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Adipose Tissue Cytokines: Effects of Social Condition

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ADIPOSE TISSUE CYTOKINES: EFFECTS OF SOCIAL CONDITION

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Social support has been demonstrated to reduce cardiovascular disease morbidity and mortality; however, the mechanisms by which social support reduces disease progression are still unclear. Oxytocin (OT) is a neuropeptide commonly associated with positive social interactions. This series of studies investigated the ability of oxytocin to attenuate atherosclerosis and its putative mediators, pro-inflammatory cytokines. Oxytocin receptors were identified by Western Blot on rat adipose tissue and rat adipocytes. OT receptor mRNA was identified in human adipocyte cDNA. In primary culture of rat abdominal adipocytes, oxytocin (10 pM) decreased LPS-induced IL-6 release by 24.9% after a six hour incubation. Adipose tissue, surgically dissected from $ApoE^{-/-}$ mice chronically infused with OT, secreted less IL-6 than mice infused with a vehicle control. In sum, the presence of OT receptors was demonstrated on adipocytes, OT was shown to reduce IL-6 release *in vitro*, and chronic OT infusion decreased IL-6 release in adipose explants immediately after sacrifice. Potential mechanisms by which adipose tissue's role in the sympathetic nervous system response may affect inflammation, metabolism, and disease are discussed.

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Chapter 1 - Rationale

This study focused on adipose tissue, which resides at the crossroads of metabolic and immune activity linked to atherosclerosis (Rajala & Scherer, 2003; Greenberg & Obin, 2006). Research has revealed that adipose tissue secretes numerous chemical messengers, such that it has been characterized by some as an endocrine organ (Schaffler, Binart, Sholmerich, & Buchler, 2005; Gimeno & Klaman, 2005). Central obesity is a risk factor for the development of atherosclerosis and metabolic syndrome, and expansion of adipose tissue is the hallmark of obesity. Dysregulation of adipokine release has been correlated with atherosclerosis and metabolic syndrome. Research on adipose tissue has identified the presence of multiple cellular constituents implicated in atherosclerosis disease progression, including adrenergic receptors, (Lafontan & Berlan, 1993), NADPH oxidase (Furukawa, Fujita, Shimabukuro et al., 2004) and OT receptors (unpublished data). The combination of central obesity as a risk factor for atherosclerosis with the presence of the above molecules on adipose tissue makes adipose tissue a candidate for investigation into the effects of stress responses on disease progression.

The implications of this study extend beyond atherosclerosis. As adipose tissue participates in the pathogenesis of metabolic syndrome, diabetes, and cardiovascular disease beyond atherosclerosis alone, the elucidation of pathways between fat tissue and disease could affect research in several fields. Further, the study includes an investigation of the role of social environment on disease progression. Although much research has demonstrated that there is a link between social environment and morbidity and mortality in humans (House, Landis, & Umberson, 1988; Berkman et al., 1992; Case et al., 1992; Orth-Gomer, 1993; Brummett et al., 2005), the mechanisms of the link

remains elusive. Possible mechanisms by which positive and negative social interactions affect disease progression are investigated.

Literature Review

Atherosclerosis and Adipose Tissue

As the theory that atherosclerosis is an inflammatory disease has gained prominence, there has been extensive research conducted on the role of pro-inflammatory cytokines. Macrophages have received significant attention due to their participation in inflammation, as well as their direct role in the initiation and progression of atherosclerosis through infiltration of the endothelium and conversion to foam cells. White adipose tissue also produces large quantities of cytokines, termed adipokines, relevant in atherosclerosis. For example, plasma levels of interleukin-6 (IL-6) are correlated with adiposity (Cottam, Mattar, Barinas-Mitchell et al., 2004), as adipose tissue has been shown to produce up to thirty percent of plasma IL-6 (Mohamed-Ali, et al., 1997). Whereas macrophages are directly involved in the pathogenesis of atherosclerosis in the vessel wall, excessive adiposity is widely recognized as one of the primary risk factors for cardiovascular disease (Nanchahal, Morris, Sullivan & Wilson, 2005). While much research has been conducted on the effects of adipokines on cardiovascular disease, further research is necessary to clarify the underlying mechanisms. While not all types of adipose tissue are equally harmful, in atherosclerosis, metabolic syndrome, and type 2 diabetes, visceral fat is especially implicated. Its increased lipolytic activity and proximity to the portal blood supply significantly affects hepatic function and systemic metabolic activity.

Adipose tissue secretes dozens of adipokines, including leptin, adiponectin, resistin, visfatin, and many others, but leptin and adiponectin have been the targets of the most extensive research (Rajala & Scherer, 2003; Greenberg & Obin, 2006; Beltowski, 2006). Leptin has been described as having several potentially atherogenic properties, including causing oxidative stress, inducing endothelial dysfunction, promoting smooth muscle cell proliferation, and increasing plasma CRP (Beltowski, 2006). Moreover, plasma leptin increases as adiposity increases. Adiponectin is an adipokine with potentially anti-atherogenic properties (Greenberg & Obin, 2006). It increases insulin sensitivity and increases free fatty acid oxidation. With respect to atherosclerosis, plasma adiponectin is lower in patients with coronary artery disease (Hotta et al., 2000), it inhibits lipopolysaccharide (LPS)-induced NF-κB activation and IL-6 production in adipocytes (Ajuwon & Spurlock, 2005), it inhibits TNF-α-induced expression of adhesion molecules (Ouchi, Kihara, Arita, et al., 1999), and it prevents macrophages from becoming foam cells (Ouchi, Kihara, Arita, et al., 2001). As leptin and adiponectin are the two most widely researched adipokines (leptin with potentially pro-atherogenic properties, and adiponectin with potentially anti-atherogenic properties), they may provide indications as to whether social condition has a pro-atherogenic or anti-atherogenic effect in this study.

In addition to the adipokines noted above, adipose tissue also secretes other atherosclerosis-related cytokines, including IL-6, TNF-α, PAI-1, and MCP-1. IL-6 induces the acute phase response, including hepatic production of C-reactive protein (CRP) (Heinrich, Castell, & Andus, 1990; Bataille & Klein, 1992). TNF-α promotes lipolysis (Zhang, Halblieb, Ahmad, Manganiello, & Greenberg, 2002), adhesion

molecule expression (Ouchi, Kihara, Arita, et al., 1999), and is correlated with insulin resistance (Kern, Ranganathan, Li, Wood, & Ranganathan, 2001). PAI-1 is a known risk factor for thrombosis and plasma PAI-1 levels correlate with visceral adiposity (Gimeno & Klaman, 2005). MCP-1 is a chemokine that attracts circulating monocytes and is implicated in the process of macrophages infiltrating into adipose tissue.

Adipose tissue is composed of multiple types of cells, including adipocytes, macrophages, preadipocytes, and fibroblasts (Gimeno & Klaman, 2005). As a result, researchers have been working to determine which adipokines and pro-inflammatory cytokines are secreted by macrophages and which are secreted by adipocytes. Currently, there are conflicting reports in the literature (Fried, Bunkin, & Greenberg, 1998; Mohamed-Ali et al., 2001; Fain, Madan, Hiler, Cheema, & Bahouth, 2004). Macrophages accumulate in adipose tissue as adiposity increases, and some researchers claim that obesity is categorized by a chronic state of low-grade inflammation (Yudkin, Kumari, Humphries, & Mohamed-Ali, 2000; Wellen & Hotamisligil, 2005). Regardless of which cell type is responsible for the secretion of which cytokine, there appear to be paracrine effects with the other cells comprising adipose tissue such that secretion from one cell type affects cytokine release from the other (Gimeno & Klaman, 2005).

Adipose Tissue and Free Fatty Acids

All cells contain fatty acids, as they are used to form the phospholipids bilayers of cell membranes. More relevant to energy homeostasis, fatty acids are one of the body's main sources of fuel. In a homeostatic intracellular environment, the quantity of fatty acids that each cell contains equals its reserve for the phospholipid bilayer plus whatever fatty acids are needed to undergo oxidation to meet the metabolic needs of the cell.

Adipose tissue is the primary storage site of free fatty acids (FFAs), which are combined with glycerol to be stored in the form of triglycerides. Dysregulation of FFAs contributes to the progression of atherosclerosis.

Lipolysis is the process by which stored triglycerides are broken down into FFAs, which are released into the circulation to provide energy. A primary target for the FFAs is muscle tissue, which uses them for energy. If more FFAs are released than the muscle requires, then the liver absorbs the FFAs and converts them into triglycerides. Excess triglycerides are packaged into very low density lipoproteins (VLDL) and returned to circulation. Lipoprotein lipase on endothelial cells in adipose tissue hydrolyzes the VLDL to facilitate storage of the lipid in the fat cells. In obese states, adipocytes expand and proliferate to accommodate increased lipid storage needs. Despite these structural changes in the adipose tissue, increased lipid storage demands eventually result in a state where the adipose tissue can no longer store all of the plasma lipids. Obesity is characterized by elevated plasma FFAs (Campbell, Carlson, & Nurghan, 1994). The circulating FFAs enter non-adipose tissues and are sequestered.

In several of the tissues (e.g., muscle, liver, pancreatic islet cells) that experience significant elevations in triglyceride content, there can be pathogenic responses, including insulin resistance (Schaffer, 2003; Defronzo, 2004). The accumulation of surplus lipid in non-adipose tissue leads to cell dysfunction or death and is termed lipotoxicity (Schaffer, 2003). In the skeletal muscle tissue, increased triglycerides are correlated with insulin resistance. In the liver, increased triglyceride storage is related to insulin resistance. Additionally, increased circulating FFAs are utilized by the liver for synthesis of very low density lipoproteins. Further, insulin suppresses hepatic glucose production, but

insulin resistance prevents insulin from doing so, leading to hyperglycemia. Consequently, hepatic glucose production increases in an insulin resistant state. In pancreatic β cells, increased islet lipid exposure has been associated with impaired insulin secretion (Zhou et al., 1995; Yu et al., 2002) and β cell apoptosis in primary rat pancreatic β cell culture (Shimabukuro et al., 1998). Thus, obesity contributes to metabolic and cardiovascular diseases (including atherosclerosis) by promoting excess circulating FFAs, which have effects on insulin resistance in muscle, impaired suppression of hepatic glucose production, and impaired insulin secretion by islets.

Lipolysis affects insulin resistance by increasing circulating FFAs and free glycerol, which inhibit the responsiveness of fat and liver cells to insulin. FFAs promote insulin resistance by altering the insulin transduction signal via inhibition of several key components of the receptor system, which facilitates insulin's action of promoting cellular glucose uptake (Griffin et al., 1999). Specifically, increased FFAs lead to a serine kinase cascade that ultimately inhibits insulin receptor substrate-1 tyrosine phosphorylation and reduced GLUT4 glucose transporter translocation to the cell membrane. FFAs also induced reactive oxygen species formation in human vascular endothelial cells (Chinen et al., 2007). Reactive oxygen species (ROS) may be involved in the initial insult to vascular cells and tissue to which immune cells respond. This may trigger the process which leads to eventual macrophage infiltration, foam cell formation, and eventual atherosclerosis.

Thus, as adipose tissue expands, it becomes insulin resistant. Since insulin is anti-lipolytic, increased insulin resistance leads to greater lipolysis and higher circulating FFAs. Higher circulating FFAs result in peripheral tissues oxidizing the FFAs. Over

time, this may result in insults to the tissues, as they cannot sustain oxidizing FFAs over prolonged periods. A proposed mechanism by which increased oxidation of FFAs damages tissues is via reactive oxygen species produced by mitochondria (Schrauwen & Hesselink, 2004). Increases in mitochondrial activity secondary to an increased need to oxidize FFAs result in an increase in intracellular ROS. FFAs exposed to ROS undergo oxidative damage, resulting in lipid peroxides, which may damage the mitochondria and other cellular constituents. Damage to the mitochondria further reduces the cell's ability to oxidize FFAs. The downstream effects of more circulating FFAs lead to increased adipose tissue expansion, increased insulin resistance, and a chronic low-grade inflammatory state, all of which contribute to the development of atherosclerosis.

Sympathetic Nervous System, Atherosclerosis, Social Condition, and Adipose Tissue

In a review by Black (2003), the author notes that acute and chronic stress responses have immune components that include inflammation. Of particular interest is the role that IL-6 plays in stimulating the acute phase immune response, and that psychosocial stressors have been demonstrated to activate the acute phase response. IL-6 secretion occurs to a significant extent in endothelial cells, the liver, and adipocytes. As inflammation is considered by many to be central to CVD, CVD may stem from repeated episodes of the acute phase response due to chronic stress, in part mediated by IL-6 production.

SNS activation involves numerous components, which includes both mobilization of lipid stores and secretion of immune agents. SNS hormones epinephrine and norepinephrine, and structurally similar agents, induce lipolysis (Nakamura, 2006; Aitchison, Clegg, & Vernon, 1982; Morimoto, Kameda, Tsujita, & Okuda, 2001) and IL-

6 release from adipose tissue (Keller, Keller, Robinson, & Pedersen, 2004). A downstream effect of IL-6 release is the stimulation of C-reactive protein release by the liver. In a previous study conducted by this laboratory, CRP across all social conditions at three and five months predicted levels of aortic atherosclerosis at seven months in Watanabe Heritable Hyperlipidemic (WHHL) rabbits (Brooks et al., 2006). This study examines whether there is a relationship between IL-6 secretion and disease across social conditions, which may explain the previously observed relationship between CRP secretion and disease across social conditions.

Further support for the hypothesis that SNS activation is related to disease progression comes from a series of studies on cynomolgous monkeys fed a high fat diet, in which an unstable social environment led to development of atherosclerosis in dominant monkeys (Kaplan et al., 1991). The administration of a β-blocker led to a lowering of heart rate and blood pressure, as well as an attenuation of the amount of atherosclerosis, indicating an SNS role in lesion progression. This finding demonstrates that the sympathetic nervous system plays an important role in disease progression in these monkeys, and it is possible that unstable WHHLs also experience similar sympathetic involvement in their disease course. In summary, research from our own laboratories, as well as parallel research in a different animal model, suggests that disease progression in stressful social conditions results from increased sympathetic nervous system activity.

In summary, two separate but related roles of adipose tissue are implicated in the progression of atherosclerosis. As obesity increases and adipose tissue begins to experience difficulty storing excess energy, circulating levels of lipids arise. One long-

term consequence of increased plasma FFAs is insulin resistance in skeletal muscle and liver tissues via inhibition of insulin signal transduction. This inhibition of insulin signal transduction prevents insulin from inhibiting hormone sensitive lipase's lipolytic effects, thus triggering further release of FFAs. Additionally, macrophages increasingly infiltrate into adipose tissue of obese individuals such that more pro-inflammatory cytokines are released by adipose tissue. The initial mechanism which induces macrophages to infiltrate adipose tissue is poorly understood, but it may be related to an autocrine response by adipocytes related to the hypertrophy that occurs as fat tissue expands (Ferrante Jr., 2007). As adipose tissue expands, adipose tissue cells secrete greater amounts of TNF-α and MCP-1, which attract circulating macrophages into the adipose tissue (Wellen & Hotamisligil, 2003). Beyond local inflammatory and chemotactic effects of macrophage- and adipocyte-derived pro-inflammatory cytokines, there are systemic effects of increased inflammation in obesity, such as increased circulating IL-6 and altered regulation of leptin, adiponectin, and other adipokines which act in an endocrine fashion.

Human Studies Linking Behavior, Weight, and Disease

Investigators have sought to implement the findings from basic science research on obesity, atherosclerosis, and stress to reduce disease prevalence by lifestyle interventions. In humans, these efforts have focused primarily on changing diet and exercise patterns. In animals, it has been shown that manipulation of the social environment in rabbits with dyslipidemia influences the progression of atherosclerosis such that stable social conditions can slow disease progression (McCabe et al., 2002).

Two randomized controlled trials have demonstrated that a program focusing on improving diet and exercise behaviors can reduce type 2 diabetes mellitus risk (Tuomilehto et al., 2001; Diabetes Prevention Program Research Group, 2002). Subjects were at risk of becoming diabetic based on plasma glucose, glucose tolerance, or weight. In both studies, the lifestyle intervention reduced the risk of diabetes by 58% when compared to the control group. In the Diabetes Prevention Program (DPP) study, the lifestyle intervention was more effective in reducing diabetes incidence than a pharmacological intervention with Metformin (31% reduction) as compared to placebo. The programs resulted in reductions in body weight and improvements in glycemic control. Although the studies focused on reducing diabetes, secondary analyses of the DPP showed that the lifestyle intervention positively affected several risk factors for atherosclerosis, including reduced obesity, HDL, triglycerides, hypertension, fasting glucose, and glucose tolerance. Several smaller studies also have demonstrated the health benefits of a lifestyle intervention by reducing mortality in men with impaired glucose tolerance in Sweden (Eriksson & Lindgrade, 1991), reducing incidence of Type 2 diabetes in China (Pan et al., 1997), and reducing incidence of diabetes in India (Ramachandran et al., 2006).

Oxytocin, Social Condition, and Adipose Tissue

Much of the background information on OT has been extensively reviewed in several sources, (Gimpl & Fahrenholz, 2001; Landgraf & Neumann, 2004). To briefly summarize, OT is a neurohypophyseal, nonapeptide secreted primarily by neurons in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus. OT released through the pituitary gland is manufactured in magnocellular neurons in the PVN and

SON. In contrast, centrally released OT is made in parvocellular neurons of the PVN and SON, as well as in extra-hypothalamic locations. OT consists of a chain of nine amino acids, and it has only one identified receptor throughout the body. OT is highly conserved evolutionarily, as it exists in all mammals, and most vertebrate species have an OT analogue (Landgraf & Neumann, 2004*)*. OT can be released both peripherally via axons to the posterior pituitary and centrally via secretions by the axons (preferentially to limbic brain areas), cell body, and dendrites (preferentially to hypothalamic nuclei). OT is not recycled at the synapses, but requires new synthesis of peptide for further release.

The OT receptor is a G protein-coupled receptor with 7 transmembrane domains consisting of 389 amino acids (Gimpl & Fahrenholz, 2001). OT receptors require at least two necessary components for high affinity binding, which are membrane cholesterol and divalent cations such as Mg^{2+}. OT receptor expression occurs throughout the body, including the uterus, mammary glands, kidney, heart, vascular endothelial cells, vascular smooth muscle cells, thymus, pancreas, prostate, and adrenal glands. OT receptor expression occurs extensively within the central nervous system in humans, including the basal nucleus of Meynert, dorsal raphe nucleus, substantia nigra pars compacta, nucleus of the solitary tract, dorsal motor nucleus of the vagus nerve, lateral septal nucleus, globus pallidus, amygdala, preganglionic sympathetic neurons of the intermediolateral column of the spinal cord, and several thalamic and hypothalamic nuclei. Many of the above regions are involved in autonomic and neuroendocrine stress responses.

OT has been implicated in complex social behaviors, such as social recognition, maternal behaviors, and pair bonding. Relevant to investigations of the relationship of

social support to disease, OT has been shown to have anxiolytic properties. OT's involvement in positive social interactions leads to relaxation, reduced SAM activity, decreased HPA activity, enhanced parasympathetic activation, & anabolic metabolism (Uvnas-Moberg, 1998). OT infusions decrease anxiety-like behaviors in male voles (Bielsky & Young, 2004). Due to its identified role in social behaviors and attenuating stress response, our laboratory has been investigating OT as potentially involved in the decrease in disease progression that occurs in stable social environments.

Research on the effects of OT on adipose tissue function has shown that OT has insulin-like effects on lipogenesis, which appear to operate through a different mechanism than insulin (Braun, Hecter, & Rudinger, 1969). Oxytocin has also been shown to inhibit β-adrenergic stimulated lipolysis in adipocytes (Muchmore, Little, & de Haen, 1981). When lipolysis was strongly stimulated, OT acted in a purely inhibitory fashion, and when lipolysis was weakly stimulated high concentrations of OT were less inhibitory than low ones. Oxytocin receptors have been implicated on rat fat cells through high affinity binding studies (Bonne & Cohen, 1975). The accumulation of evidence that there may be regulation of fat cells by hormones secreted by endocrine organs (Schaffler, Binart, Scholmerich, & Buchler, 2005), as well as the presence of OT receptors in adipose tissue (data presented below), endothelial cells, the liver, and the heart (Gimpl & Fahernholz, 2001), indicates that OT may affect these tissues which have key roles in cardiovascular disease.

OT effects on inflammation have been demonstrated by OT's ability to reduce carrageenan induced inflammation in rats' hindpaws (Petersson, Wiberg, Lundeberg, & Uvnas-Moberg, 2001), to decrease IL-6 production in human osteoblast-like cells

(Petersson, Lagumdzija, Stark, & Bucht, 2002), and to inhibit LPS-induced IL-6 release from rat neurointermediate pituitary lobe cells (Spangelo, deHoll, Kalabay, Bond, & Arnaud, 1994). In a study of oxidative colonic inflammation, OT injections significantly reduced lesion size (Iseri et al., 2005). Further, OT has been reported to prevent LDL oxidation (Moosman & Behl, 2002). To summarize, peripheral OT may bond to receptors on adipose tissue, the heart, the vasculature, and other relevant cells to attenuate disease-related inflammation and oxidation. This study will explore whether OT can attenuate SNS-related increases in lipolysis, pro-inflammatory cytokine release, and adipokine release.

Chapter 2 - Hypotheses

The overriding hypothesis which our laboratory is examining is that social environment affects disease progression and that oxytocin is involved in attenuating disease progression in stable social environments. Oxytocin is hypothesized to have anti-inflammatory properties, which can attenuate atherosclerosis. The emphasis of this dissertation is the relationship between OT and adipose tissue in the context of atherosclerosis. The smaller studies within the overall project study whether a stable social environment (thought to involve increased OT release) leads to decreased atherosclerosis in mice, whether mice chronically infused with OT exhibit less disease (Daniel Nation's dissertation), whether adipose tissue from mice infused with OT exhibit reduced stimulated and unstimulated pro-inflammatory cytokine release, and whether OT can attenuate LPS-induced pro-inflammatory cytokine release in adipocytes. The investigation of these hypotheses requires a combination of *in vivo*, *ex vivo*, and *in vitro* designed to test the grand hypothesis and elucidate its mechanisms.

Specifically, the hypotheses of the *in vivo* study are that mice maintained in a stable social environment will have less atherosclerosis than mice caged individually or in an unstable social environment. Although, the level of OT across groups is not measured in the study due to the large quantity of blood required to measure OT, it is hypothesized that three potential processes are at work among the social groups. In the stable group, affiliative behaviors are hypothesized to lead to increased OT secretion or changes in OT receptor expression resulting in lower atherosclerotic disease burden, as well as lower levels of pro-inflammatory cytokines and more adiponectin at the end of the study. In the individually-caged group, increased sedentary behavior is hypothesized

to lead to adipose tissue expansion and weight gain. As adipose tissue expands, macrophage infiltration is hypothesized to increase, thus reducing adiponectin, increasing the amount of plasma pro-inflammatory cytokines at the termination of the study, and increasing atherosclerosis in relation to the stable group. In the unstable group, increased social stressors are hypothesized to increase SNS activation with pro-atherogenic downstream effects. Specifically, unstable animals are hypothesized to have more atherosclerosis than the stable and individually-caged animals, increased plasma pro-inflammatory cytokines, and less adiponectin.

The *ex vivo* component of the study is designed to investigate the involvement of adipose tissue in atherosclerosis and to provide more data to support or to refute the *in vitro* hypotheses regarding the effects of social condition. First, *ex vivo* basal and stimulated pro-inflammatory cytokine and free fatty acid release is hypothesized to be positively correlated to total atherosclerosis. Across social conditions, the stable group is hypothesized to have the lowest basal and stimulated pro-inflammatory cytokine and free fatty acid release. The individually-caged group is hypothesized to have the strongest response to epinephrine and LPS stimulation due to increased macrophage infiltration into the adipose tissue.

The *in vitro* studies investigate the ability of OT to attenuate epinephrine- and LPS-induced IL-6 release in rat adipocytes. While all adipocytes are expected to respond to epinephrine or LPS stimulation by increasing IL-6 release, it is hypothesized that the degree of IL-6 release will be significantly reduced in cells incubated with OT.

Chapter 3 - Methods

A series of experiments were conducted to test the effects of inflammation on adipose tissue *in vitro*, to measure differences in adipokine production across social groups *in vivo*, and to determine effects of social condition on stimulated adipokine production *ex vivo*.

Experimental Animals

The first experiment was to stimulate adipocytes to release pro-inflammatory cytokines with both epinephrine and lipopolysaccharide (LPS). Fat tissue was dissected from 16 week old Sprague-Dawley female rats (Charles River Laboratories CD(SD)) sacrificed using carbon dioxide and subsequent cervical dislocation. All procedures were in accordance with protocols approved by the Animal Care and Use Committee of the University of Miami. The rats were housed individually and provided food and water *ad libitum.*

Fat Extraction and Culture

Fat (10-20 grams) from the abdominal cavity was placed in 50 ml tubes containing 20 ml DMEM/F12 buffer (Sigma catalog number D6434) containing adenosine (200 nM), 5 M Hepes, Penicillin (100 i.u.)/Streptomycin (100 ug/ml), Glutamax (2mM), and fatty acid free albumin (10 mg/ml). All instruments were sterilized via autoclave and a glass bead sterilizer. Tissue was minced coarsely upon dissection, then transported to a sterile environment for further processing. The tissue was washed once with 25 ml of DMEM/F12 buffer. All steps were conducted at room temperature. Following the initial wash, the tissue was minced for 15 minutes to obtain pieces < 1 mm using scissors inserted into the 50 ml tube. The tissue was then

centrifuged for 30 seconds at 300 x g, and the buffer was aspirated from underneath the floating tissue. The process of centrifuging, washing, and aspirating was repeated 3 times. The tissue was enzymatically digested in a shaking water bath at 37^{o} C using 1 mg/ml Type I collagenase dissolved in DMEM/F12 (Gibco cat #17100-017) for 45 minutes to obtain adipocytes. Following the collagenase digestion, the adipocytes were centrifuged and the infranatant was discarded. The tissue was washed with 50 mls media and filtered through a 200 um mesh nylon filter (Spectrum cat. # 40693) Adipocytes were collected in a 50 ml tube, which was then centrifuged and washed 3 times at 300 x g for 30 seconds. The adipocytes were placed into a petri dish at a concentration of 2-3 mls of adipocytes per well in 15 mls of media. Insulin (7 nM) was added, and the cells were incubated overnight. The media was changed after 24 hours and every 48 hours thereafter.

Drug Stimulation and Doses

On the day after isolation, the adipocyte media was replaced. On the day of the experiment (day 2 or 3 after isolation), the media was replaced. Cells were transferred from the petri dishes into a 50 ml tube with a triangular stir bar in the bottom. A stir plate was placed in the hood. The cells were stirred and aliquoted to ensure equal distribution of the cells across wells. Cells were aliquoted (0.5 ml) with a previously silanized 1 ml serological pipet and added to 0.5 mls of media pre-mixed with 2x concentrations of LPS and OT. The final concentration of cells was 100,000 cells in 1 ml in a 24-well plate.

The concentration of LPS (Sigma cat # L5293) for incubation was 100 ng/ml. Epinephrine (10^{-5} M; Sigma cat # E1635) was used to activate adrenergic receptors and initiate a pro-inflammatory response in adipose tissue. This incubation occurred with and

without OT (10 pM - 1 nM) to determine if OT was able to alter the pattern of cytokine secretion caused by pro-inflammatory stimulants as hypothesized.

Table 1 - Experimental Groups

Control Only
Control + OT
Control + Inflammatory Stimulant (LPS or Epinephrine)
Control + Inflammatory Stimulant (LPS or Epinephrine) + OT

Tissue Recovery and Assays

Initial experiments investigated IL-6 secretion over time. The time course studies showed that it takes several hours for the LPS effect to increase IL-6 release relative to the control group. Thus, subsequent studies focused on time points later than 3 or 4 hours in order to ensure a significant LPS effect. Only one time point can be accurately measured per well, as adipocytes adhere to the sides of the well. When media is aspirated out, many adipocytes remain on the sides of the well, thus confounding comparisons over time as the number of adipocytes in the media diminishes over repeated aspirations. After aspiration, the media was centrifuged. Next, the infranatant was collected and stored in four aliquots of 220 ul each in the -80^{o} C freezer. IL-6 secretion was measured by ELISA (R&D Systems cat # DY506). Assays were run in duplicate with two sets of standards per plate.

Oxytocin Pump Study

Forty-five, male, ApoE$^{-/-}$ mice (Jackson Laboratories) were 11-12 weeks of age and weighing 22-24 grams upon arrival. Mice were randomly assigned to either a control (n=21) or experimental (n=24) condition on arrival and were individually housed in a temperature- and humidity-controlled environment. The light/dark cycle was reversed

and food and water were provided ad libitum. Mice were acclimated for one week prior to the start of the experiment.

Osmotic minipumps (model 2006, DURECT Corporation, Cupertino, CA), programmed to secrete 0.15μL/hr for 6 weeks, were prepared aseptically and weighed prior to filling with either 200μg/mL OT (approximately 1μg/kg/hr) or vehicle (VH), 50mM Sodium Citrate, pH = 4. The dose was selected to provide an estimated steady state plasma OT level of between 100pM and 500pM. Following filling, pumps were weighed again. The difference in weight of each pump after loading was ≥ 90% of the expected difference. The pumps were primed in sterile saline at 37ºC for 70hrs.

Surgeries to implant the pumps were performed in a sterile environment according to the pump manufacturer's protocol (DURECT Corp.). Mice were given ketamine/xylazine (10mg ketamine/0.4mg xylazine/30g mouse) anesthesia and scrubbed with betadine and ethanol before surgery.

An incision was made subcutaneously in the mid-scapular region. The pump was then placed into the opening, previously widened with a hemostat. The wound was sealed with adhesive (Vetbond) and surgical staples (Kent Scientific Inc.). Animals then recovered from the surgical procedure in a 37ºC incubator before returning to their own cages.

The pumps were replaced at midpoint (16 weeks) because a single pump was unable to provide the amount of OT necessary for the duration of the study. The procedure was identical except for the extraction of the old pumps. Following extraction, pumps were frozen at 4°C for further analyses, specifically confirmation of OT stability and pump function.

In Vivo Study Experimental Animals

The second set of experiments was dependent upon the larger social conditioning experiment being conducted by our laboratory. This study was conducted on male ApoE$^{-/-}$ mice (Jackson Laboratories) aged six weeks at the onset of the study. All procedures were in accordance with protocols approved by the Animal Care and Use Committee of the University of Miami. Behavior and weight were measured each week of the study. Animals were randomly assigned to groups based on litter to prevent a genetic loading in a specific social condition. The stable group was composed of littermates, and the unstable group was composed of non-littermates. Pairings for the unstable group were changed twice weekly. Whenever a pairing was changed, the animals were placed in a cage with a cage divider that cut the size of a standard cage roughly in half. The introduction of the two animals was videotaped for the first 15 minutes. The recordings were later scored for the amount of affiliative, aggressive, and other behaviors. Each day, animals were confined in a smaller space with a cage divider for four hours so as to maximize the amount of contact between animals.

The animals were sacrificed at 25 weeks of age. During the course of the study, behavior, weight, blood, urine and feces were collected for measurement. Animals were sacrificed by anesthetizing them with ketamine/xylazine, followed by a cervical dislocation. Animals were shaved, cleaned with iodine, and then opened with a scalpel. The epididymal fat tissue was removed first and weighed. Subsequently, the unwanted viscera were removed, leaving part of the carcass with the aorta still attached. The carcass was submerged in 10% buffered formalin.

At a later date, the aortas were cleaned of fat and connective tissue and opened longitudinally with fine scissors. In collaboration with Ed Herderick, aortas were stained with Oil Red O and pinned on wax and digitally photographed. Points with Oil Red O stain received a binary value, resulting in the creation of a map of the aorta with pixels positive for Oil Red O standing out against a black background. Proportions of disease were calculated regionally (aortic arch, thoracic aorta, abdominal aorta) with specific landmarks used to identify the regions. Total lesion area and proportion of region with disease were provided.

In Vivo Plasma Adipokines

Plasma adipokine (Millipore cat # MADPK-71K-07) and cytokine (Millipore cat # MCYTO-70K-05) levels were determined at baseline and termination of the experiment using multiplex assays.

In Vivo Study - Fat Extraction & Ex Vivo Culture

The next experiment consisted of dissecting out the epididymal white adipose tissue from mice upon sacrifice. Prior to sacrifice day, 4 ml glass tubes were silanized with Sigmacote (Sigma SL2) to prevent the adipose tissue from adhering to the glass sides of the tube. Tubes were rinsed with water and autoclaved to ensure sterility.

At sacrifice, approximately 200-500 mgs of wet weight of fat tissue were dissected from each mouse. The entire epididymal fat pads were taken from each mouse. Between 25 and 50 mgs of tissue was saved for future RNA quantitation, homogenized, and preserved in 1 ml lysis reagent (Qiagen). An additional 25-50 mgs piece of tissue was saved for histological analyses and preserved in 10% buffered formalin. The remainder of the tissue was washed in 3 mls of media and minced for 2 minutes. The media was

removed, and the tissue was again washed with 5 mls media. The washed tissue was moved to a 1.7 ml plastic tube and centrifuged for 30 seconds at 300 x g. The tissue was then aliquoted into one of six (3 experimental conditions in duplicate) 4 ml glass tubes. The conditions were control, epinephrine (10^{-5} M), or LPS (100 ng/ml). Tissue was incubated in a 37^{o} shaking water bath for 6 hours. 500 mls of media were removed and frozen. Tissue was frozen, lyophilized, and weighed (dry weight). Cell culture media was assayed for IL-6 (R&D Systems cat # DY406), adiponectin (R&D Systems cat # DY1119), and NEFA (Wako USA). Results were normalized to tissue weight to normalize.

Statistical Analyses

Three sets of statistical calculations were employed. First, t-tests were used to compare the effectiveness of OT in altering cytokine production. In particular, comparisons were made between media from unstimulated wells or wells stimulated with epinephrine or LPS. Analysis of social condition differences in adipokine production were measured using ANOVA with post hoc comparisons with significance at the 0.05 level. Pearson correlations were also conducted to measure the relationship between plasma adipokines, *ex vivo* results, aortic disease, weight, and glucocorticoids.

Chapter 4 - Results

In Vitro Studies

The primary hypothesis being investigated in the *in vitro* studies was whether OT can attenuate pro-inflammatory cytokine (IL-6) release. As a result of a lack of conclusive studies in the literature to demonstrate the presence of OT receptors on adipocytes, experiments were conducted to confirm the presence of OT receptors on adipocytes. First, real time PCR was conducted on cDNA from human subcutaneous adipose tissue and adipocytes (ZenBio), and OT receptor mRNA was identified in both adipose tissue and in adipocytes. There was a five-fold greater expression of OT receptor mRNA in adipocytes as compared to adipose tissue, likely because OT receptors are expressed in higher concentration in adipocytes than in other cell types present in adipose tissue. Further, mRNA for OT was detected in both adipose tissue and adipocytes suggesting that these cells may generate locally produced OT. Additionally, Western Blots performed of rat adipose tissue and adipocytes showed immunoreactive bands of approximately 67 kD as would be expected for mature OT receptors (Figures 1 and 2). Moreover, the bands are at the same level as similar bands in a positive control, rat uterine tissue, known to have high levels of OT receptor expression. The combination of PCR and Western Blot provide better evidence than previous high affinity binding studies to confirm existence of OT receptors on adipocytes. These data show that both OT mRNA and OT receptor protein are expressed in adipocytes, suggesting that previous studies using high-affinity binding to identify OT receptors were likely mediated by receptor-dependent events.

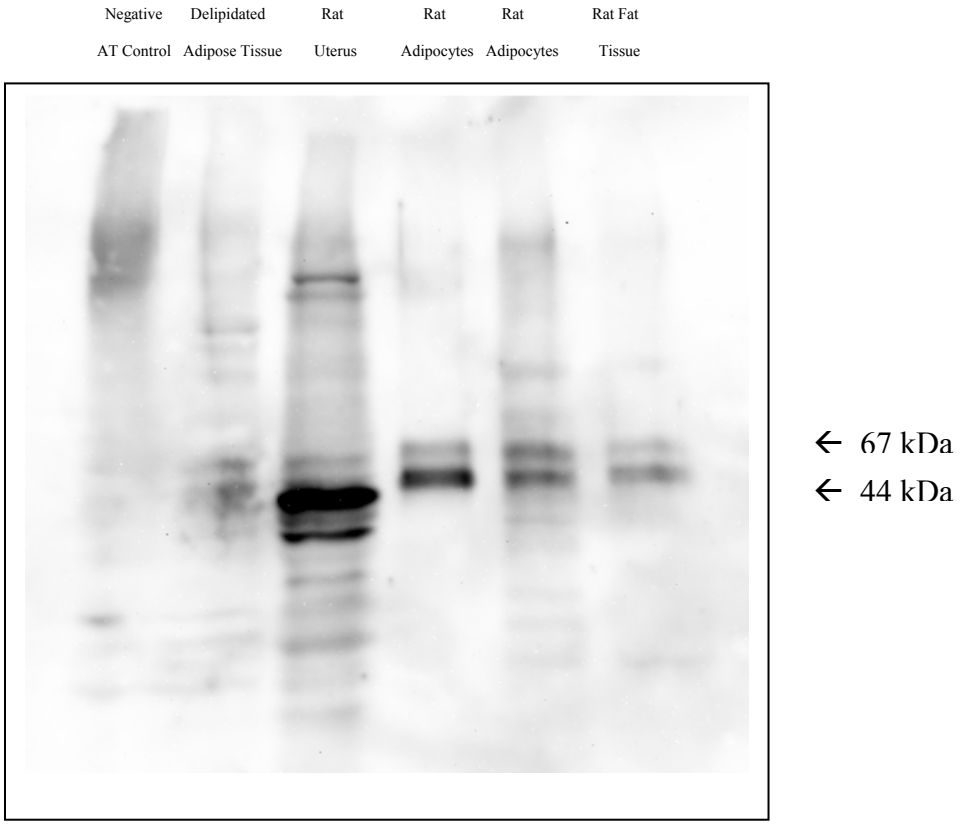

Figure 1. Comparison of OT receptor Western blot involving different tissue types.

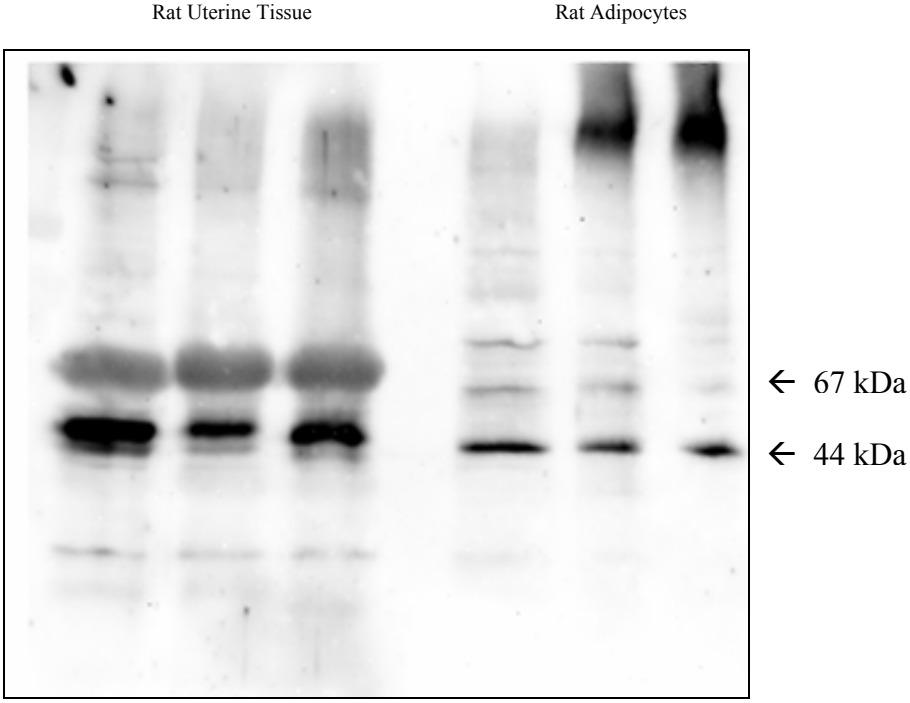

Figure 2. Comparison of OT receptor Western blot in rat adipocytes and rat uterine tissue.

Dose response experiments were conducted to determine the optimal dosages of LPS and epinephrine for stimulating IL-6 secretion in adipose tissue. An LPS dose response shows that IL-6 release increased significantly between 10 ng/ml and 100 ng/ml of LPS after six hours with no further effect at higher doses (Figure 3). Consequently, 100 ng/ml was selected as the dosage of LPS to use in the study because it was the minimum dose necessary to provide an inflammatory response would provide the best chance for an anti-inflammatory agent to attenuate that response.

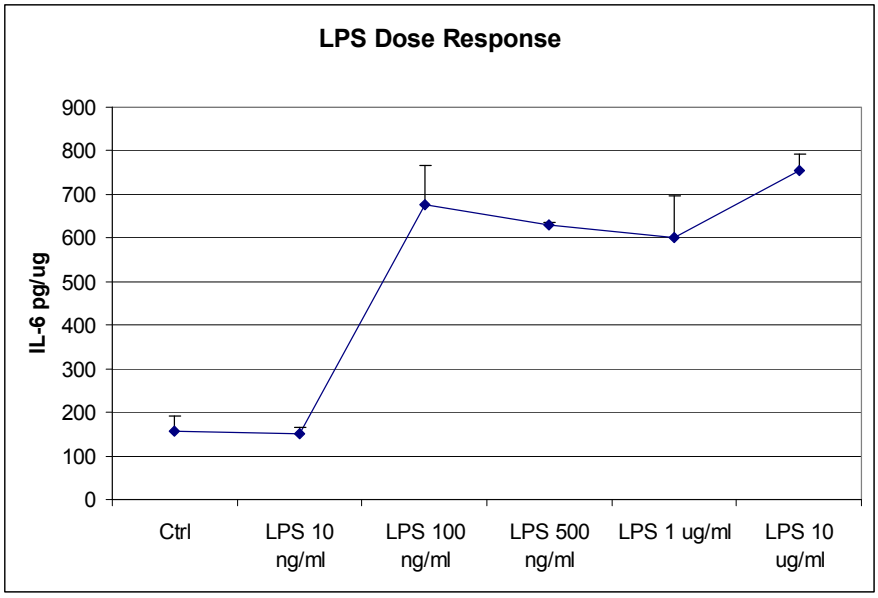

Figure 3. LPS dose response of IL-6 release in rat adipose tissue.

A time course study was conducted to examine elevations in LPS-induced IL-6 release. As shown in Figure 4, there was little difference between the media and LPS groups until the 6 hour time point. After the 6 hour time point, the differences between the media and LPS groups increased.

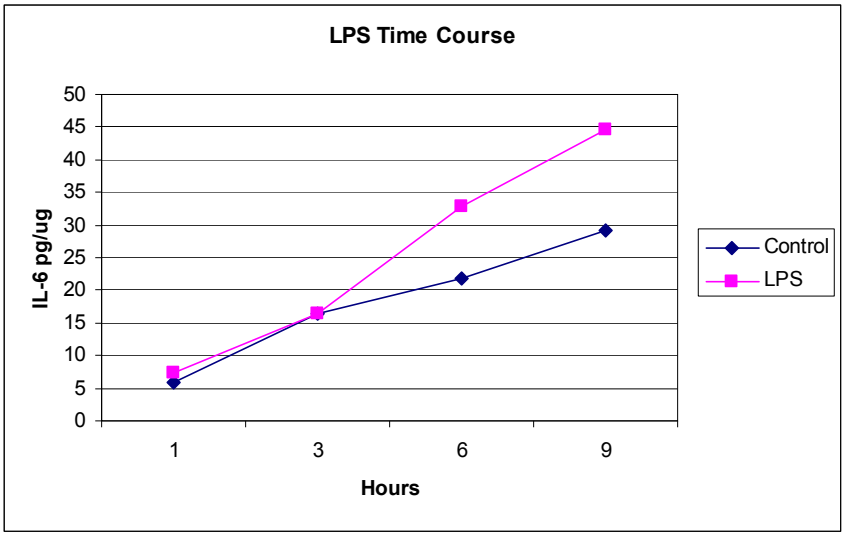

Figure 4. Time course of LPS-induced IL-6 release in rat adipose tissue.

A similar experiment was conducted to determine the optimal epinephrine dose for stimulating IL-6 release from adipose tissue. Several doses of epinephrine were incubated with adipose tissue; however, only the 10^{-5} M dose was able to significantly increase IL-6 output above the control, as shown in Figure 5. As a result, the 10^{-5} M dose of epinephrine was selected for future incubations.

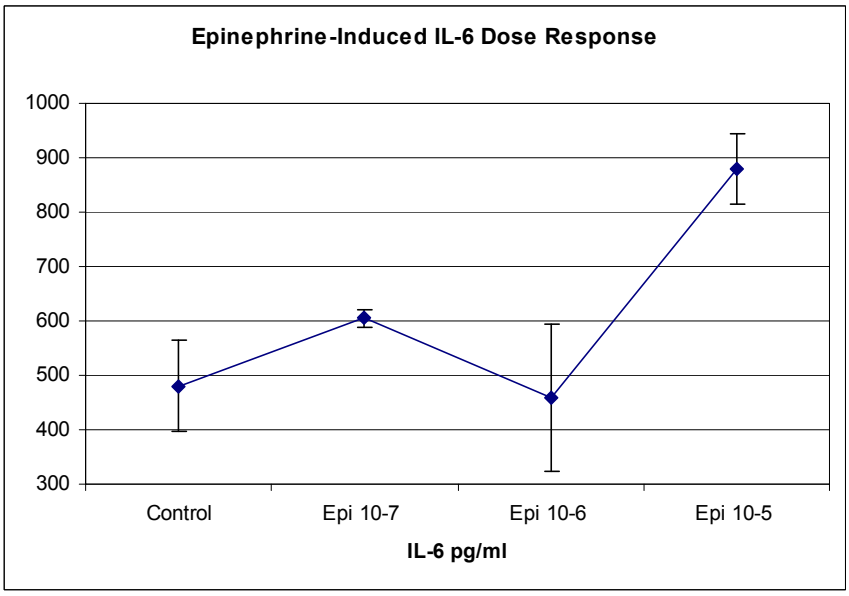

Figure 5. Time course of epinephrine-induced IL-6 release in rat adipose tissue.

Across a series of experiments, oxytocin did not alter the induction of IL-6 release in adipocytes stimulated with epinephrine. In Figure 6, stimulated IL-6 release of conditions co-incubated with epinephrine with and without OT (10 pm and 1 nM) are compared. OT did not attenuate epinephrine-induced IL-6 release (Epi vs. Epi + OT 10 pM, $p = 0.86$; Epi vs. Epi + OT 1 nM, $p = 0.38$).

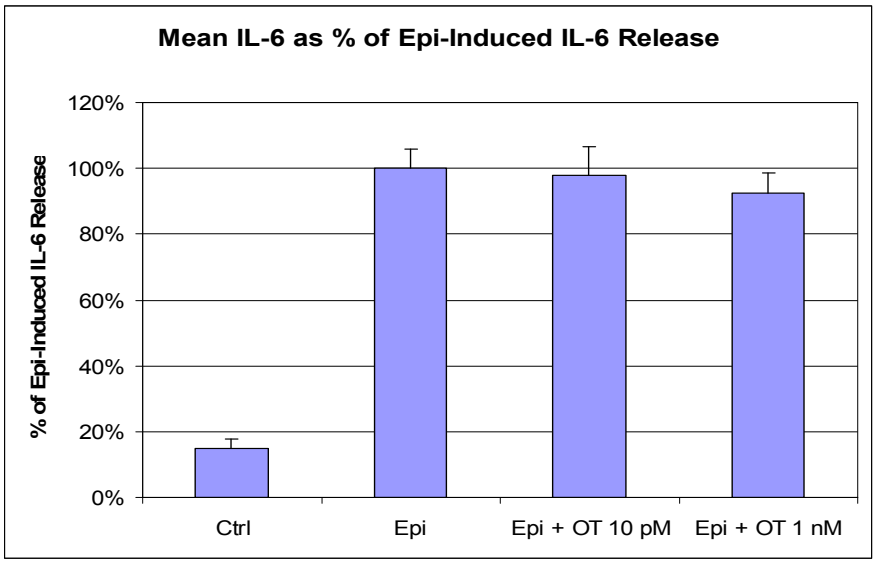

Figure 6. OT and epinephrine-induced IL-6 release at 6 hours in rat adipocytes.

When adipocytes were stimulated with LPS, oxytocin significantly reduced the LPS-induced increase in IL-6 by 24.9% ($p < .05$, Figure 7). This effect occurred primarily at 6 hours after co-incubation of adipocytes with both oxytocin (10 pM) and LPS (100 ng/ml). Co-incubation of a higher dose of OT (100 pM) did not significantly reduce LPS-induced IL-6 release ($p = 0.53$).

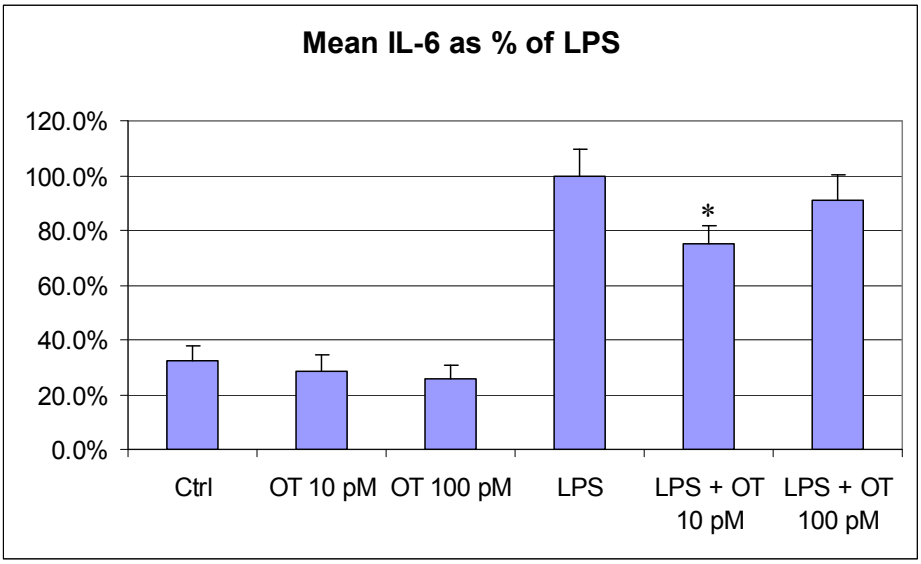

Figure 7. OT and LPS-induced IL-6 release at 6 hours in rat adipocytes.
* $p < 0.05$

In sum, the *in vitro* studies demonstrate that OT is able to attenuate LPS-induced IL-6 release from adipocytes by 25%. OT did not attenuate an epinephrine-induced IL-6 release.

Ex Vivo Data – Pump Study

This study was conducted in collaboration with Daniel Nation as part of his dissertation in order to determine whether mice chronically infused with OT exhibit less atherosclerosis than control mice. Adipose tissue from control mice or mice that received chronic oxytocin infusions over 15 weeks was collected at sacrifice and the organ cultures were incubated for 6 hours to evaluate cytokine production. OT-treated mice had significantly lower basal, adipose tissue IL-6 release in culture 6 hours after sacrifice than did mice that received saline control ($p < .01$, Figure 8). There were no differences in IL-6 release when fat was stimulated by epinephrine or LPS between the oxytocin and control conditions.

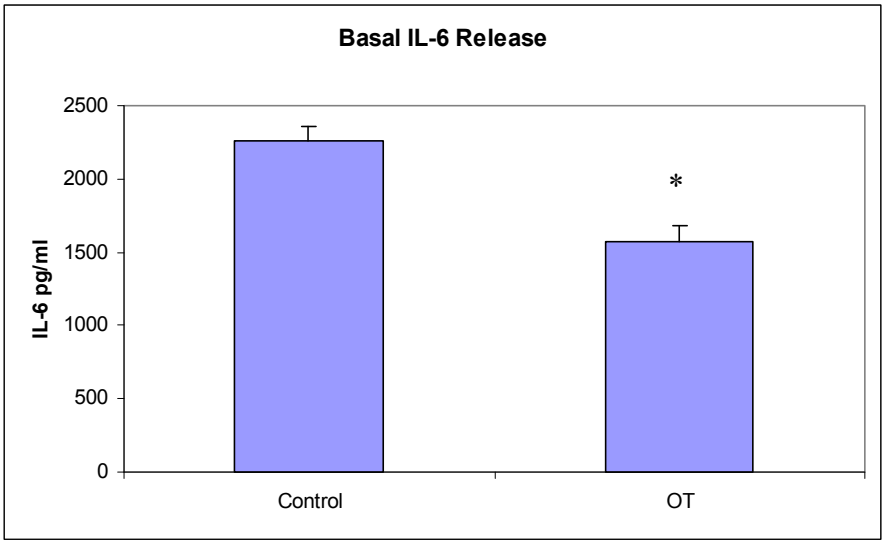

Figure 8 – IL-6 release from adipose explants of mice chronically infused with OT or vehicle control.
* $p < 0.05$

Ex Vivo Data – Mouse Social Environment Study

There were two primary hypotheses of the *ex vivo* component of the series of studies: whether social environment leads to differential regulation of adipose tissue cytokine and free fatty aid release and whether stimulated or unstimulated release relates to disease. Adipose explants removed from mice and incubated in epinephrine, LPS, or control media did not show any group-related differences in IL-6 or NEFA release (Table 2). No group differences were found in terms of basal (control) secretion. Responsiveness to epinephrine and LPS did not vary across social groups.

Table 2 - Comparison of *Ex Vivo* Data Across Groups (ANOVA)

	F	*p*
IL-6 Control	0.772	0.47
Epi-Induced IL-6	0.154	0.86
LPS-Induced IL-6	0.124	0.88
Adiponectin Control	0.258	0.77
Epi-Induced Adiponectin	0.286	0.75
LPS-Induced Adiponectin	1.245	0.30
NEFA Control	0.655	0.53
Epi-Induced NEFA	0.357	0.70

Across groups, the responsiveness of adipose tissue to epinephrine and LPS was significantly correlated with disease. Epinephrine-induced IL-6 release was negatively correlated with disease in the arch and thoracic regions (r = -.302, p < .05). LPS-induced IL-6 release was also negatively correlated with disease in the arch and thoracic regions (r = -.285, p < .05).

Lipolysis was measured by the increase in free fatty acids released to the media. No significant differences in free fatty acid levels were apparent between social conditions nor were basal *ex vivo* free fatty acid levels correlated with disease. Epinephrine-induced free fatty acid levels showed a trend toward a significant negative correlation with disease in the aortic arch (r = -.273, p = .07).

Ex Vivo Variables of Interest

In table 3, correlations between *ex vivo* data and lipids, the pro-inflammatory cytokine PAI-1, and final weight are displayed.

Table 3 – Correlation Matrix of *Ex Vivo* Data

	Baseline Cholesterol	*Endpoint Total PAI-1*	*Final Weight*
IL-6 Control	r = 0.293* p < .05	r = -0.112 p = .45	r = -0.100 p = .50
Epi-Induced IL-6	r = 0.289* p < .05	r = -0.262 p < .08	r = -0.056 p = .71
LPS-Induced IL-6	r = 0.382* p < .01	r = -0.343* p < .02	r = -0.135 p = 0.36
NEFA Control	r = 0.267 p = 0.07	r = 0.352* p < .02	r = -0.200 p = .18
Epi-Induced NEFA	r = 0.167 p = 0.27	r = 0.209 p = 0.16	r = -0.384* p < .01

Mouse 1 Results

Social Group and Disease Differences

Social condition was hypothesized to affect disease progression. Studies were performed in which mice were placed in of 1 of 3 social conditions (individually-caged, stable, unstable), but the results did not support that hypothesis. In Figures 9-11, mean disease in the arch, thoracic, and abdominal regions of the aorta are compared across social condition.

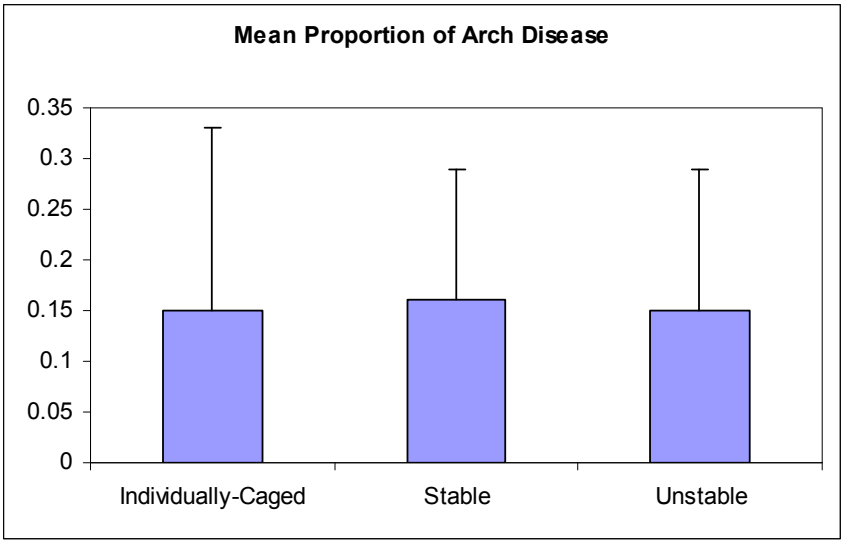

Figure 9. Aortic arch disease across social conditions.

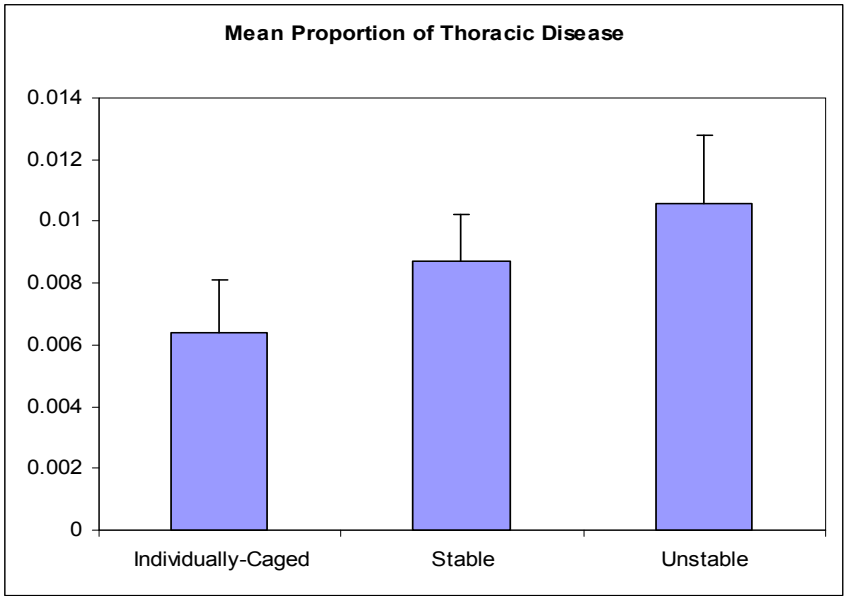

Figure 10. Thoracic aorta disease across social conditions.

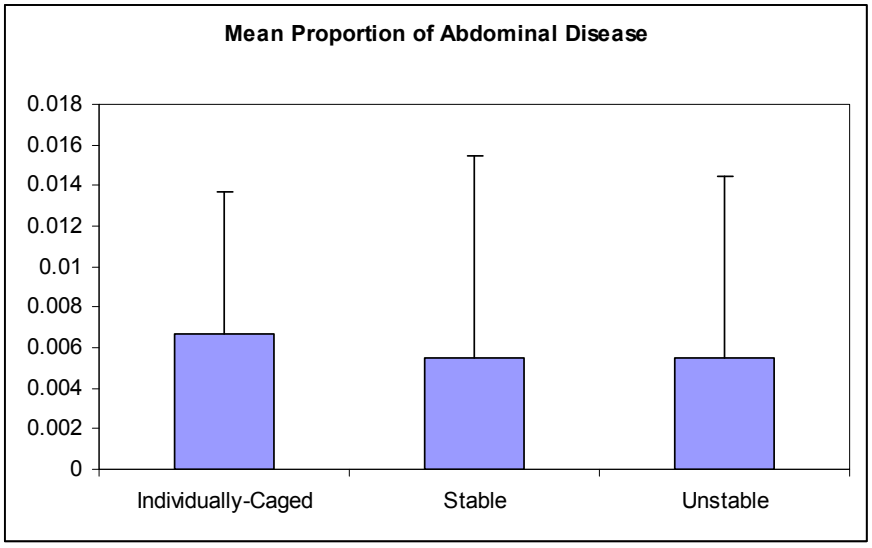

Figure 11. Abdominal aorta disease across social conditions.

This study hypothesized that social condition would also affect weight gain, which was a primary factor in adipose tissue's role in disease progression. There were no group differences in baseline weight, final weight ($F = 1.04$, $p = 0.36$), or weight gain ($F = 1.46$, $p = 0.24$).

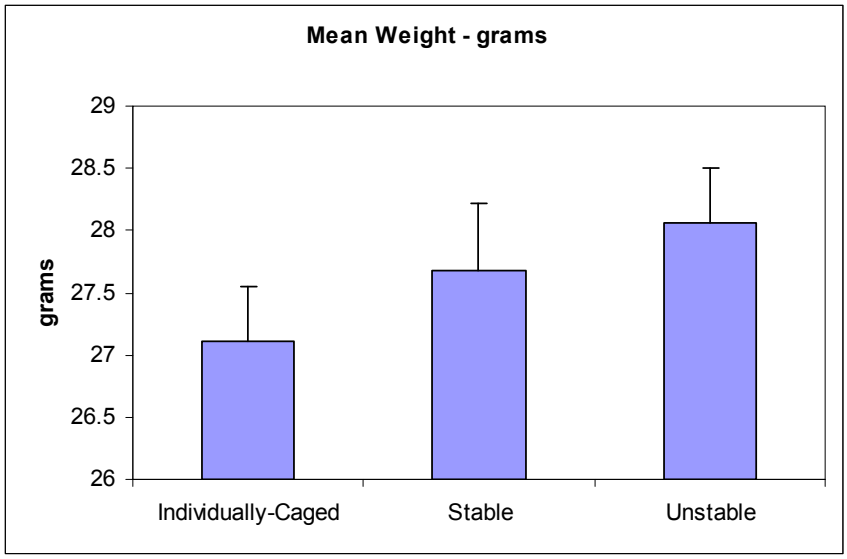

Figure 12. Final weight across social conditions.

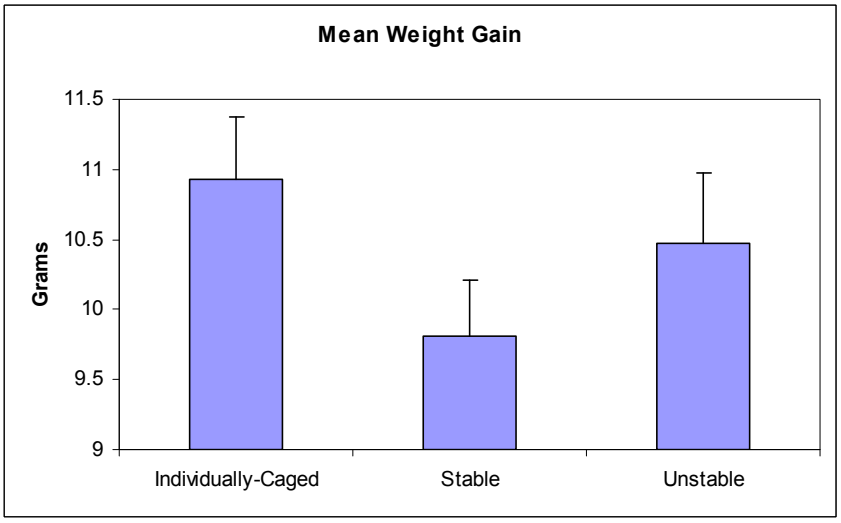

Figure 13. Mean weight gain across social conditions.

Across groups, weight was significantly negatively correlated to disease in the aortic arch (r = -0.34, p = 0.02) and with cumulative disease in the arch and thoracic aorta (r = -0.36, p = .01). Additionally, there was a significant correlation between each animal's final weight and epididymal weight (r = .620, p <.001).

Table 4. Correlation Matrix of Weight Variables and Disease

	Final Weight	Weight Gain
Aortic Arch Disease	r = -0.342* p = 0.02	r = -0.216 p = 0.15
Thoracic Aorta Disease	r = -0.015 p = 0.92	r = -0.093 p = 0.53
Arch & Thoracic Aorta Disease	r = -0.363* p = 0.01	r = -0.159 p = 0.28

In Vivo Studies – Plasma Lipids, Cytokines, & Adipokines

Cytokine and adipokine concentrations were compared across social condition at the baseline, midpoint, and endpoint of the study. At baseline, there were no significant differences in any of the cytokine or adipokine concentrations, which was in line with expectations. At endpoint, there were no group differences in most cytokine and adipokine expression between any of the social conditions. There was a significant difference in adiponectin expression at endpoint (F = 3.88, p < .03). The Individually-Caged animals had significantly higher concentrations of adiponectin than the Stable group (Figure 14). Endpoint adiponectin was significantly negatively correlated with weight at the onset of the study (r = - 0.34, p < .02), but not with weight at the end of the study (r = - 0.21, p = .15). Endpoint adiponectin was also negatively correlated with baseline cholesterol (r = - 0.42, p < .01) and midpoint cholesterol (r = - 0.33, p < .02).

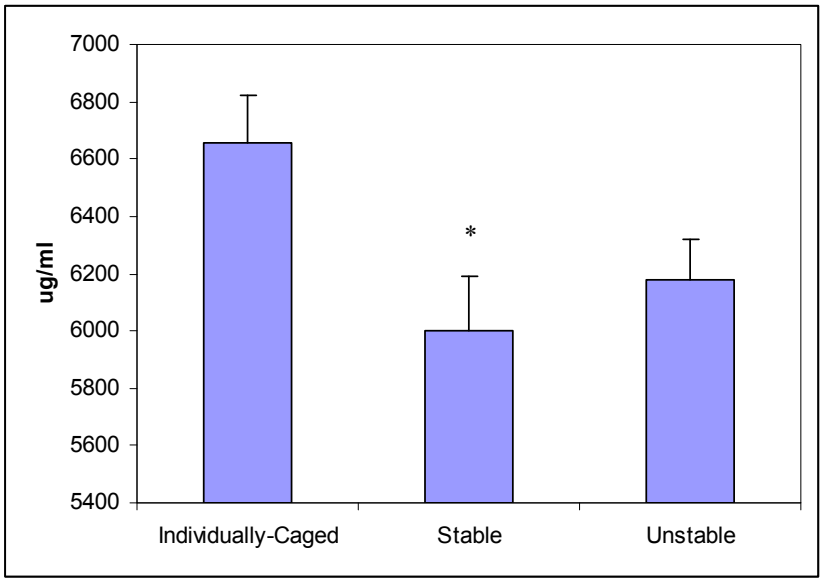

Figure 14. Adiponectin differences by social condition.
* $p < 0.05$.

Table 5. Correlation Matrix of Lipids with Disease

	Arch Disease	Thoracic Disease	Arch + Thoracic Disease
Baseline Cholesterol	$r = 0.167$ $p = 0.26$	$r = -0.134$ $p = 0.37$	$r = 0$ .020 $p = 0.90$
Midpoint Cholesterol	$r = 0.141$ $p = 0.35$	$r = 0.018$ $p = 0.90$	$r = 0.101$ $p = 0.50$
Baseline Triglycerides	$r = 0.229$ $p = 0.13$	$r = -0.072$ $p = 0.63$	$r = 0$ .090 $p = 0.55$
Midpoint Triglycerides	$r = 0.263$ $p = 0.08$	$r = -0.167$ $p = 0.26$	$r = -0.008$ $p = 0.96$

Significant correlations between most plasma adipokines (PAI-1, leptin, resistin, insulin) and disease were not found at baseline or midpoint. A trend toward significance was found between endpoint IL-6 and aortic arch disease ($r = 0.256$, $p = .09$) and between IL-6 and cumulative disease in the arch and thoracic aorta ($r = 0.284$, $p < .06$).

Circulating monocytes, as measured by CBC, were significantly correlated with disease in the thoracic aorta ($r = 0.426$, $p < .01$).

In sum, no disease differences were identified by social condition. However, across social conditions, several variables were found to be associated with disease. In particular, final weight, midpoint triglycerides, and endpoint IL-6 were all correlated with disease, which was consistent with hypotheses.

Predictors of Disease

Several *in vivo* and *ex vivo* variables were fitted into a regression equation to significantly predict disease ($F = 8.175$, $p < .001$) in the aortic arch. Midpoint triglycerides ($\beta = 0.275$, $p < .03$), final weight ($\beta = -0.529$, $p < .001$), *ex vivo* epinephrine-induced IL-6 response ($\beta = -0.249$, $p < .05$), and *ex vivo* epinephrine-induced NEFA response ($\beta = -0.481$, $p = .001$) all combined to predict disease in the aortic arch. Again, social environment did not affect disease in these mice.

Chapter 5 - Discussion

The primary aims of the study were to examine the relationship between social condition and disease progression in ApoE$^{-/-}$ mice and to investigate anti-inflammatory effects of OT in adipose tissue and adipocytes. Despite previous studies in WHHL rabbits and cynomolgous monkeys that demonstrated the effect of social environment on disease progression, social condition did not significantly affect disease progression in ApoE$^{-/-}$ mice. However, the studies demonstrated the ability of oxytocin to reduce pro-inflammatory cytokine release from adipocytes and adipose tissue.

While OT receptor mRNA had been demonstrated in the 3T3-L1 cell line, protein expression has not previously been shown in the literature. In this study, the expression of OT receptors was confirmed in adipose tissue and adipocytes from primary culture. Second, incubation of adipocytes with oxytocin was shown to reduce LPS-induced IL-6 release *in vitro*. Third, the research was extended to an animal model through the infusion of oxytocin over months. Fat tissue removed from mice infused with oxytocin secreted lower levels of IL-6 release than fat tissue from vehicle controls during an *ex vivo* incubation. Together, the data indicate that oxytocin attenuates adipocyte-derived IL-6 release. This complements previous work from our laboratory, which demonstrated that OT reduces LPS-induced IL-6 release in THP-1 macrophages and endothelial cells (Szeto et al., 2008) and that ApoE-/- mice chronically infused with OT exhibit significantly lower disease in the thoracic aorta (Nation et al., in preparation).

Additional findings potentially relating adipose tissue inflammatory responses to atherosclerosis are also presented. Adipose tissue explants from ApoE$^{-/-}$ mice were incubated with epinephrine and LPS. There were significant inverse relationships

between epinephrine-induced and LPS-induced IL-6 release and disease. Moreover, there was an inverse relationship between disease and epinephrine-induced lipolysis. While the mechanisms are not presently clear, the relationship between atherosclerosis and SNS hormone-induced adipose tissue activity is especially interesting. It suggests a possible mechanism linking the effects of OT on IL-6 and reduced disease morbidity and mortality in individuals with higher levels of social support. However, the *in vitro* studies did not find that OT inhibits epinephrine-induced IL-6 release. In the light of the overall study results, perhaps more research on the relationship of OT and epinephrine in adipocytes is warranted.

An additional potential mechanism by which OT may attenuate LPS-induced IL-6 production is in fact suggested by the discrepant effects of OT on LPS- and epinephrine-induced IL-6 release. These findings indicate that the mechanism by which OT affects *in vitro* IL-6 release may be specific to the LPS pathway. One potential mechanism present in the literature is indicated by previous research that has demonstrated that the OT receptor gene has an IL-6 response element that results in the upregulation of the receptor as a consequence of inflammation (Schmid, Wong, & Mitchell, 2001). Increased IL-6 secretion by adipose tissue may act locally to increase OT receptor expression, which may provide a mechanism by which OT can attenuate the inflammatory response.

The effects of stress on the initiation and progression of disease have been extensively discussed. One significant finding is that social support can decrease disease morbidity and mortality (Berkman & Syme, 1979; House, Landis, & Umberson, 1988; Berkman et al., 1992; Case et al., 1992; Orth-Gomer, 1993; Brummett et al., 2005). In 1988, House et al. noted that "the evidence on social relationships is probably stronger,

especially in terms of prospective studies, than the evidence which led to the certification of the Type A behavior pattern as a risk factor for coronary heart disease." One possible mechanism by which social support leads to diminished morbidity and mortality is the effects of OT on inflammatory processes. Significantly, an OT effect on adipose tissue may have treatment implications for cardiovascular disease and Type II Diabetes Mellitus.

Mouse Social Environment Study

The purpose of the study was twofold. First, the study aimed to replicate previous work regarding the effects of social environment on atherosclerosis. By replicating the previous work in the WHHL rabbit in mice, many more opportunities to discover mechanisms underlying the effects of social condition on disease would have become available due to the proliferation of techniques designed for mouse models. Second, the contribution of adipose tissue to mechanisms broadly related to disease, as well as adipose factors underlying the effect of social condition on disease, was investigated. Given the role of adipose tissue in metabolic and immunologic variables involved in atherosclerosis, diabetes, and metabolic syndrome, adipose tissue was hypothesized to be influenced by social condition.

In the Mouse 1 study, social condition did not influence disease progression, which was the central hypothesis of the series of studies. As a result, hypotheses related to the effects of social condition on adipose tissue's relation to social environment became moot.

As this was the first mouse study conducted by the lab, we experienced a learning curve in certain unexpected areas. First and foremost, the number of animals alive at the

end of the study was significantly below expectations, thus reducing the study's power. The animals died for several reasons. The initial order of 80 animals that was placed to Jackson Laboratories was not filled to specifications. A considerable number of animals died due to hydrocephalus prior to shipment. Upon receiving the animals several more animals died of hydrocephalus. An additional problem soon occurred in that multiple animals developed malocclusion, which resulted in death and weight loss early in the study. An additional animal developed a tumor and died. Another animal was trapped by a cage divider and died, and its pair had to be excluded from the study as well.

Second, the study did not extend long enough for sufficient disease to develop. The study ended at 25 weeks of age, and the amount of disease in the aortic arch was approximately 15% of total area. In the thoracic aorta, roughly 1% of the aorta had lesions. Because there was little disease, it was difficult to find differences in disease across social conditions. Further, little disease makes it difficult to predict and model mechanisms by which disease occurs.

The third problem in study methodology was the lack of metabolic cages. A considerable degree of variability was introduced into the study because of the lack of standardized metabolic cages.

Fourth, the arena model for videotaping new pairings did not sufficiently capture the degree of aggressive behavior in the unstable group. Anecdotally, considerable fighting was witnessed in the unstable group outside of the videotaping period. No fighting was ever witnessed in the stable group in or out of the videotaping period. In future studies, a different model of introducing new pairings and videotaping should be considered.

Fifth, methodological problems occurred in assaying blood, particularly with the Lincoplex assays. The Linco assays were supposed to maximize results with minimal blood volume; however, the concentration for many cytokines fell below the limit of detection. As a result, the reliability of the Linco assays was disappointingly low. In the future, Linco assays should not be used because they were not sensitive enough to detect cytokines levels and were relatively unreliable for biomarkers with detectable levels.

Sixth, this experiment was designed to replicate the WHHL model in the most commonly used mouse model for atherosclerosis. However, the ApoE$^{-/-}$ model is not ideal for conducting research into adipose tissue. Mice that are genetically altered to be more susceptible to obesity may be a better model for the effects of social condition on functional and structural changes in adipose tissue.

Limitations & Future Research

A primary limitation of the present series of studies is the lack of a demonstration of the mechanism which mediates OT's effect on IL-6 release. Future research should investigate critical proteins in the NF-κB cascade and the expression of transcription factors known to affect IL-6 expression.

A secondary limitation is the emphasis on IL-6 as a marker of inflammation. Adipose tissue releases dozens of adipokines, and many of them have significant paracrine and endocrine effects that influence multiple disease states, including diabetes mellitus, cardiovascular disease, and obesity. Consequently, the next series of studies would benefit by studying other significant adipokines and pro-inflammatory cytokines, including TNF-α, resistin, leptin, visfatin, and adiponectin.

Third, the study of OT and inflammation would benefit through its extension into human cells. Already, we have demonstrated that human adipocytes express mRNA for both OT and OT receptors. Previous research by our laboratory has demonstrated a similar effect of OT on human endothelial cells and THP-1 macrophages (Szeto et al., 2008).

Fourth, the effect of OT on epinephrine should be re-investigated. One possibility is that the effect of OT tended to occur initially after six hours of incubation. As epinephrine is a fast-acting hormone, perhaps it would be useful to investigate further the dosage and timing of incubation with epinephrine and OT. A secondary measure would be the presence of adrenergic receptors before and after incubation with OT.

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