bothersome after a period of time. We will remove the cuff every hour for a period of about ten minutes to give your finger a rest. Every five minutes during the experiment, the arm cuff will inflate briefly and then deflate.

____ Catheter insertion and blood sampling. On each of the experiment days, a GCRC nurse will apply numbing cream and then insert a small plastic tube (catheter) into a vein on one arm and another catheter into a vein on the hand or forearm of the other arm. The catheter is about 1-2 inches long and has the thickness and consistency of a cooked piece of spaghetti. The catheters will remain in your arms for the duration of the study. During the study, we will infuse either lipid/heparin or saline/glycerol (see below) in the arm catheter. We will draw blood at the beginning of the study and each hour thereafter for four hours (a total of 5 draws) from the hand catheter. We will be measuring various hormones and substances (i.e., insulin, fat, glycerol) from the blood we take. The total amount removed on each study day is about 8 tablespoons (120 ml) of blood. In order to obtain the correct measurements in the blood, you will place your hand in a heated wooden box or a heated mitt (heating pad) throughout the experiment to keep your hand warm. The temperature of the air in the box will not be hot enough to burn your hand.

____ Blood Flow. Every 20-30 minutes we will measure blood flow to your calf. Velcro blood pressure cuffs will be wrapped around your thigh and around your ankle, and thin rubber tubes filled with mercury will be wrapped around the middle of your calf. The ankle cuff will be inflated to a pressure high enough to stop blood flow to your foot. This causes no problems for the 2-5 minute period the cuff will be inflated. While this cuff is inflated, the other cuff will be

inflated to a much lower pressure for 10-15 seconds, about 3 times a minute. While this cuff inflates you may feel that your calf is swelling. This sensation will cease when the cuffs are released.

____ Cardiac Output (acetylene rebreathing). We will measure your heart's pumping rate by analyzing the air you rebreathe in and out of a bag using fairly deep breaths for 15-20 seconds. The bag will contain a small concentration of two gases, acetylene and helium. The concentrations of these gases are so small that there is no risk of them catching fire. Some people report a slightly "tangy" taste from the rebreathing gas. Although you may become light-headed for a few seconds during rebreathing or develop a slight headache from repeated rebreathing, there are no other known risks to performing this procedure. The gas disappears from your lungs and blood in less than 5 minutes.

____ Neural Activity (Microneurography). During the experiment, your leg will be elevated to promote good blood flow. We will record nerve signals in the nerve located near your knee. First, we will map the nerve by exciting the nerve with a pencil-shaped electrode placed on your skin. When we electrically excite the nerve, you will notice twitching, numbness, prickling, or tingling in the lower part of your leg. These feelings will disappear when we stop exciting the nerve. Once we have mapped the nerve, we will insert two tiny, sterile, needle electrodes through the skin. We do this without local anesthesia. The needle electrodes are so small they cause little pain when inserted. We insert 1 needle electrode just under the skin a short distance from the nerve. The second needle will contact your nerve. When the second needle enters the nerve, you will notice twitching, numbness, prickling, or tingling again. Some people may feel a moment of discomfort. We will place the needle electrode so that you will have twitches without the tingling feelings. We will need to move the needle a little until we get a good recording of your nerve's signal. We may take up to 60 minutes to get a good signal, but you may stop the study at any time. We will not exceed 60 minutes regardless of our success with the signal. When we get a good signal, we will begin the experiment. Most likely, you will be unaware of the needle electrode once it has been placed and left alone. Your leg may feel like it is falling asleep. It is important that you keep your leg very still to maintain a good nerve signal throughout the experiment (about 4.5 hours). Once the electrodes are placed, the rest of the experiment will last about 4.5 hours. In total, your leg will need to be elevated, relaxed, and still for up to 5.5 hours. After the experiment, we remove the electrodes by pulling them out of the skin.

____ Lipid Infusion. Following the microneurography procedure on only one of the study days, a fat solution will be infused in the catheter in your forearm throughout the rest of the test (about 4 hours). The fat solution is composed of a few different substances including soybean oil, egg yolk, and water. The total caloric value of the fat solution that will be infused over 4 hours is about 700 to 800 calories. For comparison purposes, a large milkshake is about 750 calories. On the other study day we will infuse normal saline and glycerol as a control.

____ Heparin Infusion. On the same day that we infuse the fat solution, we will also infuse a substance called heparin that is naturally found in human tissues. Sometimes heparin is given to people in the hospital to help prevent clots from forming in the blood, but we are using it in this study because of its ability to increase the amount of free fatty acids in the blood.

____ Saline/Glycerol Infusion. On the control day following the microneurography procedure, normal saline (the same volume as lipid infusion) and glycerol infused in the catheter in your forearm throughout the rest of the test (about 4 hours). Glycerol is a safe, non-toxic substance that is found in your body. There are no known risks to this procedure.

____ Graded Exercise Test (GXT). The GXT tests your fitness level and cardiovascular system. Your blood pressure, heart rate, and the electrical activity of your heart are measured. We will apply a cuff around the upper arm to measure blood pressure and 10 ECG electrodes to the chest to measure heart rate. During the test, you will wear a nose clip and breathe into a tube to measure the oxygen and carbon dioxide you breathe out.

you are working by using a numbered scale matched to short phrases (rating of perceived exertion or RPE scale). At first, you will warm up by walking at a comfortable pace on the treadmill for about 4 minutes. Then, if you are part of the younger group, you will begin to run at a comfortable pace. If you are part of the older group, you will continue to walk. Then the grade of the treadmill will increase a little every 2 minutes. The exercise will become harder. The test will be most accurate if you do your best to exercise for as long as you can. However, you can stop at any time. The test is 10-20 minutes long.

___ Metabolic Measurements. You will breathe into a tube during the GXT so that we can collect your expired air. We will measure the volume of air that you breathe out. We will also measure the amount of oxygen and carbon dioxide in the air you breathe out.

____ Voiding Urine. Prior to instrumentation on the experiment day, you will be asked to use the bathroom. If you need to void during the experiment, the men will have the option of using a condom catheter (a condom like soft sheath that connects to a drain tube and into a urine collection bag) or a urinal. The women may use a female urinal. The urine will be immediately discarded.

3. Discomforts and risks: All procedures carry risk. Risk has two aspects: severity and frequency. Severe risk might threaten the loss of life or limb, while a mild risk might be discomfort. The frequency of a risk is the chance that a problem will occur. In this section, we have summarized the risks associated with the procedures used in this experiment. The risks in this experiment have different severity and frequency, and some could be life threatening. Please feel free to ask about the severity or frequency of these risks at any time. To help you decide whether you are willing to accept the risks associated with this experiment, the table below provides some commonly mentioned risks and the estimated chance they will occur to you:

Contracting meningitis while you are at a large university	1 in 20,000
Being struck by lightning in your lifetime	1 in 10,000
Contracting AIDS if you avoid "high risk" activity	1 in 3,000
Dying of liver disease if you drank one beer per day	1 in 1,000
Developing breast cancer by age 25	1 in 1,000
Contracting a disease caused by radon in your home	1 in 440
Being killed in a car accident in your lifetime	1 in 60
Contracting cancer at some point during your life	1 in 5

General: If we should find any problems during the screening, we will inform you as soon as we can. In that case, we will suggest that you that contact your personal doctor for treatment. Medical tests can make some people feel so nervous that they feel faint (vasovagal response). Please share your concerns with us at any time so that we may keep you informed and help to prevent your feeling nervous.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. You may become lightheaded or may faint. To keep the chance of infection minimal, the medical staff uses the same techniques used in hospitals.

Body Composition Test: The DXA procedure exposes an individual to a small amount of radiation where the x-ray beam crosses the body. The radiation involved is equivalent to a whole-body radiation dose of approximately 1.5 mrem (millirem). A mrem is a unit of whole-body radiation dose. For comparison purposes, 1.5 mrem is less than the you would receive from a routine chest x-ray, or from cosmic rays during a coast-to-coast flight, or from 5 days worth of natural background radiation in central Pennsylvania. A DXA procedure also creates unknown risk to an unborn fetus. To minimize this risk, we administer a pregnancy test prior to DXA

testing. A positive pregnancy test will exclude you from the study.

Cardiac Output (acetylene rebreathing): Because you breathe slightly deeper than normal, there is a chance you will feel light headed for a few seconds during rebreathing or develop a slight headache after repeated measurements. There are no other known risks to this procedure.

Neural activity (microneurography): When we electrically excite the nerve, you will notice twitching, numbness, prickling, or tingling in the lower part of your leg. These feelings will disappear when we stop exciting the nerve. You may feel mild pain when the needle electrodes enter the skin. When the electrode enters your nerve, you may feel "an ache," "pins and needles," "cramping," or numbness. These feelings will cease when we stop moving the electrode. About 1 in 12 subjects feel some aching at the recording site or "pins and needles" below the recording site for a few days after the study. Some people say their lower leg feels slightly weaker for a few days (similar to what you might feel after jogging). To minimize chances of any problems, you should not rub the site or perform heavy leg activity for at least 24 hours after the study. It is possible for the needle to cause permanent damage to your nerve. This could affect the function of your lower leg. However, permanent damage to a nerve has never occurred in more than 15,000 procedures worldwide. Anytime your skin is pierced, there is a chance of infection. Infections can be serious. To minimize this risk, the electrodes are sterile. Also, your skin is cleaned with betadine prior to insertion and after removal of the electrodes. There have been no serious medical problems resulting from this procedure. One week after the study, you will fill out and return a questionnaire to us. The questionnaire will inform us of any symptoms you may have experienced after the study.

Lipid Infusion: About 1 in 20 patients receiving lipid infusions report mild nausea. Less than 1 in 100 report vomiting, headache, flushing, increase in temperature, sweating, sleepiness, pain in the chest and back, slight pressure over the eyes, dizziness, pancreatitis, and irritation at the site of infusion. Allergic reactions can occur and can be managed by usual allergic means. Rarely, a serious allergic reaction which affects the entire body (anaphylaxis) may develop. An anaphylactic reaction can be life-threatening. You are free to discontinue these tests at any time. If a bad reaction should occur, medical help will be summoned right away.

Heparin Infusion: You will be asked if you are hypersensitive or allergic to heparin, or if you have received heparin within the past 3 months. If you answer "yes" to any of the questions, you will not be allowed to participate in the project. This is a necessary requirement to ensure that the slight possibility of an adverse response to heparin in a subject sensitive to heparin is alleviated. Under rare circumstances you may notice a change in skin color, chest pain, chills and/or fever, irritation or pain at the place of injection, itching and burning feeling, nausea and/or vomiting, numbness or tingling in hands or feet, peeling of skin, runny nose, and tearing of eyes. More serious reactions include abdominal or stomach pain, bleeding, unexplained bruising, nosebleeds, backaches, blood in urine, constipation, coughing up blood, dizziness, headaches (severe or continuing), joint pain, stiffness, or swelling.

Heparin-Induced Thrombocytopenia: Within a few days to 2 weeks, a condition called heparin-induced thrombocytopenia (HIT) could occur. This happens when the number of cells responsible for clotting in the blood decreases, but the chance of forming a dangerous clot increases since these cells bind to heparin. About 1 in 200 medical patients in the hospital receiving heparin develops HIT, and about 1 in 400 develops the clotting abnormalities. **In the extreme HIT can lead to loss of limb, heart attack, stroke or even death.** In patients who had surgery and receive heparin these rates are higher, but in pregnant healthy women HIT is not usually seen. What the risk of HIT is in healthy research subjects is not known, but HIT is probably much less likely in healthy people like you. Because medical patients in the hospital may receive more heparin and are more predisposed to HIT than research volunteers, **the risk for developing HIT from the lipid/heparin infusion in this study is less than 1 in 200.** Receiving heparin in this study also increases your risk of developing HIT in the future if heparin is administered to you (i.e., surgery) because you will develop antibodies specific to heparin.

However, your chance of developing HIT under those circumstances returns to the original risk (less than 1 in 200) if heparin is administered more than three months after the study. The risk increases if heparin is administered less than three months following the lipid/heparin study day. If a bad reaction should occur during the study, medical help will be summoned right away. We will call you about one month after the experiment to check on how you are doing.

Saline/Glycerol Infusion: Possible risks include fever, infection, and possibly irritation at the injection site. There are no other known risks involved with saline/glycerol infusion.

Deep venous thrombosis (DVT) or clots: DVT is when a blood clot in a vein blocks the normal flow of blood back to the heart. DVT or clots can form when blood pools in the leg and the leg is very still for a long time. An example of this is when someone sits for a long time on an airplane traveling across the ocean. In this project, it is unlikely that a DVT or clot will form because your leg is raised during the experiment so blood will not be pooling in the vessels. Also, your leg is unlikely to be still long enough for the clots to form. When you sleep at night or when you sit still watching a couple of movies, your leg may be inactive for a longer time than that experienced during this project. Although unlikely, there is still a small chance of developing DVT or clots during this project. DVT or clots could cause either leg to have pain, usually in the calf or thigh; swelling in one leg; and/or warm, reddened skin in the calf or thigh that is tender to the touch. If a DVT or clot should form, a blood clot could travel from the leg to the small vessels in the lung to cause serious or even life-threatening problems such as lung damage. Serious blood clots can occur in people who have not been in a research study and have no known reason for the problem. There are less than 5 cases per 100,000 persons less than 15 years old each year in the entire United States. About 500 cases of serious blood clotting problems per 100,000 people at 80 years old are reported each year. About 2/3 of the cases are DVT. Death occurs in about 6% of the DVT cases in the United States each year. There have been no reports of persons having DVT or clots from being in a study like this. We estimate the risk of DVT in this study to be 1 in 10,000.

Topical Anesthetic Cream: Numbing cream will not be used in those who have sensitivity to lidocaine. Eye contact should be avoided. When used, all sensations within the treated area are blocked. For this reason, unintentional trauma to the treated area, such as scratching, rubbing or exposure to hot or cold temperatures should be avoided until complete sensation has returned. During or immediately after application, mild swelling, skin redness or abnormal sensation may develop at the site of treatment. In clinical studies, no serious reactions resulted from the use of the cream. Allergic reactions can occur and can be managed by usual allergic means. Whole body adverse reactions following appropriate use are unlikely due to the small dose absorbed. If effects do occur, they are similar in nature to those seen with other local anesthetic agents and may include lightheadedness, nervousness, apprehension, dizziness, drowsiness, twitching, and vomiting. Reactions may be brief or not at all.

Betadine: Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to Betadine if you are allergic to iodine or shellfish. You will inform us if you have these allergies so that we will use alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or collapse.

Blood sampling/venous catheter: The risks of a blood sample include bruising and/or discomfort from the needle, venous inflammation from the catheter, infection (less than 1 in 10,000), or the chance that you will become lightheaded. Should you feel this way we will stop the experiment, and you will be given fluids to drink (water or juice). We ask that you remain in the laboratory until we have checked your blood pressure and we are sure that you feel OK.

Graded Exercise Test: You will likely have tiredness, sweating, and breathlessness. You will also have increased heart rate and muscle fatigue. You may also have lightheadedness, fainting, nausea, or muscle cramp, but these occur less frequently. More severe reactions include irregular heartbeat, heart attack (1 in 2,000), and death (1 in 5,000). Severe reactions are rare. It is

possible for you to stumble or fall on a treadmill leading to cuts, scrapes, dislocations, broken bones, head injury, abnormal cardiac rhythms, or even death. You will be taught the safe use of the treadmill and watched closely during exercise. All changes in speed will be made gradually, and you will be assisted in mounting and dismounting.

4. a. Benefits to me: You will receive a medical screening that could inform you about your health. This study should help improve your understanding of how your body reacts to elevated fat levels in the blood.

b. Potential benefits to society: This study will help us to know how dietary fat intake and elevated fat levels in the blood affect nerve activity and blood pressure. Knowing these changes may lead to ways to prevent and treat these impairments. This could lead to improvements in the health and quality of life for obese people and those with problems regulating fat concentration in the blood. A greater percent of our population is becoming overweight or obese. As this happens, the health and welfare of this increasing population has an even greater impact on society, as a whole.

5. Alternative procedures, which could be utilized: Recording heart rate, blood pressure, and blood flow is routine. Increasing fat concentrations in the blood can be accomplished by other means than infusion, such as eating a high fat meal, but these effects last for short periods and are not well controlled. There are other methods to obtain an index of activity in the nerves, but the data obtained from these measurements do not always reflect the amount of true nerve activity, especially with fat intake. Therefore, there is no alternative to the direct recording of electrical activity in nerves.

6. Time duration of the procedures and study: This study will require 6 visits. Days 1 will take a half hour, and day 2 will take about 1 ½ hours at the GCRC (located next to Noll Laboratory). Days 3 and 5 last about 7 hours each in the Noll Laboratory. The two study days (days 3 and 5) will be separated by a period of at least 2 weeks. Days 4 and 6 take about 30 minutes each at the GCRC.

7. Statement of confidentiality: The data are available only to the investigators. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any publication resulting from the research no personal, identifiable information will be disclosed. The researchers code your data with an identification number for statistical analyses. All records containing personal identifiable information are kept in locked cabinets and password-protected computers in secure locations. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), the Office for Research Protections at Penn State and the Biomedical Institutional Review Board may review records related to this project.

8. Right to ask questions: If you have any questions or concerns about the research or your participation in the present investigation, please contact John Florian (W: 814-865-0476, H: 861-6042) or Sandra Smithmyer (W: 814-865-0476, H: 814-357-3794). If there are findings during the research that could relate to your wanting to help with the study, you will be told of the findings. You may contact the Office for Research Protections, 201 Kern Graduate Building, University Park, PA 16802, (814) 865-1775 for additional information concerning your right as a research participant.
Please initial the statement below indicating your understanding of this right.

____ I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

9. Compensation: There will be no charge for any tests required for the study. You will receive the following compensation for participation in this study to compensate for your travel and loss of time:

Day 1 (1st screening day) – no monetary compensation.
Day 2 (2nd screening day) – no monetary compensation.
Day 3 (1st study day) - $90 total: $50 for instrumentation (e.g. catheters, microneurography) and $40 for completion.
Day 4 (1st post-study day) – no monetary compensation.
Day 5 (2nd study day) - $110 total: $50 for instrumentation and $60 for completion of the study.
Day 6 (2nd post-study day) – no monetary compensation**.**

You may be asked to repeat a trial. If you agree to repeat a trial, you will be paid for the repeated trial as stated above.

If you are an employee of Penn State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Penn State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Voluntary participation: Your participation in this study is voluntary, and you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or your refusal to participate will in no way affect your care or access to medical services. You may decline to answer specific questions. However, your acceptance into the study may be contingent upon answering these questions. Your helping with the study may be ended without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document.

11. Event of Injury: Medical care is available in the event of injury resulting from research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against the University for injury resulting from negligence of the University or the investigators. Questions regarding this statement or your rights as a subject of this research should be directed to the Office for Research Protections in 201 Kern Building, University Park, PA (814-865-1775).

12. In the event that abnormal test results are obtained, you will be apprised of the results immediately and recommended to contact your private medical provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for your participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand its contents.

____

Volunteer Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

____

Investigator Date

VITA

John P. Florian

Education

Institution	Degree	Discipline	Date Completed
Pennsylvania State University (University Park, PA)	Ph.D.	Physiology Gerontology (minor)	2007
Florida State University (Tallahassee, FL)	B.S.	Exercise Physiology	2002

Fellowships/Awards

2007	Graduate Exhibition research award
2006-2007	Pennsylvania Space Grant Consortium Fellowship
2006	ACSM Doctoral Student Research Grant
2006	Graduate Research Incentive Award
2005	Physiology Poster Presentation Award
2004-2006	Pennsylvania Space Grant Consortium Fellowship
2003-2006	NIA Gerontology Predoctoral Fellow
2002	Kappa Omicron Nu Scholarship Award

Professional Affiliations

2007	Member, American Physiological Society
2003-2007	Member, American College of Sports Medicine

Abstracts

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3. Sirolli CN, Jarvis SS, **Florian JP**, Curren MJ, Pawelczyk JA. The effect of a somatostatin analog on splanchnic blood volume during head-up tilt. *Med Sci Sports Exerc*. 38(5): S392, 2006.
4. Curren MJ, **Florian JP**, Jarvis SS, Pawelczyk JA. Effect of a somatostatin analog on splanchnic hemodynamics and tilt-table tolerance. *FASEB Journal*.
5. **Florian JP**, Curren MJ, Baisch F, Pawelczyk JA. Caloric restriction decreases orthostatic tolerance. *FASEB Journal*. 18(4): A756, 2004.
6. **Florian, JP** Curren, MJ, Baisch, F, Pawelczyk, JA. Influence of hypocaloric nutrition and bedrest on cardiovascular control during exercise. *Med Sci Sports Exerc*. 36(5): S272, 2004.
7. Curren, MJ, **Florian JP**, Minter SM, Williams JW, Pawelczyk JA. Moderate whole body skin cooling fails to alter cardiovascular hemodynamics or autonomic responses. *FASEB Journal*. 35(5): S254, 2003.