2009-06-25

The Oxytocinergic Anti-Inflammatory Pathway in Atherosclerosis

Daniel Addison Nation
University of Miami, dnation@miami.edu

UNIVERSITY OF MIAMI

THE OXYTOCINERGIC ANTI-INFLAMMATORY PATHWAY IN ATHEROSCLEROSIS

By

Daniel Addison Nation

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

June 2009

UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

THE OXYTOCINERGIC ANTI-INFLAMMATORY PATHWAY IN ATHEROSCLEROSIS

Daniel Addison Nation

Approved:

_____ Philip M. McCabe, Ph.D. Professor of Psychology	_____ Terri A. Scandura, Ph.D. Dean of the Graduate School
_____ Neil Schneiderman, Ph.D. Professor of Psychology	_____ Armando Mendez, Ph.D. Associate Professor of Medicine
_____ Edward Green, Ph.D. Professor of Psychology	_____ Julia Zaias, D.V.M., Ph.D. Assistant Professor of Comparative Pathology

NATION, DANIEL ADDISON (Ph.D., Psychology)
The Oxytocinergic Anti-Inflammatory Pathway in Atherosclerosis (June 2009)

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor Phil McCabe.
No. of pages in text. (89)

Background. Social deprivation or isolation accelerates the progression of atherosclerosis in several animal models of the disease. Conversely, stable social environment has been associated with reduction in the extent and severity of atherosclerosis. While positive social interactions are thought to be related to this protective effect, little is known about the physiological mechanisms responsible. Recently, the neurohypophyseal peptide, oxytocin (OT), has been found to play a role in both positive social interactions and cardiovascular homeostasis, suggesting that this neuropeptide may be responsible for mediating the beneficial effects of positive social environment on atherosclerosis. The first aim of the current study is to examine the potential anti-inflammatory effects of OT on *in vitro* cellular models involved in the pathophysiology of atherosclerosis. The second aim is to examine whether long-term administration of OT slows the progression of atherosclerosis in apoE$^{-/-}$ mice. The third aim is to obtain evidence *in vivo* that OT is impacting disease through novel anti-inflammatory effects on tissues important in atherogenesis.

Methods. 1) Human macrophage-like (DTHP-1) cells and human aortic endothelial cells (HAECs) were stimulated with lipopolysaccharde (LPS) alone, and in the presence of different concentrations of OT, and IL-6 secretion was measured. 2) ApoE$^{-/-}$ mice were socially isolated at 12 weeks of age and continuously infused with OT (n=24) or vehicle

(n=21) from subcutaneously implanted osmotic minipumps for 12 weeks. Plasma levels of lipids, adiponectin, insulin, and CRP were assessed pre- and post-treatment. Extent of aortic atherosclerosis (percent lesion area) was assessed post-treatment and areas of high lesion prevalence were compared between OT and vehicle (VH) control groups. Constitutive release of IL-6 from *ex vivo* adipose tissue samples taken from a subset (n=12/group) was compared between treatment groups.

Results. 1) OT demonstrated dose-dependent inhibition of LPS-induced IL-6 secretion from macrophages (35-55%, $p < 0.01$) and aortic endothelial cells (15-25%, $p < 0.01$). 2) ApoE$^{-/-}$ mice continuously infused with OT displayed decreased plasma CRP levels after 6 weeks of treatment and diminished lesion area at the thoracic aorta after 12 weeks of treatment relative to vehicle control animals (37%, $p < 0.05$). Additionally, adipose tissue samples taken from OT infused mice showed decreased constitutive release of IL-6 (30%, $p < 0.01$). These findings were unrelated to changes in plasma lipids, insulin, physical activity levels, or 24-hour corticosterone secretion.

Discussion and Conclusions. These findings demonstrate that OT is capable of inhibiting stimulated pro-inflammatory cytokine production in macrophages and aortic endothelial cells *in vitro*, and constitutive release from adipose tissue *in vivo*. OT also decreased circulating CRP levels and slowed the progression of early stage atherosclerosis in an aortic region of high lesion prevalence in socially isolated apoE$^{-/-}$ mice. Taken together, these results suggest that increased peripheral OT could be partially responsible for the beneficial effect of positive social environment on atherosclerosis.

TABLE OF CONTENTS

List of Figures

List of Tables

Chapter 1: Background

Social Isolation and Atherosclerosis

Social isolation or social deprivation has been found to affect the progression of atherosclerosis in multiple animal models of the disease. For example, in cynomolgus monkeys, the extent of atherosclerosis found in socially deprived females is greater than that of animals housed in social groups.[1] Watanabe Heritable Hyperlipidemic (WHHL) rabbits housed in isolation also show greater atherosclerotic lesion area than those in a stable social environment.[2,3] Recent research has shown that socially isolated apoE$^{-/-}$ mice also display increased atherosclerotic lesion development relative to those housed in social groups.[4] These compelling findings from animal research have prompted studies in humans examining the association between social deprivation and noninvasive measures of atherosclerosis, including ultrasound detected carotid plaque-score and ankle brachial pressure index.[5,6] Furthermore, epidemiologic studies have suggested that social support may constitute a protective factor for the development of ischemic heart disease[7] and could be associated with improved recovery from cardiovascular events,[8,9] providing further evidence that social environment may be impacting the underlying vascular disease in humans.

While the effect of social isolation or deprivation on atherosclerosis has been well established by the above animal and human studies, the specific mechanisms responsible for this effect remain unclear. Some findings suggest that social isolation is impacting atherosclerosis through mechanisms affecting endothelial function, which is one of the earliest events in the development of atherosclerosis. In support of this hypothesis, isolated WHHL rabbits exhibit elevated aortic NAD(P)H-oxidase activity relative to

rabbits housed under stable social conditions.[10] NAD(P)H-oxidase is the most important vascular source of membrane-bound superoxide in the artery. Superoxide can inactivate endothelium-derived nitric oxide (NO) and lead to endothelial dysfunction, manifested as impaired endothelium-dependent vasodilation, which is a common characteristic of the initial stages of atherosclerosis.[11-14] In addition to inactivating endothelium-derived nitric oxide, superoxide produced by macrophages, vascular smooth muscle cells (VSMC), or endothelial cells can oxidize LDL.[15] Oxidized LDL (ox-LDL) is more readily taken up by macrophage scavenger receptors, stimulating macrophage activation, adherence, and chemotaxis.[16] This can ultimately lead to the deposition of lipid-laden foam cells in the vessel wall, creating the fatty-streaks that mark the first stage of atherosclerotic lesion development.[17] Thus, the finding that socially isolated hyperlipidemic rabbits show elevations in NAD(P)H-oxidase suggests that overactivity of this enzyme could play an important role in the early stages of atherosclerotic lesion development in these animals.

One mechanism that could be responsible for the increased NAD(P)H-oxidase activity found in isolated WHHL rabbits is excessive sympathetic nervous system (SNS) activation. In support of this hypothesis, evidence suggests that norepinephrine can induce increases in NAD(P)H-oxidase activity through activation of the α_1-adrenoceptor in the injured aorta.[18] Isolated WHHL rabbits display elevated 24-hour urinary catecholamine concentrations and resting heart-rate relative to socially housed animals, suggesting that isolation-induced SNS activation could be causing the observed increases in NAD(P)H-oxidase activity in these animals.[10, 2] Cynomolgus monkeys housed in

social isolation also exhibit elevated resting heart rate relative to animals housed is social groups.[19] This is consistent with research in multiple species indicating that isolation produces physiological signs of stress, including increased SNS activation.[20, 21]

Other research in humans and animals indicates that physical contact can lower heart-rate, suggesting that isolation may lead to increased heart-rate through deprivation of physical contact. For example, studies done in humans suggest that resting and stress-induced elevations in heart-rate both decrease in response to general physical contact with other people.[22-25] Other findings indicate that the mere presence of a social partner can produce a reduction in heart-rate.[26] Research in monkeys has also found lower heart-rate in animals provided physical contact versus no contact.[27] Elevated resting heart-rate has long been associated with cardiovascular disease in humans and animals.[28-30] This suggests that the lack of physical contact associated with social isolation could be responsible for the observed increases in SNS activity, oxidative stress, and atherosclerosis in these animals. In support of this hypothesis, at least one study found that warm physical contact from experimenters inhibited atherosclerotic lesion development in cholesterol-fed rabbits.[31]

Increased SNS activation is also thought to exacerbate the development of atherosclerosis through mechanisms other than increased oxidative stress. Hypertension leads to alterations in blood flow that may lead to endothelial injury and dysfunction, particularly in sections of the aorta susceptable to low shear stress, periodic reversal of flow direction (i.e., oscillatory flow), and turbulance.[32] This hypothesis is supported by research demonstrating that chemically induced overactivation of the SNS causes endothelial injury and inflammation in cholesterol-fed rabbits.[33] Research in cholesterol-

fed monkeys indicates that social stress can cause SNS overactivation, endothelial injury, and increased atherosclerosis.[34,35] Furthermore, studies have found that blockade of adrenergic receptors with propranalol can abolish the stress-induced exacerbation of atherosclerosis in socially stressed monkeys.[35]

Other findings suggest that isolation may lead to metabolic dysfunction that could be responsible for both increased SNS activation and extent of atherosclerosis. Socially isolated WHHL rabbits are hyperinsulinemic relative to animals housed in either stable or unstable social conditions, suggesting that insulin resistance could play a role in the exacerbation of atherosclerosis in these animals.[2] Insulin-resistance can accelerate atherosclerosis through increased oxidative stress and endothelial dysfunction.[36] The reciprocal relationship between endothelial functioning and insulin-resistance is thought to be due to the role of insulin in modulating the balance of NO and endothelin-1 release in the endothelium. Thus, decreased NO and increased endothelin-1 release from endothelial cells as a result of insulin-resistance could be a critical factor mediating the relationship between insulin-resistance and atherosclerosis.[36] Diminished bioavailability of NO can also cause oxidative stress and elevations in blood pressure, leading to increased oxidant and hemodynamic strain and, consequently, atherosclerosis in vulnerable areas.[37]

The mechanism whereby social isolation leads to hyperinsulinemia in these animals in currently unknown, but one possibility is that this metabolic disturbance is related to an energy imbalance. WHHL rabbits housed in isolation display a pattern of sedentary behavior and increased weight gain relative to animals housed in social groups.[2] These findings suggest that the metabolic disturbance found in individually

housed animals may be related to an imbalance between energy storage and usage. Obesity, particularly when associated with visceral adiposity, is a known risk factor for the development of insulin resistance and atherosclerosis.[38] The connection between obesity and atherosclerosis is thought to be mediated by adipose tissue dysfunction, which may develop as a result of energy imbalance.[39] Increased energy storage disproportionate to energy usage can overwhelm energy storage mechanisms at the level of the mitochondria and endoplasmic reticulum of adipocytes and pancreatic β-cells.[39] This can lead to adipocyte dysfunction characterized by increased oxidative stress and decreased protein synthesis. Adipocyte dysfunction also leads to increased lipolysis and, consequently, free fatty acid (FFA) production within adipose tissue.[40] High FFA concentrations within the context of obesity is thought to result from the breakdown of triglyceride rich HDL.[41] These FFAs are thought to play a central role in the development of insulin-resistance and adipocyte dysfunction by overwhelming energy storage systems and causing oxidative stress within the cell.[39] Thus, the increased lipolysis characteristic of adipocyte dysfunction is part of a feedforward cycle leading to the production of more FFAs, which perpetuate the insulin-resistant state. Circulating FFAs also directly impact atherosclerotic lesion development through sequestration within the extracellular matrix of the vessel wall and activation of inflammatory pathways implicated in the disease.

Adipose tissue also plays an important role in the development of insulin resistance through hormonal modulation of insulin-sensitivity. Hormones secreted from adipose tissue, including leptin, resistin, ghrelin, and adiponectin, form an adipo-neuro-endocrine axis that can modulate energy storage/usage through pleiotropic effects on

satiety, insulin-sensitivity, and other metabolic factors.[42] Plasma adiponectin, for instance, has been found to play a particularly important role in increasing insulin-sensitivity. This adipokine is also thought to provide protection against the development of atherosclerosis through its direct anti-inflammatory actions on vascular tissues.[42] Decreased adiponectin production is related to adipocyte dysfunction and is thought to play an improtant role in the progression of atherosclerosis in obese individuals.

A less understood mechanism linking adipocyte dysfunction with atherosclerosis involves the activation of inflammatory pathways. The increased oxidative stress, FFA production, and altered hormone profile of these dysfunctional adipocytes seems to activate general inflammatory mechanisms within the adipose tissue.[37] This may be responsible for the observed association between obesity and pro-inflammatory cytokines and plasma C-reactive protein (CRP).[43,44] There is also accumulating evidence that insulin functions as an anti-inflammatory and anti-oxidant hormone, which could explain why insulin-resistance is associated with increased inflammatory signaling.[32] Inflammation is also increasingly being recognized as a critical component of the development of insulin-resistance. Evidence suggests that pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β) promote insulin-resistance through direct actions at the level of the insulin receptor and intra-cellular signaling pathways within a variety of tissues.[45] Cytokine mediated inflammatory signaling can also directly impact the development of atherosclerotic lesions, particularly during the early stages of the disease, through

activation of the endothelium and circulating leukocytes.[45] Interestingly, increased visceral adiposity has been associated with increased systemic inflammation, which may be mediating the relationship between obesity, insulin resistance, and atherosclerosis.[38]

It has been proposed that dysfunctional adipose tissue enters a pro-inflammatory state leading to the accumulation of resident adipose tissue macrophages (ATMs).[47] Recently, key aspects of adipose tissue functioning have been ascribed to ATMs, which can powerfully influence the local and systemic levels of inflammation.[48] Increased production of pro-inflammatory cytokines by ATMs within visceral adipose tissue may be largely responsible for the association between obesity and atherosclerosis. Studies focusing on the inflammatory process within adipose tissue indicate that ATMs may be distinct from macrophages recruited into adipose tissue through chemotactic signaling.[48] Monocyte chemotactic protein-1 (MCP-1) and its receptor (CCR2) are thought to be responsible for recruitment of circulating monocytes into visceral adipose tissue. Recruited macrophages display a pro-inflammatory phenotype, exhibiting higher levels of IL-6, inducible nitric oxide synthase (iNOS), and CCR2 than ATMs. These findings suggest that recruitment of macrophages in a pro-inflammatory state into adipose tissue may play an important role in the development of adipocyte dysfunction, insulin-resistance, and atherosclerosis.

To summarize, animals housed in isolation are socially deprived and display a specific physiological profile characterized by chronic SNS activation, elevated heart rate, weight gain, hyperinsulinemia, and vascular oxidative stress.[2,10,19,49] These risk factors in association with one another have been proposed to constitute a distinct "metabolic syndrome" that may represent a common underlying pathophysiological

process leading to the development of atherosclerosis. However, the complex causal relationships between these risk factors makes it difficult to determine which particular factor is ultimately responsible for the syndromal presentation and effects on lesion formation.

One approach to this problem is to focus on risk factors directly associated with social isolation. WHHL rabbits housed in isolation display a pattern of sedentary behavior that could be responsible for a number of the risk factors associated with social isolation. For example, physical inactivity has been shown to increase oxidative stress through activation of NAD(P)H-oxidase, leading to endothelial dysfunction and atherosclerosis in mice.[50] Other studies have found that physical inactivity and sedentary lifestyle is associated with increased SNS activation in rhesus monkeys and humans, respectively.[51,52] Physical inactivity has also been associated with metabolic variables related to obesity and insulin resistance, and it has been suggested that oxidative stress may be a common pathogenic mechanism linking the insulin metabolic syndrome, diabetes and cardiovascular disease.[53,54] Other research implicates a sedentary pattern of behavior in increased adipose tissue inflammation through adipocyte dysfunction and increased infiltration of adipose tissue by macrophages. Alternatively, increased SNS activation as a result of the stress of social deprivation could be responsible for the increased NAD(P)H-oxidase activity, elevated resting heart rate, and insulin-resistance.

Thus, a number of questions remain regarding the connection between social isolation and atherosclerosis. The fact that both psychosocial stress and physical inactivity can cause chronic SNS activation makes it difficult to determine the extent to which either of these factors is independently responsible for the physiological changes

associated with individual housing. It is also unclear whether inactivity and/or psychosocial stress could be causing metabolic and oxidative changes through mechanisms that are independent of SNS activation and energy imbalance. Finally, the relative importance of each of these risk factors in the exacerbation of atherosclerosis observed in these socially deprived animals remains to be determined.

It must also be noted that animals housed under socially isolated conditions are always compared with those housed in social environments. It is, therefore, possible that some of the observed differences in risk factors between these groups is a reflection of the beneficial effects of social housing. This could be due to an as yet undiscovered, protective mechanism associated with affiliative social behavior.

Affiliative Social Behavior & Atherosclerosis: The Oxytocin Hypothesis

While multiple animal models have focused on identifying the physiological mechanisms linking stressful social conditions, including unstable social environment and social isolation, with increased atherosclerotic disease, little is known about the mechanisms underlying the protective effect of stable social conditions. Cholesterol-fed monkeys, hyperlipidemic rabbits, and apoE$^{-/-}$ mice housed under stable social conditions develop less atherosclerotic disease than those housed in unstable social conditions or in isolation.[1-4,31,55,56] These results could simply be due to the lack of SNS activity and physical inactivity that characterizes these stressful housing conditions. Alternatively, they could suggest the existence of a protective mechanism that dampens the progression of atherosclerosis under stable social conditions. In WHHL rabbits, stable social environment is associated with increased affiliative social behavior, which could play a role in the activation of a hormonally driven athero-protective pathway. It has been

proposed that the neurohormone, oxytocin (OT), could represent such a protective factor and may be responsible for the beneficial effects of positive social environment on atherosclerosis.[57]

OT is a nonapeptide synthesized in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus.[58] Magnocellular oxytocinergic neurons in PVN send their terminals to the posterior pituitary where they release oxytocin into portal circulation.[59] Parvocellular oxytocinergic neurons in PVN send axons to multiple brain and spinal cord areas important for autonomic nervous system control and processing of painful stimuli. These projections include other hypothalamic nuclei, the median eminence, the amygdala, hippocampus, locus coeruleus (LC), striatum, raphe nuclei, the dorsal motor nucleus of the vagus nerve, and the nucleus tractus solitarius (NTS).[60] Release of oxytocin into portal circulation is traditionally associated with parturition and the milk ejection reflex during lactation.[61] Recent studies have demonstrated that OT also plays a role in socially-related behaviors and in the stress response.[62,63] Specifically, evidence suggests that OT is involved in affiliative social behaviors, such as maternal care, sexual behavior, monogamous social bond formation, and social recognition.[62]

While the exact role of OT in affiliative social interactions remains controversial, it has been hypothesized that OT may counter SNS activation under conditions of social recognition or affiliation, thereby buffering the stress response and facilitating affiliative behaviors.[64] Thus, affiliative social interaction may protect against atherosclerosis by increasing OT-induced inhibition of SNS activity through central autonomic circuits. Evidence for this potential "stress buffering" role of OT comes from studies in both

humans and animals. During breast feeding OT levels rise dramatically in the blood and CNS of rats and humans.[65] These increases in OT have been associated with increased calm and decreased stress, aggression, and blood pressure in mothers.[66-69] OT administration has also shown sedative and anxiolytic effects in mice, possibly through decreased HPA-axis activation.[69,70] However, it should be noted that the effects of OT administration on the HPA-axis are complex and depend on the dose, species, route of administration, and timecourse. For instance, intravenous (i.v.) injections of OT cause a transient rise in blood pressure in rats, but have the opposite effect in humans.[71-74] Intracerebroventricular (i.c.v.) administration in rats leads to a decrease in basal blood pressure, but has no effect on heart rate.[75] Repeated OT adminstration in rats through either of these routes can cause decreases in blood pressure that last for weeks after the last injection.[76] For example, one study found that repeated subcutaneous (s.c.) administration of large doses (1mg/kg) in male rats causes approximately 15 mmHg decrease in blood pressure that lasts for 10 days after the last injection.[77]

Mechanistic explanations for the potential stress buffering role of OT come from neurophysiological research suggesting that OT can enter the pituitary portal system through the median eminence to directly affect secretion of anterior pituitary hormones, including Adrenocorticotropin hormone (ACTH), growth hormone (GH), and prolactin.[78] Studies in this area have also found species-specific effects indicating that OT causes short-term increases in ACTH and corticosterone secretion in rats, but inhibits these hormones in humans and nonhuman primates.[79] Other lines of research indicate that long-term OT treatment also lowers corticosterone and neuropeptide Y concentrations, while increasing cholecystokinin (CCK) levels. This suggests that OT may be decreasing

SNS activity and increasing vagal activity, implying a general shift from sympathetic to parasympathetic activition.[80] Other research further demonstrates that OT administration causes a shift toward vegetative functions, including increased energy storage. For example, i.c.v. injections of OT can cause the release of the gastrointestinal hormones, insulin and glucagon, through activation of vagal efferents that innervate the GI tract and pancreas.[80,81] Consistent with these findings, long-term OT administration has been found to cause weight gain through increased energy storage.[82]

Many of the anti-stress and blood pressure reducing actions of OT are thought to be mediated by CNS mechanisms. One mechanism considered to underlie many of these actions is the upregulation of autoregulatory α_2-adrenoceptors in the LC and the NTS.[83] This leads to decreased release of norepinephrine throughout the brain and diminished SNS activity in the periphery.[84] OT neurons in PVN project directly to these areas and can modulate cardiovascular reponses through these connections.[85] OT also exerts its regulation of autonomic nervous system activity through the dorsal vagal complex, the amygdala, preganglionic sympathetic neurons, CRH release from PVN, and glucocorticoid receptor expression in the hippocampus.[86-92]

Behavioral research suggests that the central OT pathway inhibiting SNS activity may be activated in response to stressful stimuli. For example, WHHL rabbits housed in an emotionally stressful, unstable social environment show increased OT secretion in PVN during social interactions.[3] Other findings suggest that the stress of social deprivation leads to an increase in circulating OT. For example, prairie voles housed in social isolation show increased peripheral OT levels.[93] Rats exposed to the stress of a novel environment also show increased release of OT into portal circulation.[94] Some

have argued that these findings suggest a role for central OT pathways in diminishing stress-induced elevations in SNS activation, which could lead to decreased atherosclerosis.[64]

In addition to its CNS and systemic actions, OT is also produced locally in a variety of peripheral tissues in both sexes, suggesting that this peptide may be involved in the basic physiological functions of several organs.[95,96] It has been proposed that OT is a cardiovascular hormone that plays an important role in normal homeostatic mechanisms, as OT and its receptor (OTR) are synthesized and released in the heart and vasculature of rats.[96-99] The intrinsic OT system within the heart stimulates the local release of atrial natriuretic peptide, which has negative chronotropic (slows heart-rate) and inotropic (decreases cardiac contractility) effects, producing a reduction in circulating blood volume and pressure.[98] It has also been proposed that an OT system intrinsic to the vasculature regulates vascular tone as well as regrowth and remodeling.[99] Thus, the activation of local OT systems within cardiovascular tissues themselves may be more important than CNS mechanisms in the pathophysiology of atherosclerosis. This suggests the potential importance of peripheral OT in the effect of social context on atherosclerosis.

These considerations lead to the hypothesis that positive social environment may increase peripheral OT and, subsequently, diminish atherosclerosis in susceptible populations. However, a remaining consideration is whether peripheral OT increases during positive social interactions. At least two human studies investigating the impact of affiliative social interactions[100] and "warm" physical contact[101] on plasma OT concentrations have supported the notion that positive social contact may increase

peripheral OT levels. The observed increases in plasma OT were modest (approximately 2 fold), but one study also found a prolonged drop in systolic blood pressure in individuals treated with "warm" physical contact. Some evidence also exists within the animal literature suggesting that physical contact may increase peripheral OT levels in rats.[100-101] However, attempts to demonstrate increased plasma OT concentrations in WHHL rabbits housed under stable social conditions have met with equivocal results and methodological difficulties (unpublished findings). Additional studies are needed to firmly establish the mechanism responsible for the effect of physical contact on OT and the exact relationship between the stimulus and peripheral OT response. While increased peripheral OT is the most obvious index for activation of OT receptors within the vasculature, several different mechanisms could be responsible for the activation of these receptors in target organs. Release of oxytocin directly into the portal circulation from the posterior pituitary could travel to the heart, aorta, and other tissues to exert effects hormonally. Aside from the classic posterior pituitary portal system, peripheral OT systems could be activated through efferent parasympathetic nerve terminals[102] or modulation of peripheral levels of OTR expression.[103]

While each of these possiblities remains controversial, research investigating the activation of oxytocinergic systems through peripheral administration of OT has provided evidence for the existence of an OT pathway that could convey protection against the progression of atherosclerosis (*see* below). Thus, one possible mechanism mediating the

beneficial effect of positive social environment on atherosclerosis is activation of the a peripheral oxytocinergic system that modulates inflammatory and oxidative stress pathways.

The Oxytocin Anti-inflammatory and Anti-oxidant Pathways

Animal studies investigating the effects of peripheral OT administration in models of inflammatory diseases have provided evidence for the existence of potentially cardioprotective pathways involving OT. For example, peripheral OT administration has been shown to improve wound healing, possibly through increased insulin-like growth factor (IGF) production.[104] In the carageenan hind paw inflammation model, OT was found to increase nocioceptive thresholds and decrease both edema and tissue myeloperoxidase (MPO) activity.[105] The level of MPO activity in tissue is a marker for neutrophil recruitment into the intravascular space.[106] The production of reactive oxygen species (ROS) by MPO activity from infiltrating neutrophils contributes to many inflammatory conditions through damage to cell membranes, lipid peroxidation, and activation and recruitment of macrophages.[107] OT administration has also been shown to decrease MPO activity in acetic acid induced colitis in rats.[108] In this same study, peripheral OT administration was able to inhibit circulating TNF-α, a potent proinflammatory cytokine implicated in the progression of atherosclerosis through perpetuation of inflammatory signaling in and around atherosclerotic lesions.[109] TNF-α is thought to be responsible for increased neutrophil infiltration during conditions of acute inflammation through upregulation of the chemotactic factor, MCP-1, on circulating leukocytes and endothelial cells.[110] This suggests that OT induced decreases in TNF-α levels could be responsible for observed decreases in tissue MPO activity. This

same mechanism could be responsible for the observed decreases in tissue malondialdehyde (MDA), a marker for lipid peroxidation, and increased glutathione, a protective anti-oxidant. Finally, tissue collagen was decreased in OT treated animals, suggesting that OT may have an anti-fibrotic effect. This finding is supported by previous literature suggesting that oxytocin may influence fibrinolysis and prevent platelet aggregation through increased production of prostacyclin.[111,112] Collagen accumulation is an important component of extracellular matrix formation, which is marker for atherosclerotic plaque severity and contributes to ongoing lipid deposition and macrophage recruitment in atherosclerosis.[113] Similar results for tissue MPO activity, glutathione, MDA, collagen, and serum TNF-α have been found in animal models of thermal injury, septic shock, renal infection and ischemia-reperfusion injury, and hepatic injury.[114-117]

Collectively, these findings suggest that peripheral OT administration activates anti-inflammatory, anti-oxidant, and anti-fibrotic pathways that can be protective for a number of conditions that are exacerbated by these factors. It is well established that atherosclerosis can be accelerated by inflammation, oxidative stress, and development of extracellular matrix. Specifically, TNF-α, MPO, lipid peroxidation (MDA), collagen accumulation, and platelet aggregation are all thought to play major roles in the progression of atherosclerosis. Each of these disease promoting factors have been found to be susceptible to OT-induced down-regulation in animal models. Conversely, OT administration has been shown to promote glutathione, a potentially protective anti-oxidant factor in atherosclerosis. Findings from our laboratory[57] suggest that OT receptors are present on monocytes, macrophages, endothelial cells, and vascular smooth

muscle cells and that OT inhibits TNF-α stimulated NAD(P)H-oxidase activation in these cells *in vitro*. Determination of whether OT exerts potent inhibitory effects on other important factors in atherosclerosis, such as other pro-inflammatory cytokines (e.g., IL-6) is one of the primary goals of the current study. Some evidence suggests that OT can decrease lipopolysaccharide (LPS) induced IL-6 production in neurointermediate cells of the pituitary, but these findings have yet to be extended to other cell types involved in atherogenesis.[118]

The specific mechanism responsible for these effects requires clarification. Some experimental findings have suggested that the anti-inflammatory and anti-oxidant effects of OT were due to CNS mechanisms, while others have excluded this possibility by demonstrating that i.c.v. administration of OT has no such effects.[119] Administration of high doses of OT can induce glucocorticoid release and some authors have suggested that the observed anti-inflammatory actions of OT could be mediated by increases in endogenous steroids.[117] However, others have shown similar effects at lower doses that were unlikely to impact glucocorticoid levels.[105] Activation of the CNS OT system could reduce inflammation and oxidant stress through dampening of sympathetic nervous system activity and concomitant activation of the parasympathetic system.[120,121]

Parasympathetic nervous system activation, specifically activation of vagal efferents, has been shown to reduce inflammation and oxidative stress in similar models of acute tissue injury and inflammation.[122] These actions are mediated by acetylcholine activation of the α7-bungaro-toxin sensitive nicotinic acetylcholine receptor complex.[123] This hypothesis is consistent with the possibility reviewed above (*see*, *The Oxytocin Hypothesis*) that OT causes a shift in autonomic nervous system balance from

sympathetic to parasympathetic domination.[79] Alternatively, OT could reflexively activate these same vagal efferents through receptors on vagal afferents. Other endogenously produced peptides have been found to use a similar mechanism to produce anti-inflammatory effects. For example, the gastrointestinal peptide ghrelin has anti-inflammatory effects through activation of vagal afferents in the GI tract.[124]

Another possibility is that OT is directly acting on peripheral OTRs to dampen inflammatory and oxidative stress effects through purely local mechanisms. As mentioned above, support for this hypothesis comes from research suggesting OTR activation can inhibit NAD(P)H-oxidase activation in response to TNF-α in macrophages and endothelial cells.[57] Similar research in neurointermediate cells of the pituitary suggests that OT can inhibit pro-inflammatory cytokine release.[118] It should also be noted that the OTR promotor region is colocalized with those of pro-inflammatory cytokines, suggesting that this peptide plays a role in modulating transcription of inflammatory genes.[125] Thus, the most likely explanation for the observed anti-inflammatory and anti-oxidant effects of OT *in vivo* is the down-regulation of pro-inflammatory cytokine and ROS production within vascular and immune cells important in the acute phase response. However, at high but physiologically relevant doses, OT has significant affinity for the vasopressin receptor (V_1).[126] Consequently, the effects of V_1 receptor activation may be equally relevant to those of OTR in research investigating the peripheral effects of high doses of OT.

While previous studies have found that administration of super-physiological doses of OT in the context of acute injury results in anti-inflammatory and anti-oxidant effects, no research to date has investigated the impact of chronic OT administration at

physiologically relevant doses on low-grade inflammation and atherosclerosis. The primary objective of the current study is to demonstrate the ability of increased peripheral OT to slow the progression of atherosclerosis in the apoE$^{-/-}$ mouse through anti-inflammatory mechanisms.

The ApoE$^{-/-}$ Mouse Model of Hypercholesterolemia

Apolipoprotein E (apoE) is a large (34kd) glycoprotein that is a main apoprotein responsible for clearance of chylomicron and very low density lipoprotein (VLDL) by the liver and other tissues.[127] It is synthesized within the liver, but also locally within a variety of tissues and by circulating macrophages.[127] ApoE$^{-/-}$ mice display plasma total cholesterol levels >500mg/dL, most of which is carried by chylomicrons and VLDL.[128] Mice normally show high levels of HDL and low LDL, but apoE$^{-/-}$ mice show a shift towards increased total cholesterol, VLDL, and triglycerides, with a marked drop in HDL levels.[129] ApoE$^{-/-}$ mice also display extensive atherosclerosis even when kept on a low fat diet.[129] ApoE$^{-/-}$ mice on a C57BL background are widely used in CVD research and are currently being used by our laboratory and others to examine the influence of social environment on atherosclerosis.[4]

One advantage of the apoE$^{-/-}$ mouse model of atherosclerotic lesion development is the relatively rapid progression of disease found in these animals. Electron microscopy of the proximal aorta in apoE$^{-/-}$ mice can reveal adhesion to the endothelium and transendothelial migration of blood monocytes as early as 5 to 6 weeks of age. It has been reported that by 6 to 10 weeks of age formation of foam cell containing fatty streaks, along with some migration of smooth muscle cells, is evident under light microscope.[128] These mice may have visible atherosclerosis by 2 to 3 months of age,

with lesions grossly observable in the aortic root under dissection microscope.[129] By 4 to 5 months of age mice show more advanced lesions with fibrose caps and necrotic cores surrounded by macrophage laden shoulders. A high-fat or "Western" diet accelerates the process of lesion development.[128] Progression of these proximal lesions has been well characterized by studies examining cross-sections at the aortic root.[129] This method is useful for determining the stage and other histological features of lesion progression, but makes a gross estimation of total lesion area in atherosclerosis prone regions throughout the aorta more challenging and laborious. Recently, the *en face* method has been increasingly utilized in older mice to examine measures of lesion area throughout the aorta.[130] Despite substantial differences in methodology between these measures, regression analyses suggest that they are closely related.[128]

Atherosclerotic disease in apoE$^{-/-}$ mice bears considerable similarity to that of humans, but there are notable exceptions, particularly concerning lipid profile and hemodynamics. Other differences involve the relative importance of specific inflammatory mediators. For instance, while TNF-α clearly plays a major role in accelerating atherosclerosis in numerous models of atherosclerosis, results concerning its role in apoE$^{-/-}$ mice are equivocal.[131] Other important cytokines, such as interferon-gama (IFN-γ) and MCP-1 have been found to accelerate disease in these animals through upregulation of adhesion molecules and LDL scavenger receptors on macrophages.[132]

Importantly, overexpression of CRP has been shown to increase atherosclerosis in apoE$^{-/-}$ mice, confirming the association between this inflammatory marker and the disease found in humans and other animal models.[133]

Lesions in these mice consist mainly of macrophage and T cell infiltrates, both of which have a demonstrated impact on disease development in this model.[134] This indicates that recruitment of circulating leukocytes into the vessel wall by inflammatory mediators is likely to play an important role in disease development in these animals. Extracellular matrix has also been shown to play a significant role in the progression of disease in these mice, with increased collagen levels being associated with more extensive atherosclerosis.[135] This could be due to the increased sequestration of ox-LDL species within areas of dense extracellular matrix. Studies have demonstrated increases in ox-LDL species in and around lesions in apoE$^{-/-}$ mice, indicating that oxidation of lipids is associated with lesion formation in these animals.[136] Finally, numerous studies have implicated NAD(P)H-oxidize activity and other ROS generating enzymes in the progression of disease in this model.[137] Thus, many of the important pathophysiological processes that have been impacted by differential social environment, and possibly OT, are similarly important in the progression of the disease in apoE$^{-/-}$ mice.

Recently, increased attention has been paid to the role of adipose tissue functioning in disease development in apoE$^{-/-}$ mice. In a study examining the effect of visceral fat transplanted subcutaneously into apoE$^{-/-}$ mice, Öhman and colleagues found that transplanted mice displayed increased atherosclerosis and adipose tissue

inflammation.[138] This suggests that visceral adiposity and macrophage infiltration of adipose tissue may play a role in modulating the extent of atherosclerosis in these animals.

Other work has focused on the importance of physical activity in the course of disease development in apoE$^{-/-}$ mice. Laufs and colleagues compared cholesterol-fed apoE$^{-/-}$ mice housed under sedentary conditions with those provided a running wheel on multiple measures of NAD(P)H-oxidase activity and extent of atherosclerosis.[50] Results indicated that physically inactive animals displayed elevated NAD(P)H-oxidase activity and increased atherosclerotic lesion area. Subsequent studies utilizing a swimming paradigm have provided further support for the anti-oxidant and anti-atherogenic effects of physical activity in these animals.[139] Specifically, swimming was found to increase endothelial functioning through increased vascular sensitivity to acetylcholine-induced vasodilation in cholesterol-fed mice.[140] The beneficial effect of swimming exercise in apoE$^{-/-}$ mice was blocked by administration of the nitric oxide synthase (NOS) inhibitor, L-NAME, and endothelial NOS (eNOS) activity was inversely correlated with disease in these animals.[139] Additional findings included decreased lipid peroxidation and monocyte accumulation within lesions of the exercise group.

Together these findings indicate that physical activity may affect the progression of atherosclerosis in apoE$^{-/-}$ mice through several related mechanisms, including decreased oxidative stress and inflammation and improved endothelial and adipose tissue functioning. These mechanisms are thought to be affected by social isolation through increased SNS activation and decreased levels of general physical inactivity. Their

importance in the apoE$^{-/-}$ model provides further evidence that this model of atherosclerosis is appropriate for the study of the effect of social isolation on the disease.

The extent to which social deprivation may be impacting any of the disease mechanisms associated with physical inactivity remains to be determined. Only one study has examined the effect of social environment on atherosclerosis in apoE$^{-/-}$ mice.[4] This study found that socially deprived animals displayed elevated plasma lipids, decreased plasma G-CSF, and increased extent of atherosclerosis in the innominate artery when compared with animals housed in social groups. This suggests that social isolation can exacerbate atherosclerosis in apoE$^{-/-}$ mice, providing an excellent model for studying the pathophysiological mechanisms underlying the effects of social environment on the disease. The principal aim of the present study was to determine whether increased activation of the peripheral oxytocinergic system could diminish the extent of atherosclerosis in apoE$^{-/-}$ mice through anti-inflammatory effects on cells critically involved in disease progression. This would suggest that increases in peripheral OT related to positive social interactions could be responsible for the established protective effect of positive social environment on the development of atherosclerosis.

Summary

Physical contact and positive social interactions associated with stable social environment are thought to play a role in slowing the progression of atherosclerosis in these animals relative to those kept in social isolation. Some evidence indicates that warm physical contact and affiliative behavior are associated with increases in plasma OT. Recent research has demonstrated that peripheral OT plays a role in cardiovascular, immune, and adipose tissue functioning. Other lines of research have demonstrated that

administration of exogenous OT can have anti-inflammatory and anti-oxidant effects on vascular tissues. The initial experiments of the present study sought to further establish the anti-inflammatory effects of OT on immune and vascular cells. The principal objective of the current study is to examine the effect of chronic elevations of peripheral OT levels on atherosclerotic disease and inflammation in apoE$^{-/-}$ mice. The ability of peripheral OT to inhibit the progression of atherosclerosis would suggest that this neuropeptide may be partially responsible for the beneficial effect of positive social environment on atheroslcerosis.

Chapter 2: Hypotheses

1. The oxytocin anti-inflammatory pathway has observable effects *in vitro* on biological mechanisms implicated in cardiovascular disease.

    a. Oxytocin inhibits LPS-induced secretion of IL-6 from DTHP-1 macrophages and human aortic endothelial cells.

2. Chronic activation of peripheral oxytocinergic systems slows the progression of atherosclerotic disease.

    a. Continuous peripheral infusion of OT over 12 weeks reduces the extent of atherosclerosis in apoE$^{-/-}$ mice.

3. These reductions in atherosclerosis are associated with decreased markers of inflammation.

    a. Continuous infusion of OT over 12 weeks diminishes constitutive release of the pro-inflammatory cytokine, IL-6, from *ex vivo* adipose tissue.

4. The observed anti-inflammatory and anti-atherogenic effects are not due to changes in lipids, physical activity levels, or stress hormone production.

    a. Animals treated with OT over 12 weeks show no differences in plasma lipids, activity levels, or 24-hour corticosterone secretion relative to vehicle control animals.

Chapter 3: Methods

Cell Culture Studies

THP-1 Macrophages.

The human monocytic THP-1 cells are a leukemic cell line derived from a patient with acute monocytic leukemia.[141] THP-1 cells acquire phenotypic and functional features of macrophages after incubation with a stimulant such as phorbol myristate acetate (PMA). PMA stimulation of THP-1 cells prompts their differentiation into macrophage-like cells and they become adherent.[142] All experiments were performed in PMA stimulated THP-1 cells after differentiation due to low basal levels of pro-inflammatory cytokine production in unstimulated cells (unpublished findings).

THP-1 cell lines were grown in suspension culture in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 μM mercaptoethanol. To induce differentiation, 1.0×10^5 cells were plated in 35 mm culture dishes in complete medium containing 100 ng/ml PMA for 24 to 48 hours. The adherent cells were maintained in culture at 37°C in a humidified 95% air/5% CO_2 incubator for 7 or 8 days before experimental manipulations.

Human Aortic Endothelial Cells.

Human aortic endothelial cells (HAECs) were cultured in Medium 200 supplemented with low serum growth supplement (Invitrogen) at 37°C in a humidified

95% air/5% CO_2 incubator. Cells were used between the third and eighth passage. For experiments, cells were plated into 35 or 60 mm dishes at 25,000 or 50,000 cells/dish and grown until ~80% confluent.

Experiments.

On the day of the experiment, wells were incubated in fresh media supplemented with 1% FBS and conditional reagents. Serum supplementation is necessary to facilitate LPS (Sigma, St. Louis, MO) binding to the macrophage toll-like receptor complex (CD14/MD2/TLR4) through assistance of the lipopolysaccharide binding protein (LBP) chaparone.[143] A 1% supplementation was found to be the optimal concentration based on results of preliminary experiments (unpublished observations). Conditions included the following: 1) supplemented media alone 2) LPS alone 3) LPS and oxytocin (Phoenix Peptides, Belmont, CA). LPS concentration was 100 ng/mL and oxytocin concentrations were 10pM and 100pM for each experiment. Experimental conditions were run in triplicate and data was collapsed across three separate but identical experiments. After six hours of incubation, media was aliquoted and centrifuged at 10,000 x g. Supernatants were transferred to a fresh microcentrifuge tube and frozen at -20ºC. Cells remaining adherent to wells were washed three times with PBS and frozen at -20ºC.

Cells were digested with 0.1M NaOH and homogenate protein concentrations were determined by the BCA Protein Assay (Pierce Corp., Rockford, IL). Media IL-6 concentrations were measured by a commercially available enzyme-linked immunosorbant assay (ELISA) kit (BD Bioscience, San Diego, CA) and were normalized for cellular protein concentrations. Preliminary studies showed minimal IL-6 secretion in the media alone and oxytocin alone conditions without LPS stimulation (data not shown).

Pilot Study: Infusion of ^{3}H-Oxytocin

Preparation of Materials.

A solution containing 1.09μg of tritiated-oxytocin (^{3}H-OT, 46.3 Ci/mmol) in 50μL of water:ethanol (8:2) was added to 24μL of a stock solution of 120μg cold OT and mixed in sterile saline to a total volume of 1mL. This resulted in a total OT concentration of approximately 120μg/mL. A 5μL sample of this solution was diluted into 500μL PBS/0.1% BSA and two samples of 50μL and 100μL were counted in the presence of 4mL of scintillation fluid in a beta radiation counter for 10min to obtain counts per minute (cpm).

The specific activity (counts/unit mass) of the OT solution was calculated by using the following equation:

$$\text{Specific Activity} = \frac{\text{Counts/}\mu\text{L pump solution}}{\text{OT/}\mu\text{L pump solution}} = \frac{50{,}000\text{cpm/}\mu\text{L}}{120\text{ng OT/}\mu\text{L}} = 417\text{cpm/ng OT}$$

Four osmotic minipumps (model 2006, DURECT Corporation, Cupertino, CA), set to infuse at 0.15μL/hr for 6 weeks, were maintained under sterile conditions and weighed before being loaded with 200μL of the 120μg/mL ^{3}H-OT solution. Pumps were reweighed after loading. The change in mass of each pump after filling was confirmed to be $\geq$ 90% of the expected change. The pumps were then primed in a sterile saline bath at 37°C for 70hrs.

Experimental Animals and Surgeries.

Immediately after priming, the pumps were implanted into four male C57BL mice, 11-12 weeks of age and weighing 22-24g (Jackson Laboratories). Surgeries were performed under sterile conditions according to pump manufacturers instructions

(DURECT Corp.). Briefly, mice were anesthetized with a ketamine/xylazine (10mg ketamine/0.4mg xylazine/30g mouse) cocktail and scrubbed with ethanol and betadine sterilization solutions prior to surgery. A small subcutaneous incision was made in the mid-scapular region and a hemostat was used to create a pocket for the pump. The pump was then inserted into the subcutaneous pocket, flow modulator first, and the wound was sealed with adhesive (Vetbond) and surgical staples (Kent Scientific Inc.). Animals were allowed to recover in a 37°C incubator prior to returning to their home cages.

OT Pharmacokinetics and Stability.

Estimation of the pharmacokinetic parameters of OT allows for calculation of the predicted steady state plasma concentration for any given concentration loaded into the minipump. The pump infusion rate is constant and known ($k_o = 0.15\mu L$). The presumed dispersion volume for a 22g mouse (blood volume = ~7% of mass) is approximately 1.5mL. However, studies of the pharmacokinetic properties of oxytocin in multiple species have consistently found that a two compartment model fits the data, with an apparent volume of distribution (V_D) at steady state of approximately 150mL/kg (for 22g mouse, $V_D = 3.5mL$).[144] Based on the literature regarding OT pharmacokinetics in other species, the plasma half-life and volume of distribution at steady state may be estimated for mice ($t_{1/2}$ = ~1.5 min).

Assuming 100% absorption (i.e., intravenous infusion) we can calculate the predicted plasma steady state (Cp^{ss}) of OT based on the following equation (Cp^{ss} = concentration in plasma at steady state; $k_{el} = \ln 2/t_{1/2}$; k_0 = infusion rate; V = volume of distribution at steady state; t = time):

$$Cp^{ss} = \frac{k_o}{k_{el} \times V} \times (1 - e^{-kel \times t})$$

as $t \rightarrow \infty$, $e^{-kel \times t} \rightarrow 0$

Thus, with continuous infusion the equation simplifies allowing calculation of the theoretical steady state concentration of OT:

$$Cp^{ss} = \frac{k_o}{k_{el} \times V} = \frac{(18ng/hr)}{(\ln 2)/(1.5min/60min))(3.5mL)} = 190\ pg/mL$$

Despite the simplicity of the model provided by the known continuous infusion rate, studies of the pharmacokinetic properties of OT in some animal species have found that its volume of distribution may be larger than the water volume of the entire animal.[144] This indicates that OT is differentially distributed into tissue compartments and is likely to show a higher affinity for skeletal muscle and/or adipose tissue, which may then serve as a reservior for continued OT diffusion into circulation.[145] Given the size of OT it is unlikely that this peptide is directly entering into circulation through capillaries in the subcutaneous space.[146] It is more probable that OT is entering the venous system through lymphatic drainage. Considering these factors impacting the absorption and distribution of OT, as well as the presence of generic "oxytocinases" in

multiple tissue compartments, it is expected that the actual Cp^{ss} for subcutaneous infusion may be substantially lower than that predicted by the single compartment, i.v. infusion model.

For empirical determination of OT pharmacokinetics in C57BL mice, blood was collected from animals implanted with osmotic minipumps containing ^{3}H-OT at weeks 1, 3, and 4 after surgery and plasma samples were extracted. Extracted samples were subjected to analysis by high pressure liquid chromatography (HPLC) and fractions were collected every minute for 30 minutes. Scintillation fluid (4mL) was added to each fraction and counts were obtained from a beta counter after 30 minutes of counting per fraction. A 50μg OT standard was used to confirm which fraction contained intact OT by chromatography at 450nm.

After the animals were sacrificed each pump was removed and pump contents were analyzed by HPLC to determine whether any OT degradation had occurred during the incubation.

Infusion of OT into apoE$^{-/-}$ Mice

Experimental Animals.

Forty-five male apoE$^{-/-}$ mice, 11-12 weeks of age and weighing 22-24g, were purchased from Jackson Laboratories. Mice were randomized to control (n=21) and experimental (n=24) groups on arrival and were individually housed in a tempurature- and humidity-controlled environment on a reverse 12hr light/dark cycle and given food

and water *ad libitum*. Mice were acclimated for 7 days before initiation of experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami.

Behavioral Monitoring.

Videotape samples of home cage individual behavior were obtained at study midpoint (week 6) for a subset of animals from each group (n=12 per group). During nocturnal behavioral sampling each animal was videotaped for 5 minutes in its home cage under red light. Tapes were later reviewed and time spent in active (e.g., cage exploration, rearing) and sedentary (e.g., grooming, sitting) behaviors was recorded to examine potential effects of peripheral OT administration on activity levels. Behaviors were identified as described previously.[10] Time spent in active versus sedentary behaviors was then converted into percent time based on the 5 minute behavioral sampling.

Feces Collection.

For assessment of 24-hour corticosterone levels at baseline and midpoint (6 weeks of treatment), mice were placed in clean metabolic cages, containing wire mesh floors and liquid nutrients, for 24-hours of feces collection. Fecal samples were gathered from cage bottoms and stored in 15mL conical tubes at -80°C for later extraction and assay.

Blood Draws.

Mice were fasted for 7 hrs prior to blood draws at baseline (week 0), midpoint (week 6), and endpoint (week 12) for measurement of plasma lipids, CRP, insulin, and adiponectin. At baseline and midpoint blood was drawn from the submandibular vein and at endpoint by cardiac puncture with heparinized needles. After submandibular

blood draws all animals were immediately hydrated with 1mL of Ringer's lactate. Samples were collected in EDTA coated tubes and centrifuged (5000 x g for 15min) and plasma was aliquoted and stored at -80°C.

Surgeries.

Primed (70hrs) osmotic mini-pumps containing either 200μg/mL OT (approximately 1μg/kg/hr) or vehicle (VH), 50mM Sodium Citrate, pH = 4, were surgically implanted into each animal. The dose was chosen based on pharmacokinetic calculations and stability data gathered from the literature and a pilot study to ensure a minimum estimated steady state plasma OT level of 100pM and a maximum of 500pM. Assuming 100% absorption (i.e., i.v. infusion), this dose of OT should produce the following plasma concentration at steady state:

$$Cp^{ss} = \frac{k_o}{k_{el} \times V} = \frac{(30ng/hr)}{((\ln 2)/(1.5min/60min))(3.5mL)} = 300\ pg/mL$$

This figure likely represents an overestimation due to imperfect absorption and the presence of extra-hepatic oxytocinases.

Surgeries were identical to those described in the preliminary study (see, *Pilot Study: Infusion of ^{3}H-Oxytocin*, above). While the animals were under anesthesia they were also implanted with a radio-transponder assigned a random 12 digit number.

Pump-exchange surgeries performed at midpoint were also identical except for the removal of previously implanted pumps. After surgical removal, pumps were stored at 4°C until analyses could be performed to confirm OT stability and pump function.

Feces Extraction and Assay.

Fecal samples were weighed and extracted prior to assaying corticosterone levels as described previously.[147,148] Breifly, each sample was boiled twice in 10 mL of 95% ethanol for 20 min. The solvents from these extractions were then combined and dried in a water bath. Tubes were then rinsed with ethyl acetate:hexane solution (3:2) and again dried prior to resuspending the residue in 95% ethanol. Suspensions were assayed using the ^{125}I-radiolabelled corticosterone kit (ICN Pharmeceuticals, Costa Mesa, CA) and results were corrected by sample weight.

Plasma Assays.

Plasma lipid levels were measured using an automated analyzer (Roche Diagnostics). Plasma CRP (Immunological Consultants Inc.), insulin (Mercodia), and adiponectin (R & D Diagnostics) were measured using commercially available ELISA kits.

Tissue Collection.

On the date of sacrifice (12 weeks after beginning OT infusion) mice were deeply anesthetized with an i.p. injection of a ketamine/xylazine cocktail. After induction of

anesthesia was confirmed, the animals were exanguinated by cardiac puncture with a heparinized needle. Samples were transferred to an EDTA coated tube and centrifuged (2000 x *g* for 15min) and plasma was aliquoted and stored at -80°C.

After sacrifice the epididimal fat was dissected, weighed, and prepared for assessment of *ex vivo* adipokine secretion. The femoral arteries were also freshly dissected, homogenized with a manual stator-rotor system in PBS/DTPA buffer, and frozen for later analysis of NAD(P)H-oxidase activity.

The mice torsos were then fixed in 10% buffered formalin. The heart and aorta were later removed, stripped of adventitia, and cut longitudinally for *en face* preparation. Tissues were stored in 10% buffered formalin for later histological analysis and quantification of atherosclerotic disease.

Ex Vivo Adipokine Secretion.

After dissection, epididimal fat samples taken from a subset of animals (n=12 per group) were minced for 3min and washed with 5mLs of DMEM. Infranatants were aspirated and tissue was centrifuged for 45sec at 1000rpm. Fat tissue was then transferred to pre-weighed glass tubes and incubated in 1.5mL of DMEM culture media with or without stimulation with epinephrine (10μM) or LPS (100ng/mL). Media was supplemented with 1% FBS for LPS-stimulated samples. After 6 hours of incubation on a water bath shaker at 37°C, culture media was centrifuged and supernatants were aliquoted and stored at -80°C. Tubes containing adipose tissue were lyophilized overnight and re-weighed. The dry weight of the adipose tissue sample was determined by taking the difference between the pre-sample tube weight and the tube weight with

dried tissue sample. Both epinephrine and LPS increase IL-6 production several fold in adipose tissue,[149] providing an index of stimulated pro-inflammatory cytokine release. Epinephrine also stimulates the release of non-esterified fatty acids (NEFAs).[149]

Tissue Assays

Culture media samples from the *ex vivo* adipose tissue experiment were used to measure IL-6 concentrations, using a comercially available ELISA kit (R & D). NEFA concentrations were also obtained from these samples using an automated analyzer. Both IL-6 and NEFA values were corrected for sample dry-weight.

NAD(P)H-oxidase activity in arterial tissue homogenate was estimated with lucigenin-enhanced chemiluminescence (5 μM lucigenin concentration, 100 μM NAD(P)H) as previously described.[150] The number of superoxide free-radicals produced by NAD(P)H oxidase activity is directly related to the effect these free-radicals have on lucigenin derived chemiluminescence. Chemiluminescence was quantified by a luminometor (Berthold) to measure oxidase activity and results were expressed in relative light units per microgram protein (RLUs/ μg protein). Homogenate protein concentrations were assessed using the BCA protein method.

Quantitation of Atherosclerosis.

All histomorphometric procedures will be performed blinded. The method for preparation of mice aortas and quantification of disease was performed as described previously.[151] Breifly, aortas previously fixed in formalin were stained with the lipid staining agent, oil-red-O, and photographed. A reference aorta template, created from the

average size and shape of all the aortas in the sample, was overlayed onto each aorta image. Percent lesion area was calculated from the proportional area of pixels stained with Oil Red O for a given aortic section.

Statistical Analyses

ANOVA was used as an omnibus test for *in vitro* experiments involving multiple doses and a control condition. Post-hoc Dunnett's tests were utilized to determine which doses differed from control.

ANOVA with repeated measures was used to examine change in plasma measures (CRP, insulin, adiponectin, cholesterol, and triglyceride levels) over time and between groups. In instances where the sphericity assumption was violated, appropriately adjusted degrees of freedom were used to determine significance. Where group x time interactions were found to be significant, post-hoc t-tests of midpoint and endpoint measures were used to examine the time course of group differences. Welch or studentized t-tests were used to compare groups on all baseline plasma measures, midpoint behavioral measures, baseline and midpoint 24-hour corticosterone, and endpoint tissue measures, including NAD(P)H-oxdiase activity, *ex vivo* IL-6 secretion, and percent lesion area. Measures of atherosclerosis were highly skewed and kurtotic, consequently, Logarithm base 10 transformed variables were used for all analyses of these measures. One animal in the vehicle control group was removed from all lesion area analyses due to having extremely high levels of disease (> 5.5 standard deviations) that substantially influenced all statistical parameters and significance tests.

Exploratory analyses using Pearson correlations were performed to examine which variables significantly predicted extent of atherosclerosis overall and in the arch

and thoracic sections of the aorta. When multiple variables significantly predicted disease, multiple regression analyses were used to determine the nature of the observed relationships. Additionally, ANCOVA analyses were utilized to examine whether group differences in disease were influenced by variables predicting disease. All significance tests were two-tailed and $p < 0.05$ was considered significant.

Chapter 4: Results

Cell Culture Studies

THP-1 Macrophages & HAECs.

Combined results from 3 separate, but identical experiments revealed that OT dose-dependently diminished LPS-induced IL-6 secretion by 35% (10pM) to 55% (100pM) in DTHP-1 cells, $p < 0.01$ (Figure 4.1A). Comparable experiments in HAECs revealed that the low OT dose (10pM) nonsignificantly reduced IL-6 secretion by approximately 15%, $p > 0.10$, but the high dose (100pM) produced a statistically significant reduction of approximately 25%, $p < 0.01$ (Figure 4.1B). Examination of individual well data from all experiments in both cell types across doses reveals a high range of variability in response to OT (Figure 4.2A & B).

Pilot Study: Infusion of ^{3}H-Oxytocin

OT Stability in Plasma & Osmotic Mini-pumps

Priming fluid was collected from the minipump incubation solution and beta radiation was counted at 24hrs, 48hrs, and 67hrs to determine pump extrusion rate. Results were compared with theoretical predictions based on manufacturer's specifications (Figure 4.3).

Results of plasma analyses indicated that most of the ^{3}H-OT absorbed into systemic circulation was rapidly metabolized (Figure 4.4A-C). From the beta counts data the plasma concentration of intact ^{3}H-OT can be estimated using the specific activity of the pump solution (417cpm/ng). After subtracting background (~5 CPM) and controlling for quenching due to sample acetonitrile (~25% of signal), plasma ^{3}H-OT concentations were estimated by dividing the appropriate fraction counts by the specific activity. The

resulting empirical estimates (< 60 pg/mL) were significantly lower than our theoretical estimates and provided only a minimal signal above background. Interestingly, the plasma concentration of OT also appeared to diminish over time, suggesting that OT was not being infused at a constant rate (Figure 4.4D).

HPLC analysis of OT degradation in osmotic minipumps *in vivo* indicated that the percent intact OT diminished linearly over time. Peaks presumed to be associated with degradation products increased in proportion to the degradation of intact OT (Figure 4.5A-D). These data were comparable to those obtained from OT pumps stored *in vitro* at 37°C for a similar duration (Figure 4.6A & B).

The findings from this preliminary study suggested that OT was not stable in the pumps at 37°C *in vivo* or *in vitro*. Other researchers have suggested that OT may be more stable at low pH (~4) which helps maintain its ring forming Cys-Cys disulfide bridge. A brief stability study was carried out by comparing the degradation of OT in sterile saline to that in 50mM sodium citrate titrated to pH = 4 with HCl. Solutions containing 200μg/mL OT were placed in vials and pumps and incubated at 37°C and 60°C for 7 days. Results indicated that OT showed two times as much degradation (18% vs. 6%) in neutral saline solution versus the more acidic (pH = 4) sodium citrate buffer.

Infusion of OT into apoE$^{-/-}$ Mice

OT Stability

Osmotic minipumps taken from two to four apoE$^{-/-}$ mice per group were examined after removal at midpoint and endpoint of the study to confirm OT stability and pump function after 6 weeks of *in vivo* incubation. HPLC analysis revealed that all assayed pumps contained the appropriate solution for their group (OT vs. VH; Figure

4.7A-B) and the expected quantity of residual solution (~20uL). Analysis of residual solution by HPLC revealed that approximately 80% of the OT remained intact after 6 weeks in the pump *in vivo* (Figure 4.7C). Two pumps loaded with OT contained residual solution that was opaque. Examination of the solution under a light microscope revealed cellular debris and mononuclear and polymorphonuclear cells, suggesting infiltration of plasma contents into the pump solution. Residual solution taken from these pumps was centrifuged at high speed (10,000 x g) through a microfilter to purify the contents before HPLC analysis. Results of HPLC revealed that even in these compromised pumps OT showed comparable stability (~80%).

Behavior

After 6 weeks of infusion, OT treated animals spent an average of 15.0% (SEM = 1.4%) of their time in sedentary behaviors and 85.0% (SEM = 1.4%) of their time in active behaviors. Vehicle control animals spent an average of 13.3% (SEM = 1.7%) of their time in sedentary behaviors and 86.8% (SEM = 1.7%) of their time in active behaviors. There was no significant difference between groups in their level of behavioral activity, $p > 0.10$ (data not shown).

Corticosterone

Animals in the OT treatment group demonstrated significantly greater 24-hour fecal corticosterone concentrations at baseline, prior to the initiation of treatment,

7846ng/g (SEM = 683ng/g) vs. 5523ng/g (SEM = 795ng/g), t (39) = 2.224 p = 0.032, but were no longer different after 6 weeks of treatment, 7913ng/g (SEM = 707) for OT vs. 7291ng/g (SEM = 678) for VH animals, $p > 0.10$.

Plasma Assays and Weight

No significant group differences were detected between groups on baseline measures of weight, cholesterol, triglycerides, CRP, or insulin, $p > 0.10$ for all (Table 4.1). Analysis of plasma total cholesterol and triglycerides showed no significant group x time interaction or main effect for group, but cholesterol significantly increased, F (2,84) = 5.525, p = 0.002, and triglycerides significantly decreased over time, F (2,84) = 24.206, p = 0.0005. Plasma CRP showed no significant group x time interaction or main effect for group, but CRP did increase significantly overall during the study, F (2,86) = 16.026, p = 0.0001. Though there was no significant group x time interaction or baseline differences for plasma CRP levels, OT treated animals showed significantly lower plasma CRP levels after 6 weeks of treatment, t (41) = -2.646, p = 0.022 (Figure 4.8A). Plasma insulin levels also displayed no significant group x time interaction or main effect for group, but increased significantly overall during the study, F (2,86) = 8.119, p = 0.001. Weight showed no significant group x time interaction or main effect for group, but did increase significantly overall during the study, F (2,88) = 472.904, p = 0.0008.

Change in adiponectin levels showed a significant group x time interaction over the study, F (2,86) = 7.187, p = 0.001, and main effects were detected for both time, F (2,86) = 64.175, p = 0.001, and group, F (1,43) = 8.378, p = 0.006. Post-hoc t-tests revealed that the OT treated animals had significantly lower adiponectin levels at midpoint, t (43) = -4.756, p = 0.00002, but not at baseline or endpoint (Figure 4.8B).

NOX activity

Analysis of arterial NAD(P)H-oxidase activity by chemiluminescence assay revealed no significant group differences after controlling for sample protein, $p > 0.10$ (data not shown).

Ex Vivo Adipokine Secretion

T-tests comparing a subset of animals from each group on basal, epinephrine-stimulated, and LPS-stimulated IL-6 secretion over six hours revealed group differences in basal IL-6 secretion, $t\ (22) = -2.957$, $p = 0.007$. Adipose tissue samples taken from OT treated animals showed greater than 30% reduction in IL-6 release relative to samples taken from vehicle control animals (Figure 4.9).

No statistically significant group differences in IL-6 were detected in stimulated conditions (Figure 4.9) or in NEFAs in any condition, $p > 0.10$ for all (data not shown).

Atherosclerosis

Aortic atherosclerosis showed a bimodal distribution throughout the aorta, with most disease occuring in the aortic arch and a second area of lesion prevalence near the thoracic aorta. Visual comparison of cumulative lesion prevalence maps indicates apparent group differences in the extent of atherosclerosis at the thoracic aorta (Figure 4.10). Results of t-tests examining group differences in log-transformed measures of percent lesion area revealed that OT treated animals displayed significantly

(approximately 40%) less atherosclerosis within the thoracic aorta, t (26.201) = -2.156, p = 0.04 (Figure 4.11). No significant group differences were detected in the aortic arch or total lesion area.

Correlations, Multiple Regression, and ANCOVAs

Correlational analyses revealed that only baseline adiponectin (r = .550, $p <$ 0.001) showed a positive correlation and only baseline weight (r = -.389, $p <$0.05) showed a negative correlation with total lesion area (Figure 4.12). Both of these baseline measures were also negatively correlated with each other (r = -.401, $p < 0.01$). Multiple regression analyses indicated that after controlling for baseline adiponectin, baseline weight no longer significantly predicted total lesion area. ANCOVA indicated that there were still no significant differences in total percent lesion area between treatment groups after controlling for baseline adiponectin.

A similar pattern of results was found in the aortic arch, which comprised the majority of the total lesion area. However, endpoint triglycerides also showed a modest positive correlation with lesion area in this section of the aorta (r = .306, $p < 0.05$). Endpoint triglycerides did not show a significant correlation with baseline weight or adiponectin, $p > 0.10$. There were no significant differences between treatment groups in percent lesion area in the aortic arch after controlling for either baseline adiponectin or endpoint triglycerides, $p > 0.10$.

None of these measures correlated significantly with percent lesion area within the thoracic aorta, $p > 0.10$ for all. The only statistically significant predictor of disease in this secondary area of lesion prevalence was endpoint insulin, which showed only a

modest correlation ($r = .332$, $p < 0.05$). Even after controlling for endpoint insulin, animals in the OT treatment group still showed significantly diminished extent of atherosclerosis in this section of the aorta, $p < 0.05$.

Chapter 5: Discussion

To date no studies have examined the effect of chronic OT administration on markers of inflammation and atherosclerosis in an *in vivo* model of the disease. Findings from the current study suggest that OT may slow the progression of atherosclerosis in a site-specific manner, even in animals with strong genetic determinants. Importantly, these differences were found to be independent of changes in plasma lipids, weight, physical activity levels, stress (corticosterone), or insulin levels. Additional findings, both *in vivo* and *in vitro*, indicate that OT reduces the secretion of the pro-inflammatory cytokine, IL-6, from vascular, immune, and adipose tissues, and may cause decreased CRP production *in vivo*. Increased production of pro-inflammatory cytokines by these tissues has been implicated in the initial stages of atherosclerotic lesion development, which include endothelial cell dysfunction, macrophage activation, and foam cell formation and transmigration.[46] Additional research from our laboratory indicates that OT reduces NAD(P)H-oxidase activity in stimulated macrophages and endothelial cells *in vitro*.[57] The mechanism underlying these anti-inflammatory and anti-oxidant effects and the extent to which they are responsible for the observed differences in atherosclerotic lesion development are important areas for future research.

The current study involved apoE$^{-/-}$ mice fed a chow diet for 24 weeks, yielding relatively low levels of disease with nearly all lesions being in the earliest stages of development. The fact that OT was able to slow the initial development of these lesions in a region of high lesion prevalence suggests that it may be working through mechanisms important during lesion initiation. This is consistent with the *in vitro* finding that OT is capable of reducing pro-inflammatory cytokine release from macrophages, as

early lesions are characterized by intravasation of activated macrophages in a pro-inflammatory state.[46] Another important aspect of the initial stages of lesion development is endothelial dysfunction, characterized by endothelial cell release of pro-inflammatory cytokines and increased activation of NAD(P)H-oxidase. The present study and other recent studies from our laboratory have found that OT is capable of dampening pro-inflammatory cytokine release and NAD(P)H-oxidase activation in endothelials cells, which could prevent the endothelial dysfunction characteristic of the early stages of lesion formation. While the current study was unable to demonstrate a treatment effect of OT on arterial NAD(P)H-oxidase activity *in vivo*, the activity of this enzyme was measured in femoral artery samples due to the need to fix aortic samples for quantification of atherosclerosis. These more distal arteries are unlikely to show any pathological changes at this early age.[152] Future studies focusing on measures of oxidase activity in regions of high lesion prevalence are more likely to obtain results consistent with *in vitro* findings.

The finding that chronic infusion of OT decreases constitutive release of IL-6 from adipose tissue during *ex vivo* incubation suggests that OT may have prolonged effects on low-grade inflammation associated with pro-inflammatory adipokine activity. This is consistent with the findings of a recent study comparing oxytocin receptor-deficient mice ($OTR^{-/-}$) with wild-type mice on measures of energy balance and adipose tissue functioning.[153] Results indicated that $OTR^{-/-}$ mice display increased weight gain, visceral and epididimal fat pad mass, adipocyte size, and lipid droplet accumulation despite similar activity levels and food intake. They also exhibited a lower core body temperature and alterations in brown adipose tissue morphology, suggesting that

decreased thermogenesis may have contributed to their weight gain. While these findings cannot be directly compared with those of the current study due to differences in methodology, they do suggest that OTR activation may shift the energy balance toward increased energy expenditure and decreased visceral and epididimal lipid accumulation and mass. These effects could be responsible for improved adipose tissue functioning, decreased inflammation, and diminished atherosclerosis in these hyperlipidemic animals.

While the present study could not investigate the specific cellular source of the observed reduction in adipose tissue IL-6 secretion, preliminary work from our laboratory has suggested that adipocytes are not directly implicated in the effects of OT on adipose tissue IL-6 release *in vitro*.[154] This suggests that resident macrophages within adipose tissue may be responsible for the observed effects of OT on adipokine secretion. This is consistent with the *in vitro* data demonstrating an anti-inflammatory effect of OT on human macrophages. This mechanistic explanation suggests that the observed decrease in *ex vivo* IL-6 secretion from adipose tissue could be due to chronic activation of an OT anti-inflammatory pathway affecting IL-6 release from resident ATMs. Alternatively, chronic infusion of OT may have led to decreased recruitment of circulating monocytes into adipose tissue over the 12 weeks of treatment. The number of resident macrophages has been found to change over time and respond to inflammatory conditions within adipose tissue.[47] Macrophages recruited from circulation tend to be in a pro-inflammatory state and exhibit increased constitutive expression of IL-6.[48] High numbers of such recruited macrophages residing within adipose tissue are thought to be responsible for the association between visceral adiposity and atherosclerosis.[155,156] It is possible that the 12 weeks of OT treatment dampened inflammatory conditions within

adipose tissue, leading to decreased recruitment of macrophages. This hypothesis is supported by the observation that adipose tissue taken from OT treated mice continued to secrete lower levels of IL-6 after removal from the treated animal. The mechanism responsible for this observation and its implications in atherosclerotic lesion development requires further research, but may involve modulation of chemotactic (e.g., MCP-1) or cytokine (e.g., IL-10) factors.[138]

The present study found that OT treatment led to diminished atherosclerotic lesion area in the thoracic aorta, but no effect was found in the aortic arch. Site specific differences in hemodynamics, including low shear stress, cyclic reversal of flow direction (i.e., oscillatory stress), stretch stress, and turbulance could be responsible for differential treatment effects at various locations within the aortic tree.[32] Different atherosclerosis-prone regions have been described in the literature on murine models of hyperlipidemia and frequently vary in their responses to treatment and development over time. It has also been shown that differential gene expression profiles exist throughout the aorta and that these differences can convey varying susceptability to specific atherogenic mechanisms.[32] For example, a study examining the effects of irradiation and bone marrow transplantation (BMT) on atherosclerosis in $LDL^{-/-}$ mice found that BMT greatly reduced thoracic lesion area, while increasing the extent of disease in the aortic sinus.[157] The authors attributed these disparate findings to the possible effects of irradiation on endothelial cell functioning in areas of altered flow (i.e., aortic sinus) versus laminar flow (i.e., thoracic aorta).

Another recent study examined monocyte accumulation in different sections of the aorta by injecting $apoE^{-/-}$ mice with radiolabelled monocytes and later obtaining

radiographic films of the entire aorta *en face*.[158] Results indicated that monocytes tended to accumulate in specific sites that overlapped well with oil-red-O staining. These areas included the aortic arch and a lower section of the thoracic aorta corresponding to the secondary area of lesion prevalence found in the current study. This suggests that OT may be affecting monocyte accumulation in this section of the aorta characterized by laminar flow patterns, which is consistent with the anti-inflammatory effects of OT on macrophages and endothelial cells *in vitro*. However, in this study few radiolabelled monocytes were visualized in 24 week old, chow-fed mice. Only older chow-fed animals and younger animals fed a high cholesterol diet showed increased monocyte accumulation. This suggests that alterations in endothelial permeability or other atherogenic effects preceding monocyte adherence and chemotaxis may have been impacted by OT treatment.

The differential treatment effects observed in the present study could also be due to variation in expression of OTR throughout the aorta. The distribution of these receptors in the mouse aorta has yet to be investigated. Another possibility is that unintended activation of vasopressin receptors (V_1) may have caused a tonic increase in blood pressure, exacerbating atherosclerosis in regions particularly vulnerable to alterations in blood flow (i.e., aortic arch). This may have masked the beneficial anti-inflammatory effects of OTR activation. In the thoracic aorta, laminar flow patterns may convey relative resistance to alterations in hemodynamic parameters, which could explain the observed treatment effect in this aortic section. Future studies utilizing V_1 receptor

antagonists or OTR agonists that do not exhibit affinity for the V_1 receptor could provide more information regarding the differential treatment effects observed in the present study.

The finding that plasma CRP levels were significantly lower in OT treated animals at midpoint suggests a mechanism whereby visceral adipose tissue IL-6 secretion could be influencing levels of low grade inflammation systemically. This low-grade systemic inflammation is thought to directly impact lesion development through activation of macrophages and endothelial cells. The observed decrease in plasma CRP was not sustained after 12 weeks of treatment, suggesting the possibility of compensatory mechanisms. Given the fact that CRP levels tended to rise with age, regardless of treatment, it is also possible that increased levels of this protein eventually overwhelmed any treatment effect produced by OT.

An unexpected finding was that plasma adiponectin levels decreased after 6 weeks of infusion with OT, but then returned to control values by the end of the study. This suggests that OT may be affecting adipokine physiology, which is consistent with the observed effects of OT on adipose tissue IL-6 secretion and with other studies implicating OT in adipose tissue homeostatic functioning.[159] The return to control values at 12 weeks could reflect compensatory changes in the circulating levels of adiponectin or increased degradation of plasma OT. Numerous prior studies have established adiponectin as a protective factor in the development of atherosclerosis in apo$E^{-/-}$ mice.[160] Thus, diminished plasma levels of this protein are not likely to be mediating the beneficial effect of OT on lesion development. However, contrary to previous studies, the current study found that baseline adiponectin values were positively predictive of

endpoint atherosclerosis. While the significance of this paradoxical result is unclear at this time, at least one study in apoE$^{-/-}$ mice has found that increased plasma adiponectin levels may be associated with adipose tissue inflammation.[138] In this study inflammation of adipose tissue was also associated with increases in other, atherogenic, adipokines, including leptin and resistin. This suggests that adipocyte dysfunction and inflammation may be associated with a general increase in adipokine concentrations in these animals, which could explain the potentially spurious association between adiponectin and disease found in the present study. Thus, the anti-inflammatory actions of OT on adipose tissue may decrease levels of adipokines generally. Future examination of the effects of OT on circulating levels of other adipokines could test this hypothesis. Only modest to minimal correlations were detected between other known risk factors, including plasma total cholesterol and triglycerides, and extent of atherosclerosis at this early stage of disease. This is likely due to the relatively low levels of disease found in the aorta at 24 weeks of age in chow-fed mice, but underscores the potential importance of markers of adipose tissue functioning in these animals.

The findings of the present study are consistent with prior studies involving acute bolus administration of OT in models of systemic shock and vascular injury. Results of *in vitro* experiments in endothelial cells and macrophages suggest a cellular mechanism by which high doses of OT could be protective in these acute injury models through anti-inflammatory actions on vascular and immune cells. Previous studies involving models of acute injury and extremely high, super-physiological doses (e.g., 1mg/kg) of OT have found mixed results in terms of the peripheral versus central effects of OT.[104,105,108] In the current study, peripheral administration of a relatively low dose was chosen so that

some mechanisms could be partially ruled-out on a theoretical basis. Relatively low doses of peripheral OT (<1ng/mL) are not thought to cross the blood-brain-barrier in sufficient concentrations to exert any direct CNS effects.[95] A gross measure of general physical activity also showed no differences between treatment groups, suggesting that OT did not affect CNS function. Importantly, this confirms that the observed differences in inflammation and atherosclerosis were not due to changes in activity levels. This suggests that the observed changes in inflammatory markers and disease progression are likely due to direct activation of peripheral OT receptors. However, OT receptors are also present at the circumventricular organs, including the pituitary gland itself, which could exert effects on peripheral organs through CNS autoregulatory mechanisms.[95] It is also possible that OT receptors in peripheral afferent nerves could activate similar autoregulatory mechanisms. While these indirect effects of OT on the CNS cannot be totally ruled-out, general CNS mechanisms activated by endogenous oxytocinergic pathways in the brain, or central administration of OT, are unlikely to be playing a role in the differences observed in this study. This is further supported by the fact that after 6 weeks of treatment the two groups showed no differences in 24-hour corticosterone secretion. This finding indicates that observed differences in inflammation and disease were not due to stress-buffering mechanisms.

It is also possible that OT is working through mechanisms other than direct receptor activation, potentially including vasopressin receptor (V_1) activation. However, the predicted plasma concentration of OT at steady state is lower than theoretical expectations for increased V_1 receptor affinity.[161] Thus, while these indirect effects of OT are possible, it is more likely that OT exerted its effects through direct receptor

activation at the individual tissue level. Future studies utilizing OT receptor knockout models, OT receptor antagonists, and chronic central administration of low doses could shed more light on questions regarding the specific mechanism of action responsible for the observations of the current study.

Previous studies have suggested that high doses of OT may have anti-inflammatory effects through unknown mechanisms.[104,105,108] The present study extended *in vivo* findings to a disease model involving chronic low levels of inflammation and slowly progressive development of vascular endothelial dysfunction and atherosclerosis. Additional experiments expanded *in vitro* research to cells involved in atherosclerotic lesion development. OT produced anti-inflammatory effects in all major tissues involved in atherosclerosis and diminished the extent of disease in an area of high lesion prevalence after 12 weeks of infusion at doses approaching physiological levels. A gross measure of general level of physical activity indicated no difference between the treatment groups, suggesting that the observed effects of OT were not mediated by changes in physical activity. The exact mechanism by which OT is exerting its anti-inflammatory and anti-atherogenic effects remains to be elucidated.

Taken together, the findings of the current study suggest that OT is capable of modulating pathophysiological mechanisms thought to be involved in the exacerbation of atherosclerosis in socially isolated animals. Results have also demonstrated that increased peripheral OT levels can slow the progression of the disease in animal models known to be influenced by social environment. This finding, together with studies demonstrating that positive social contact can increase peripheral OT levels, provides the possibility that OT may be partially responsible for protecting socially-housed animals

from atherosclerotic lesion development. However, the relevance of exogenous administration of relatively high doses of OT to endogenously produced responses to prosocial contact remains unclear. The current study sought to definitively determine whether OT could demonstrate a protective effect through a peripheral mechanism. A supra-physiological dose (estimated plasma steady state concentration of approximately 100-300 pg/mL) was intentionally selected to maximize the potential treatment effect in the periphery without causing direct activation of CNS pathways. Future research must demonstrate that positive social behavior is associated with sustained activation of peripheral oxytocinergic systems in order to further establish this mechanism as a potential mediating factor in the effect of social environment on atherosclerosis.

Summary

The findings of the current study demonstrate that OT dose-dependently inhibits pro-inflammatory cytokine (IL-6) production from macrophages and endothelial cells *in vitro* and leads to sustained decreases in basal IL-6 secretion from visceral adipose tissue from OT treated apoE$^{-/-}$ mice *ex vivo*. After 6 weeks of OT treatment, these same apoE$^{-/-}$ show decreased circulating levels of the pro-inflammatory complement component, CRP, and after 12 weeks they have diminished atherosclerosis lesion area within the thoracic aorta. None of these differences were attributable to changes in plasma lipids, insulin, physical activity levels, or stress hormones. Taken together these findings suggest that activation of a peripheral, oxytocinergic anti-inflammatory pathway through systemic OT treatment leads to decreased atherogenesis in socially-isolated animals. This supports the

hypothesis that chronic increases in peripheral OT through positive social interactions could be partially responsible for the protective effect of positive social environment on atherosclerosis.

References

1. Shively CA, Clarkson TB, Kaplan JR. Social deprivation and coronary artery atherosclerosis in female cynomolgus monkeys. Atherosclerosis. 1989;77(1):69-76.

2. McCabe PM, Gonzales JA, Zaias J, Szeto A, Kumar M, Herron A, Schneiderman N. Social environment influences the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Circulation. 2002;105:354-359.

3. Paredes J, Szeto A, Levine JE, Zaias J, Gonzales JA, Mendez AJ, Llabre MM, Schneiderman N, McCabe PM. Social experience influences hypothalamic oxytocin in the WHHL rabbit. Psychoneuroendocrinology. 2006;31:1062-1075.

4. Bernberg E, Andersson IJ, Gan LM, Naylor AS, Johansson ME, Bergström G. Effects of social isolation and environmental enrichment on atherosclerosis in ApoE-/- mice. Stress. 2008;11(5):381-9.

5. Rosvall M, Engström G, Hedblad B, Janzon L, Berglund G. Area social characteristics and carotid atherosclerosis. Eur J Public Health. 2007;17(4):333-9.

6. Whiteman MC, Deary IJ, Fowkes FG. Personality and social predictors of atherosclerotic progression: Edinburgh Artery Study. Psychosom Med. 2000;62(5):703-14.

7. Vogt TM, Mullooly JP, Ernst D, Pope CR, Hollis JF. Social networks as predictors of ischemic heart disease, cancer, stroke, and hypertension: Incidence, survival, and mortality. J Clin Epidemiol. 1992;45:659.

8. Ruberman W, Weinblatt E, Goldberg J, Chaudhary BS. Psychosocial influences on mortality after myocardial infarction. N Engl J Med. 1984;371:552.

9. Rozanski A, Blumenthal JA, Kaplan J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy. Circulation. 1999;99(16):2192-217.

10. Nation DA, Gonzales J, Szeto A, Mendez AJ, Paredes J, Brooks L, Schneiderman N, McCabe PM. The effect of social environment on markers of vascular oxidant stress and inflammation. Psychosom Med. 2008;70(3):269-75.

11. Mohazzab-H KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary endothelium. Am J Physiol. 1994;266:H2568-H2572.

12. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest. 1993;86:2109-2116.

13. Zeiher AM, Drexler H, Wollschlager H, Just H. Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. Circulation. 1991;84:1984-1992.

14. Chobanian AV, Haudenschild CC, Nickerson C, Drago R. Antiatherogenic effect of Captopril in the Watanabe Heritable Hyperlipidemic rabbit. Hypertension. 1990;15(3):327-331.

15. Aviram M. Modified forms of low density lipoprotein and atherogenesis. Atherosclerosis. 1993;98:1.

16. Jialal I. Evolving lipoprotein risk factors: lipoprotein and oxidized low-density lipoprotein. Clinical Chemistry. 1993;44(8B):1827-1832.

17. Getz GS. 2005. Thematic review series: the immune system and atherogenesis. J Lipid Res. 46(1):1-10.

18. Bleeke T, Zhang H, Madamanchi N, Patterson C, Faber JE. Catecholamine-induced vascular wall growth is dependent on generation of reactive oxygen species. Circ Res 2006;94:37-45.

19. Watson SL, Shively CA, Kaplan JR, Line, S.W. Effects of chronic social separation on cardiovascular disease risk factors in female cynomolgus monkeys. Atherosclerosis. 1998;137(2):259-66.

20. Itoh S, Yamada S, Mori T, Miwa T, Tottori K, Uwahodo Y, Yamamura Y, Fukuda M, Yamamoto K, Tanoue A, Tsujimoto G. Attenuated stress-induced catecholamine release in mice lacking the vasopressin V1b receptor. Am J Physiol Endocrinol Metab. 2006;291(1):E147-51.

21. Kaplan JR & Manuck SB. Antiatherogenic effects of β-adrenergic blocking agents : theoretical, experimental, and epidemiologic considerations. American Heart Journal. 1994;128(6):1316-1328.

22. Drescher VM, Gantt WH, Whitehead WE. Heart rate response to touch. Psychosom Med. 1980;42:559.

23. Fishman E, Turkheimer E, DeGood DE. Touch relieves stress and pain. J Behav Med. 1995;18:69.

24. Goebel MU & Mills PJ. Acute psychological stress and exercise and changes in peripheral leukocyte adhesion molecule expression and density. Psychosom Med. 2000;62(5):664-70.

25. Patronik GJ & Glickman LT. Pet ownership protects against the risks and consequences of coronary heart disease. Med Hypotheses. 1993;40:245.

26. Kamarck TW, Manuck SB, Jennings JR. Social support reduces cardiovascular reactivity to psychological challenge: a laboratory model. Psychosom Med. 1990;52:42.

27. Manuck SB, Kaplan JR, Clarkson TB. Atherosclerosis, social dominance, and cardiovascular reactivity. In: Schmidt T, Dembroski TM, Blumchen T, editors. Biological and Psychological Factors in Cardiovascular Disease. Berlin: Springer-Verlag, 1986:459.

28. Beere PA, Glagov S, Zarins CK. Retarding effect of lowered heart rate on coronary atherosclerosis. Science. 1984;226:180.

29. Berkson DM, Stamler J, Lindberg HA et al. Heart rate: an important risk factor for coronary mortality—ten-year experience of the Peoples Gas Co. In: Jones RJ, editor. Atherosclerosis.Proceedings of the Second International Symposium. Berlin:Springer-Verlag, 1970:382.

30. Kaplan JR, Manuck SB, Clarkson TB. The influence of heart rate on coronary artery atherosclerosis. J Cardiovasc Pharmaco. 1987;10(Suppl 2):S100.

31. Nerem RM, Levesque MJ, Cornhill JF. Social environment as a factor in diet-induced atherosclerosis. Science. 1980;27;208(4451):1475-6.

32. VanderLaan, PA., Reardon, CA., and Getz, GS. Site specificity of atherosclerosis: site-selective responses to atherosclerotic. Arterioscler Thromb Vasc Biol. 2004;24:12-22.

33. Pettersson K, Bejne B, Bjork H, Strawn WB, Bondjers G. Experimental sympathetic activation causes endothelial injury in the rabbit thoracic aorta via B1-adrenoceptor activation. Circ Res. 1990;67:1027-1034.

34. Kaplan JR, Manuck SB, Adams MR, Weingand KW, Clarkson TB. Inhibition of coronary atherosclerosis by propranolol in behaviorally predisposed monkeys fed an atherogenic diet. Circulation. 1987;76:1364-1372.

35. Strawn WB, Bondjers G, Kaplan JR, Manuck SB, Schwenke DC, Hansson GK, Shively CA, Clarkson TB. Endothelial dysfunction in response to psychosocial stress in monkeys. Circ Res. 1991;68(5):1270-9.

36. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation. 2006;113(15):1888-904.

37. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R.. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation. 2005;111(11):1448-54.

38. Harman-Boehm I, Bluher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E, et al. Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. J Clin Endocrinol Metab 2007;92:2240 –7.

39. de Ferranti S & Mozaffarian D. The perfect storm: obesity, adipocyte dysfunction, and metabolic Consequences. Clinical Chemistry. 2008;54(6):945–955.

40. Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, Dandona P. Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. Diabetes. 2003;52:2882–2887.

41. Campbell PJ, Carlson MG, Nurjhan N. Fat metab-adipocyte dysfunction and systemic effects review. Clinical Chemistry. 2008;54:6, 953.

42. Szmitko PE, Teoh H, Stewart DJ, Verma S. Am J Physiol Heart Circ Physiol. 2007. 292(4):H1655-63. Adiponectin and cardiovascular disease: state of the art?

43. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol. 2001;280:E745–E751.

44. Park HS, Park JY, Yu R. Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. Diabetes Res Clin Pract. 2005;69:29–35.

45. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259:87–91.

46. Libby P. Inflammation in atherosclerosis. Nature. 2002;420:868-874.

47. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003;112:1821–1830.

48. Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes. 2007;56(1):16-23.

49. Gonzales JA, Szeto A, Mendez AJ, Zaias J, Paredes J, Caperton CV, Llabre MM, Levine JE, Goldberg RB, Schneiderman N, McCabe PM. Effect of behavioral interventions on insulin sensitivity and atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Psychosom Med. 2005;67(2):172-8.

50. Laufs U, Wassmann S, Czech T, Munzel T, Eisenhauer M, Bohm M, Nickenig G. Physical inactivity increases oxidative stress, endothelial dysfunction, and atherosclerosis. Arterioscler Thromb Vasc Biol. 2005;25:809-814.

51. Wolden-Hanson T, Davis GA, Baum ST. Insulin levels, physical activity, and urinary catecholamine excretion of obese and non-obese rhesus monkeys. Obes Res 1993;1:5-17.

52. Palatini P, Pessina AC, Graniero GR, Canali C, Mormino P, Dorigatti F, Accurso V, Ferrarese E, Vriz O. et al. The relationship between overweight, lifestyle and casual 24-hour pressures, in a population of male subjects with mild hypertension. The results of the HARVEST study. G Ital Cardiol. 1995;25:977-989.

53. Ceriello A & Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arterioscler Thromb Vasc Biol. 2004;24(5):816-23.

54. Shinozaki K, Hirayama A, Nishio Y, Yoshida Y, Ohtani T, Okamura T, Masada M, Kikkawa R, Kodama K, Kashiwagi A. Coronary endothelial dysfunction in the insulin-resistant state is linked to abnormal pteridine metabolism and vascular oxidative stress. J Am Coll Cardiol. 2004;38(7):1821-8.

55. Kaplan JR, Adams MR, Clarkson TB, Koritnik DR. Psychosocial influences on female "protection" among cynomolgous macaques. Atherosclerosis. 1984;53, 283 295.

56. Kaplan JR, Adams MR, Clarkson TB, Manuck SB, Shively CA. Social behavior and gender in biomedical investigations using monkeys: studies in atherogenesis. Lab Anim Sci. 1991;41(4):334-43.

57. Szeto A, Nation DA, Mendez AJ, Dominguez-Bendala J, Brooks LG, Schneiderman N, McCabe PM. Oxytocin attenuates NADPH-dependent superoxide activity and IL-6 secretion in macrophages and vascular cells. Am J Physiol Endocrinol Metab. 2008; 295(6):E1495-501.

58. Argiolas A & Gessa GL. Central functions of oxytocin. Neurosci Biobehav Rev. 1991;15:217-231.

59. Kombian SB, Hirasawa M, Mouginot D, Pittman QJ. Modulation of synaptic transmission by oxytocin and vasopressin in the supraoptic nucleus. Prog Brain Res. 2002;139:235-46

60. Soironiew MW. Vasopressing and oxytocin in the mammalian brain and spinal cord. Trends Neurosci. 1983;6:467-472.

61. Mackenzie IZ. Induction of labour at the start of the new millennium. Reproduction. 2006;131(6):989-98.

62. Carter CS. Neuroendocrine perspectives on social attachment and love. Psychoneuroendocinology. 1998;23(8):779-818

63. Uvnäs-Moberg K. Oxytocin linked antistress effects--the relaxation and growth response. Acta Physiol Scand Suppl. 1997;640:38-42.

64. Uvnas-Moberg K, Petersson M. Oxytocin, a mediator of anti-stress, well-being, social interaction, growth and healing. Z Psychosom Med Psychother. 2005;51(1):57-80.

65. Kendrick KM, Da Costa AP, Broad KD, Ohkura S, Guevara R, Levy F, Keverne B. Neural control of maternal behavior and olfactory recognition of offspring. 1997;44(4):383-395.

66. Mezzakapa ES and Katkin ES. Breast-feeding is associated with reduced perceived stress and negative mood in mothers. 2002;21(2):187-193.

67. Light KC, Smith TE, Johns JM, Brownley KA, Hofheimer JA, Amico JA. Oxytocin responsivity in mothers of infants: a preliminary study of relationships with blood pressure during laboratory stress and normal ambulatory activity. Health Psychol. 2000;19:560-567.

68. Kendrick KM, Da Costa AP, Broad KD, Ohkura S, Guevara R, Levy F, Keverne B. Neural control of maternal behavior and olfactory recognition of offspring. 1997;44(4):383-395.

69. Petterson M & Uvnas-Moberg, K. Effects of an acute stressor on blood pressure and heart rate in rats pretreated with intracerebroventricular oxytocin injections. 2007

70. Uvnas-Moberg, Ahlenius V, Hillegaart V, Alter P. High doses of oxytocin cause sedation and low doses cause an anxiolytic-like effect in male rats. 1994;49(1):101-106.

71. Kitchen AH, Lloyd SM, Pickford M. Some actions of oxytocin on the cardiovascular system in man. Clin Sci. 1959;18:399-407

72. Katz RL. Antiarrhythmic and cardiovascular effects of synthetic oxytocin. Aesthesiology. 1964;25:653-661.

73. Petty MA, Lang RE, Unger T, Ganten D. The cardiovascular effects of oxytocin in conscious male rats. 1995;112:203-210.

74. Costa-e-Sousa RH, Pereira-Junior PP, Oliveira PF, Olivares EL, Werkeck-de-Castro JPS, Mello DB, Nascimento JH, Campos-de-Carvalho AC. Cardiac effect of oxytocin: is there a role for this peptide in cardiovascular homeostasis? Regul Pept. 2005;132:107-112.

75. Petterson M & Uvnas-Moberg, K. Effects of an acute stressor on blood pressure and heart rate in rats pretreated with intracerebroventricular oxytocin injections. 2007

76. Knox SS, Uvnäs-Moberg K. Social isolation and cardiovascular disease: an atherosclerotic pathway? Psychoneuroendocrinology. 1998;23(8):877-90.

77. Uvnas-Moberg K. Antistress pattern induced by oxytocin. New Physiol Sci. 1998;22-26.

78. Kotwica G, Staszkiewicz J, Skowroński MT, Siawrys G, Bogacka I, Franczak A, Kurowicka B, Kraziński B, Okrasa S.Effects of oxytocin alone and in combination with selected hypothalamic hormones on ACTH, beta-endorphin, LH and PRL secretion by anterior pituitary cells of cyclic pigs. Reprod Biol. 2006;6(2):115-31.

79. Uvnas-Moberg K. Physiological and endocrine effects of social contact: role of oxytocin. NY Acad. Sci. 1997;807:146-163.

80. Bjorkstrand E, Eriksson M, Uvnas-Moberg K. Evidence of a peripheral and a central effect of oxytocin on pancreatic hormone release in rats. Neuroendocrinology. 1996;63:377-383.

81. Dreifuss J, Raggenbass M, Charpak S, Dubois-Dauphin M, Tribollet E. A role of oxytocin in autonomic functions: its action in the motor nucleus of the vagus nerve. Brain Res. Bulletin. 1988;20:765-770.

82. Uvnas-Moberg K. Endocrinologic control of food intake. Nutr Rev. 1990 Feb;48(2):57-63

83. Petersson M, Lundeberg T, Uvnas-Moberg K. Oxytocin enhances the effects of clonidine on blood pressure and locomotor activity in rats. J. Auton. Nerv. Syst. 1999b;78:49-56.

84. Braga DC, Mori E, Higa KT, Morris M, Michelini LC. Central oxytocin modulates exercise-induced tachycardia. Am. J. Physiol. Integr. Comp. Physiol. 2000;278:R1474-R1482.

85. Gilbey MP, Coote JH, Fleetwood-Walker S, Peterson DF. The influence of the paraventriculo-spinal pathway, and oxytocin and vasopressin on sympathetic preganglionic neurones. Brain Res. 1982;251(2):283-90.

86. Neuman ID, Wigger A, Torner L, Holsboer F, Landgraf R. Brain oxytocin inhibits basal and stress induced activity of the hypothalamic-pituitary-adrenal axis in male and female rats: partial action within the paraventricular nucleus. J. Neuroendocrinol. 2000;12:235-243.

87. Higa KT, Mori E, Viana FF, Morris M, Michelini LC. Baroreflex control of heart rate by oxytocin the solitary-vagal complex. Am J Physiol Regul Integr Comp. Physiol. 2002;282;R537-R545.

88. Lancel M, Kromer S, Neumann ID. Intracerebral oxytocin modulates sleep-wake behaviour in male rats. Regul Pept. 2003;114:145-152.

89. Petterson M, Uvnas-Moberg K. Systemic oxytocin treatment modulates glucocorticoid and mineralocorticoid receptor mRNA in the hippocampus of rats. Neurosci Lett. 2003;343:97-100.

90. Martins AS, Crescenzi A, Stern JE, Bordin S, Michelini LC. Hypertension and exercise training differentially affect oxytocin and oxytocin receptor expression in the brain. Hypertension 2005;46:1004-1009.

91. Petterson M, Diaz-Cabiale Z, Fuxe K, Uvnas-Moberg K. Oxytocin increases the density of high affinity alpha 2-adrenoreceptors within the hypothalamus, the amygdata and the nucleus of the solitary tract in female ovariectomized rats. Brain Res. 2005;1049:234-239.

92. Uvnäs-Moberg K. Oxytocin may mediate the benefits of positive social interaction and emotions. Psychoneuroendocrinology. 1998;23(8):819-35.

93. Grippo AJ, Cushing BS, Carter CS. Depression-like behavior and stressor-induced neuroendocrine activation in female prairie voles exposed to chronic social isolation. Psychosom Med. 2007;69(2):149-57.

94. Romero LM, Levine S, Sapolsky RM. Adrenocorticotropin secretagog release: stimulation by frustration and paradoxically by reward presentation. Brain Res. 1995;676(1):151-6.

95. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. Physiol Rev. 2001;81:629-83.

96. Gutkowska J, Jankowski M, Lambert C, Mukaddam-Daher S, Zingg HH, McCann SM. Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart. PNAS. 1997;94:11704-11709.

97. Jankowski M, Hajjar F, Kawas SA, Mukaddam-Daher S, Hoffman G, McCann SM, Gutkowska J. Rat heart: A site of oxytocin production and action. PNAS. 1998;95:14558-14563.

98. Jankowski M, Wang D, Hajjar F, Mukaddam-Daher S, McCann SM, Gutkowska J. Oxytocin and its receptors are synthesized in the rat vasculature. PNAS. 2000;97:6207-6211.

99. Gutkowska J, Jankowsi M, Mukaddam-Daher S, McCann SM. Oxytocin is a cardiovascular hormone. 2000;33:625-633.

100. Grewen KM, Girdler SS, Amico J, Light KC. Effects of partner support on resting oxytocin, cortisol, norepinephrine, and blood pressure before and after warm partner contact. Psychosom Medicine. 2005;67(4):531-8.

101. Light KC, Grewen KM, Amico JA. More frequent partner hugs and higher oxytocin levels are linked to lower blood pressure and heart rate in premenopausal women. Biol Psychol. 2005;69(1):5-21.

102. Thibonnier M, Conarty DM, Preston JA, Plesnicher CL, Dweik RA, Erzurum SC. Human vascular endothelial cells express oxytocin receptors. Endocrinology 1999;140(3):1301-9.

103. Carter CS, Developmental consequences of oxytocin. 2003;79:383-397.

104. Petersson M, Lundeberg T, Sohlstrom A, Wilberg U, Uvnas-Moberg U. Oxytocin increases the survival of musculocutaneous flaps. 1998;357:701-704.

105. Petersson M, Wiberg U, Lundeberg T, Uvnäs-Moberg K. Oxytocin decreases carrageenan induced inflammation in rats. Peptides. 2001;22(9):1479-84.

106. Schierwagen C, Bylund-Fellenius A-C, Lundberg C. Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. J Pharmacol Meth 1990;23179-86.

107. Mazor R, Shurtz-Swirski R, Farah R, Kristal B, Shapiro G, Dorlechter F, Cohen-Mazor M, Meilin E, Tamara S, Sela S. Primed polymorphonuclear leukocytes constitute a possible link between inflammation and oxidative stress in hyperlipidemic patients. Atherosclerosis. 2007.

108. Iseri SO, Sener G, Saglam B, Gedik N, Ercan F, and Yegan BC. Oxytocin ameliorates oxidative colonic inflammation by neutrophil-dependent mechanism. 2005;26:483-491.

109. Popa C, Netea MG, van Riel PL, van der Meer JW, Stalenhoef AF. The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. J Lipid Res. 2007;48(4):751-62.

110. Gordon JR, Li F, Zhang X, Wang W, Zhao X, Nayyar A. The combined CXCR1/CXCR2 antagonist CXCL8(3-74)K11R/G31P blocks neutrophil infiltration, pyrexia, and pulmonary vascular pathology in endotoxemic animals. J Leukoc Biol. 2005;78(6):1265-72.

111. Williams KI, El Tahir KEH. Effects of uterine stimulant drugs on prostacyclin production by the pregnant rat myometrium. I. oxytocin, bradykinin, and $PGF_{2\alpha}$. 1980;19(1):31-38.

112. Breil RC, Kunz S, Kidess E. Platelet function, coagulation and fibrinolysis during termination of missed abortion and missed labor by $PGF_{2\alpha}$ and oxytocin. Acta Obstet Gynecol Scand. 1979;58:361-364.

113. Hultgårdh-Nilsson A, Durbeej M. Role of the extracellular matrix and its receptors in smooth muscle cell function: implications in vascular development and disease. Curr Opin Lipidol. 2007;18(5):540-5.

114. Biyikli NK, Tugtepe H, Sener G, Velioglu-Ogunc A, Cetinel S, Midillioglu S, Gedik N, Yegen BC. Oxytocin alleviates oxidative renal injury in pyelonephritic rats via a neutrophil-dependent mechanism. Peptides. 2006;27(9):2249-57.

115. Işeri SO, Sener G, Saglam B, Gedik N, Ercan F, Yegen BC. Oxytocin protects against sepsis-induced multiple organ damage: role of neutrophils. J Surg Res. 2005;126(1):73-81.

116. Düşünceli F, Işeri SO, Ercan F, Gedik N, Yeğen C, Yeğen BC. Oxytocin alleviates hepatic ischemia-reperfusion injury in rats. Peptides. 2008;29(7):1216-22.

117. Tugtepe H, Sener G, Biyikli NK, Yuksel M, Cetinel S, Gedik N, Yegen BC. The protective effect of oxytocin on renal ischemia/reperfusion injury in rats. Regul Pept. 2007;140(3):101-8.

118. Spangelo BL, De Holl D, Kalabay L, Bond BR, and Arnaud P. Neurointermediate pituitary lobe cells synthesize and release interleukin-6 in vitro: effects of lipopolysaccharide and interleukin-1β. Endocrinology 1994;135(2):556-563.

119. Işeri SO, Gedik IE, Erzik C, Uslu B, Arbak S, Gedik N, Yeğen BC. Oxytocin ameliorates skin damage and oxidant gastric injury in rats with thermal trauma. Burns. 2007.

120. Zhou D, Kusnecov AW, Shurin MR, DePaoli M, Rabin BS. Exposure to physical and psychological stressors elevates plasma interleukin 6: relationship to the activation of hypothalamic-pituitary-adrenal axis. Endocrinology. 1993 133(6):2523-30.

121. Pavlov VA, Tracey KJ. The cholinergic anti-inflammatory pathway. Brain Behavior and Immunity. 2005;19:493-499.

122. Giebelen IA, van Westerloo DJ, LaRosa GJ, de Vos AF, van der Poll T. Stimulation of alpha 7 cholinergic receptors inhibits lipopolysaccharide-induced neutrophil recruitment by a tumor necrosis factor alpha-independent mechanism. Shock. 2007;27(4):443-7.

123. Parrish WR, Rosas-Ballina M, Gallowitsch-Puerta M, Ochani M, Ochani K, Yang LH, Hudson L, Lin X, Patel N, Johnson SM, Chavan S, Goldstein RS, Czura CJ, Miller EJ, Al-Abed Y, Tracey KJ, Pavlov VA. Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. Mol Med. 2008;14(9-10):567-74.

124. Wu JT, Kral JG. Ghrelin: integrative neuroendocrine peptide in health and disease. Ann Surg. 2004;239(4):464-74.

125. Blanks AM, Shmygol A, Thronton ST. Regulation of oxytocin receptors and oxytocin receptor signaling. Semin Reprod Med 2007;25:52-59.

126. Jovanović A, Jovanović S, Tulić I, Grbović L. Effect of oxytocin as a partial agonist at vasoconstrictor vasopressin receptors on the human isolated uterine artery. Br J Pharmacol. 1997;121(7):1468-74.

127. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 1988;240: 622-30.

128. Meir KS, Leitersdorf E. Atherosclerosis in the Apolipoprotein E–Deficient Mouse: A Decade of Progress. Arterioscler Thromb Vasc Biol. 2004;24;1006-1014.

129. Daugherty A, Rateri DL. Development of experimental designs for atherosclerosis studies in mice. Methods 2005;36:129-138.

130. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. J Lipid Res. 1995;36(11):2320-8.

131. Emeson EE, Shen ML, Bell CG, Qureshi A. Inhibition of atherosclerosis in CD4 T-cell-ablated and nude (nu/nu) C57BL/6 hyperlipidemic mice. Am J Pathol 1996;149:675-685.

132. Feingold KR, Grunfeld C. Role of cytokines in inducing hyperlipidemia. Diabetes 1992;41(Suppl 2):97-101.

133. Paul A, Ko KWS, Li L, Yechoor V, McCrory MA, Szalai AJ, Chan L. C-reactive protein accelerates the prorgression of atherosclerorsis in apolipoprotein E-deficient mice. Circulation. 2004;109:647–655.

134. Schreyer SA, Peschon JJ, Leboeuf RC. Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem. 1996;271:174-178.

135. Gupta S, Pablo AM, Jiang X, et al. IFM-gamma potentiates atherosclerosis in ApoE knock-out mice. J Clin Invest. 1997;99:2752-2761.

136. Dawson TC, Kuziel WA, Osahar TA, Maeda N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 1999;143:205-211.

137. Silence J, Lupu F, Collen D, Lijnen HR. Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. Arterioscler Thromb Vasc Biol. 2001;21:1440-1445.

138. Ohman MK, Shen Y, Obimba CI, Wright AP, Warnock M, Lawrence DA, Eitzman DT. Visceral adipose tissue inflammation accelerates atherosclerosis in apolipoprotein E-deficient mice. Circulation. 2008;117(6):798-805.

139. Okabe TA, Kishimoto C, Murayama T, Yokode M, Kita T. Effects of exercise on the development of atherosclerosis in apolipoprotein E-deficient mice. Exp Clin Cardiol. 2006;11(4):276-9.

140. Pellegrin M, Berthelot A, Houdayer C, Gaume V, Deckert V, Laurant P. New insights into the vascular mechanisms underlying the beneficial effect of swimming training on the endothelial vasodilator function in apolipoprotein E-deficient mice.Atherosclerosis. 2007;190(1):35-42.

141. Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, Tada K. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res. 1982;42:1530-6.

142. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer. 1980;26:171-6.

143. Dunzendorfer S, Lee HK, Soldau K, Tobias PS. Toll-like receptor 4 functions intracellularly in human coronary artery endothelial cells: roles of LBP and sCD14 in mediating LPS responses. FASEB J. 2004;18(10):1117-9.

144. Tsartsaris V, Cabrol D, Cabronne B. Pharmacokinetics of tocolytic agents. Clin Pharmacokinet. 2004;43(13):833-844.

145. Principles of pharmacology the pathophysiologic basis of drug therapy. Eds Golan DE, Tashjian AH, Armstrong EJ, Galanter JM, Armstrong AW, Arnaout RA, Rose HS. LippincottWilliams & Wilkins. Philadelphia, PA. 2005.

146. Circulatory physiology—the essentials. 3rd edition. Eds Smith JJ, Kampine JP. Williams & Wilkins, Baltimore MD, 1990.

147. Harper JM, Austad SN. Effect of capture and season on fecal glucocorticoid levels in deer mice (Peromyscus maniculatus) and red-backed voles (Clethrionomys gapperi). Gen Comp Endocrinol. 2001;123(3):337-44.

148. Touma C, Sachser N, Mostl E, Palme R. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. General and Comparative Endocrinology. 2003;130:267-278.

149. Hoch M, Eberle AN, Peterli R, Peters T, Seboek D, Keller U, Muller B, Linscheid P. LPS induces interleukin-6 and interleukin-8 but not tumor necrosis factor-alpha in human adipocytes. Cytokine. 2008;41(1):29-37.

150. Warnholtz, A., Nickenig, G., Schulz, E., Macharazina, R., Brasen, J.H., Skatchkov, M., Heitzer, T., Stasch, J.P., Griendling, K.K., Harrison, D.G., Bohm, M., Meinertz, T., Munzel, T. Increased NADH-oxidase—mediated superoxide production in the early stages of atherosclerosis. Circulation. 1999;99:2027-2033.

151. Karra R, Vemullapalli S, Dong C, Herderick EE, Song X, Slosek K, Nevins JR,West M, Goldschmidt-Clermont PJ, Seo D. Molecular evidence for arterial repair in atherosclerosis. Proc Natl Acad Sci U S A. 2005;102(46):16789-94.

152. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb. 1994;14(1):133-40.

153. Takayanagi Y, Kasahara Y, Onaka T, Takahashi N, Kawada T, Nishimori K. Oxytocin receptor-deficient mice developed late-onset obesity. Neuroreport. 2008;19(9):951-5.

154. Brooks et al unpublished findings.

155. Fantuzzi G, Mazzone T. Adipose tissue and atherosclerosis: exploring the connection. Arterioscler Thromb Vasc Biol. 2007;27(5):996-1003.

156. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr Pharm Des. 2008;14(12):1225-30.

157. Schiller NK, Kubo N, Boisvert WA, Curtiss LK. Effect of g-irradiation and bone marrow transplantation on atherosclerosis in LDL receptor–deficient mice. Arterioscler Thromb Vasc Biol. 2001;21:1674-1680.

158. Swirski FK, Mikael J. Pittet, Moritz F. Kircher, Elena Aikawa, Farouc A. Jaffer, Peter Libby, Ralph Weissleder. Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. PNAS. 2006;103(27):10340–10345.

159. Egan JJ, Saltis J, Wek SA, Simpson IA, Londos C. Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in adipocyte plasma membranes. Proc Natl Acad Sci U S A. 1990;87(3):1052-6.

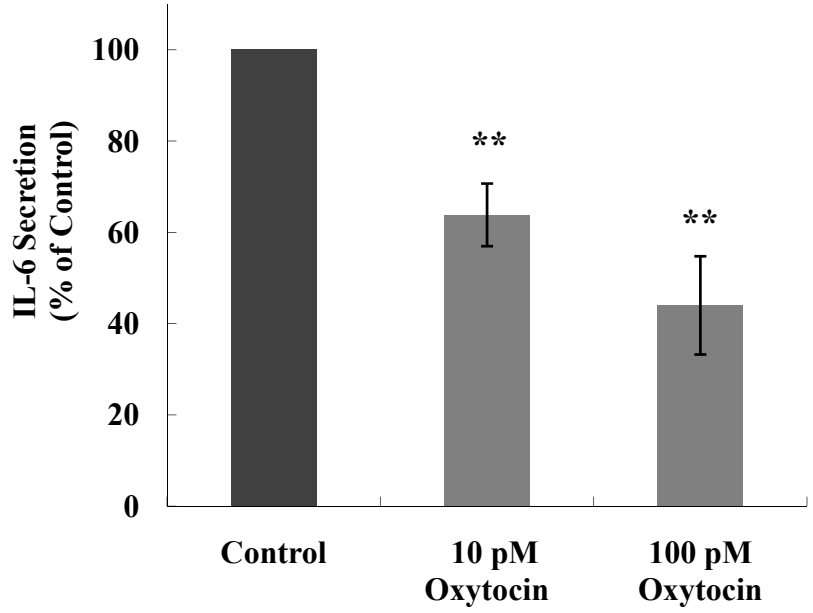
160. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R, Kadowaki T. Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. J Biol Chem. 2003;278(4):2461-8.

161. Chini B, Manning M, Guillon G. Affinity and efficacy of selective agonists and antagonists for vasopressin and oxytocin receptors: an "easy guide" to receptor pharmacology. Prog Brain Res. 2008;170:513-7.

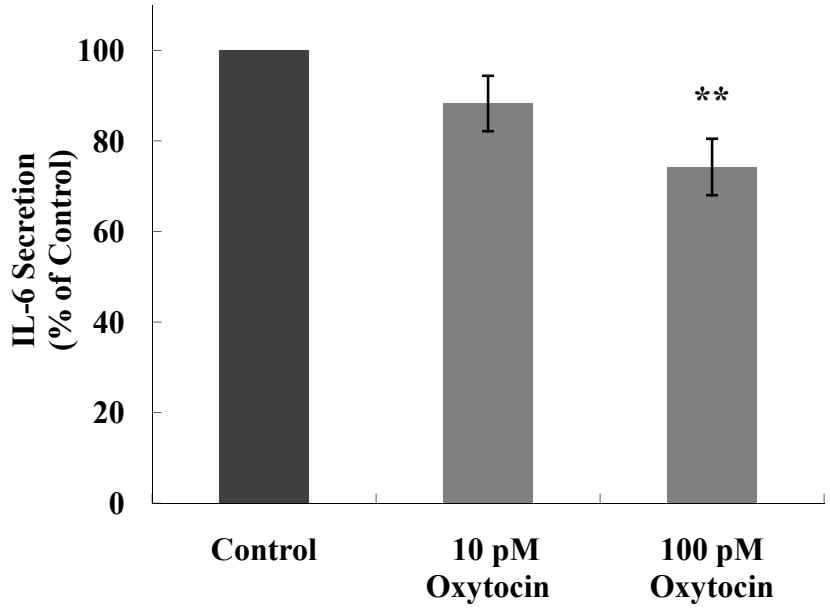
Cell Culture Studies

Figure 4.1 In the combined results of 3 separate experiments oxytocin demonstrated a dose-dependent reduction of LPS-induced IL-6 secretion over six hours in (A) THP-1 macrophages at 10pM and 100pM doses and in (B) HAECs at the 100pM dose.

A


IL-6 Secretion
(% of Control)
100
80
60
40
20
0
**
**
Control
10 pM
Oxytocin
100 pM
Oxytocin

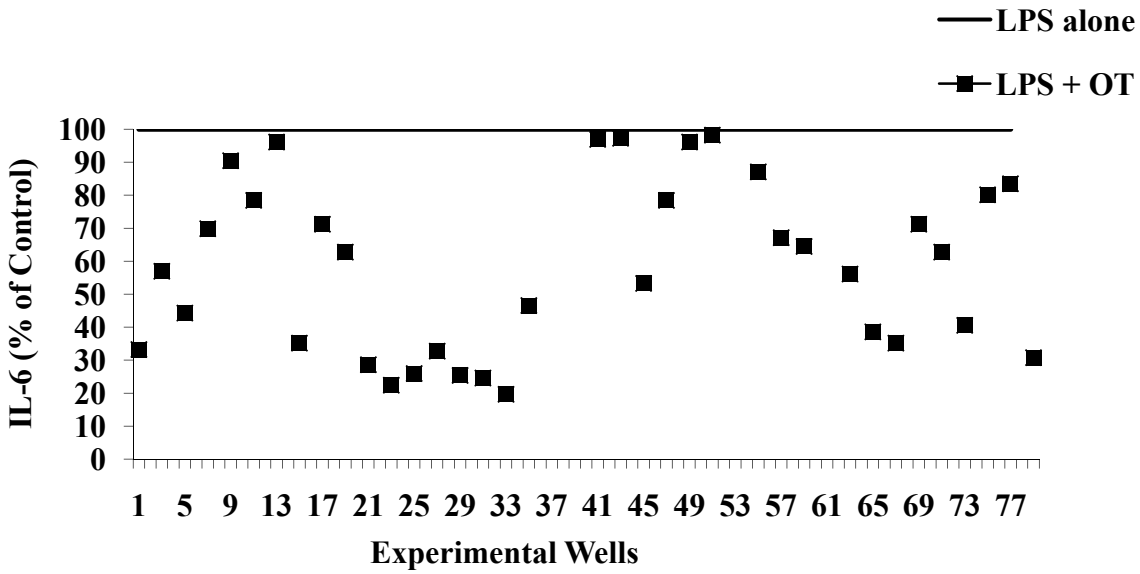
B


IL-6 Secretion
(% of Control)
100
80
60
40
20
0
**
Control
10 pM
Oxytocin
100 pM
Oxytocin

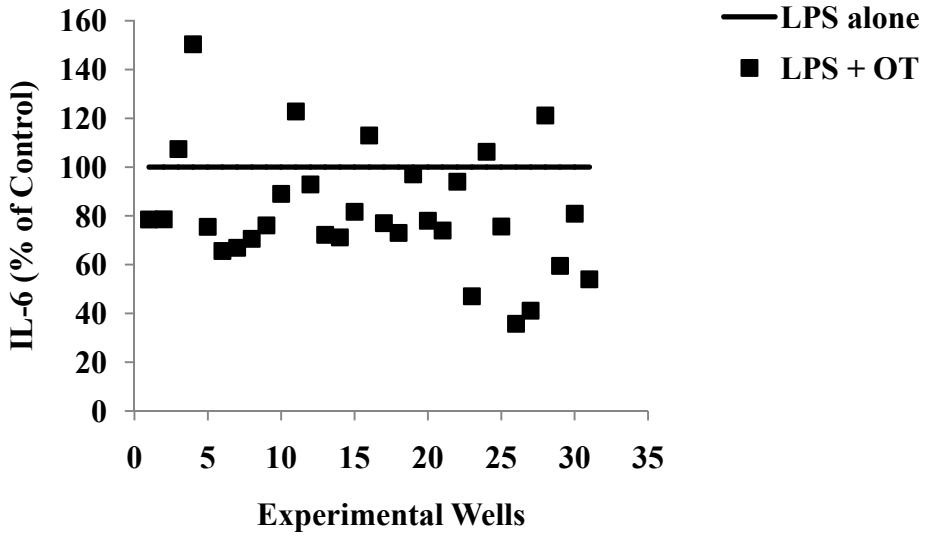
**$p<0.01$

Figure 4.2 IL-6 values expressed as percent of control for individual wells taken from every experiment regardless of dose are shown for (A) DTHP-1 macrophages and (B) HAECs.

A


LPS alone
LPS + OT
IL-6 (% of Control)
100
90
80
70
60
50
40
30
20
10
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77
Experimental Wells

B


LPS alone
LPS + OT
IL-6 (% of Control)
160
140
120
100
80
60
40
20
0
0 5 10 15 20 25 30 35
Experimental Wells

Pilot Study: Infusion of ^{3}H-Oxytocin

Figure 4.3 Extrusion of H^{3}-OT during 70hrs of pump priming *in vitro* at 37°C approximated expectations based on manufacturer specifications.

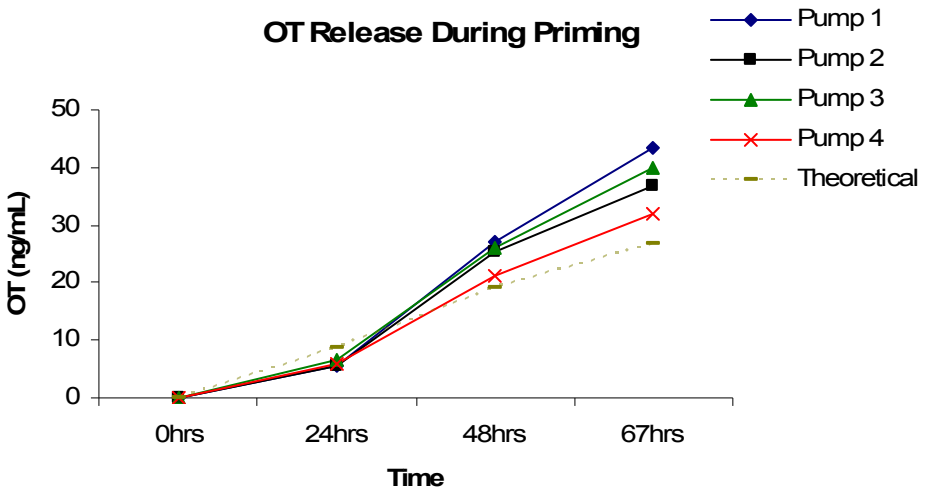
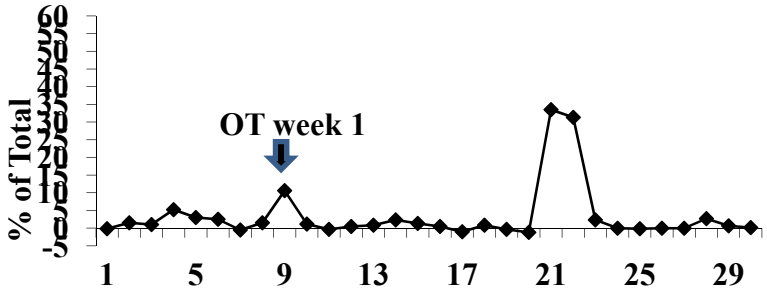
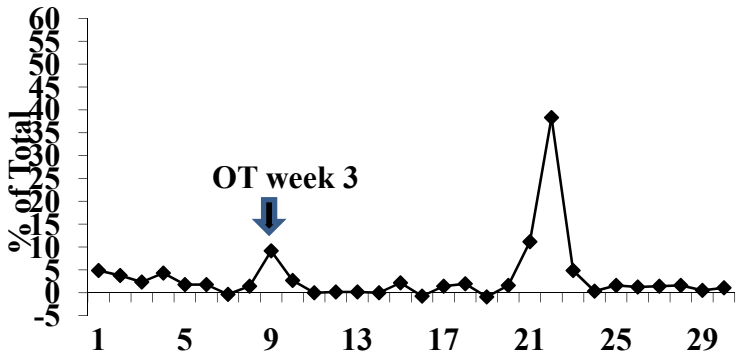


Figure 4.4 Semi-quantitative analysis of plasma OT concentrations by HPLC at (A) 1, (B) 3, and (C) 4 weeks in chronically infused animals revealed low values relative to presumed metabolites. (D) Comparison of estimated OT concentrations based on HPLC analysis indicated that OT concentrations dropped substantial from weeks 1 to 3 and remained at low levels through week 4.

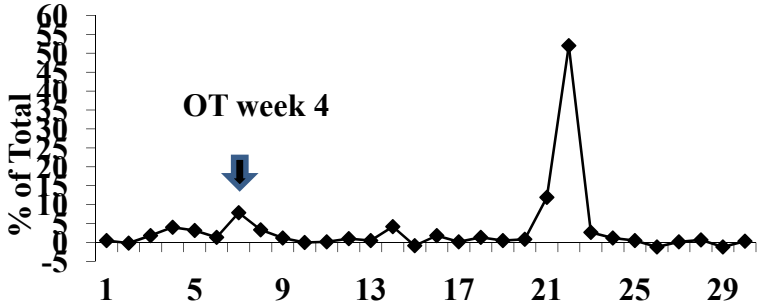
A


% of Total
OT week 1
1
5
9
13
17
21
25
29

B


% of Total
OT week 3
1
5
9
13
17
21
25
29

C


% of Total
OT week 4
1
5
9
13
17
21
25
29

D

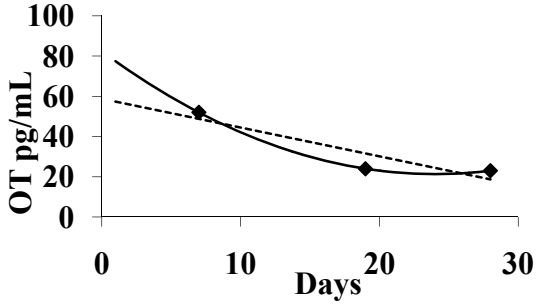
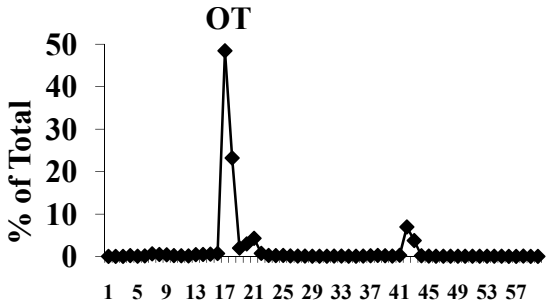
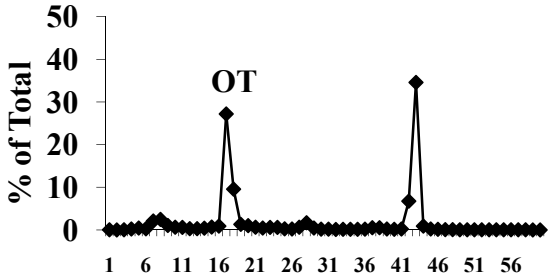

OT pg/mL
100
80
60
40
20
0
0
10
20
30
Days

Figure 4.5 HPLC analysis of pump contents after (A) 1, (B) 3, and (C) 4 weeks *in vivo* indicated that concentrations of intact OT decreased within the pump over time (D) and that presumed metabolite concentrations increased accordingly.

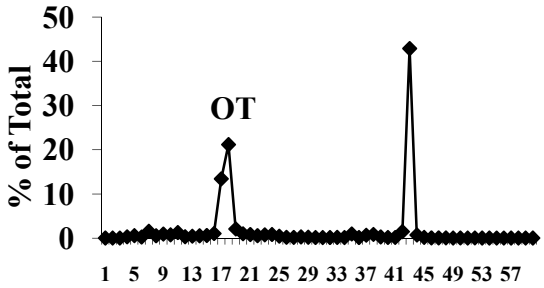
A


OT
% of Total
50
40
30
20
10
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57

B


OT
% of Total
50
40
30
20
10
0
1 6 11 16 21 26 31 36 41 46 51 56

C


OT
% of Total
50
40
30
20
10
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57

D

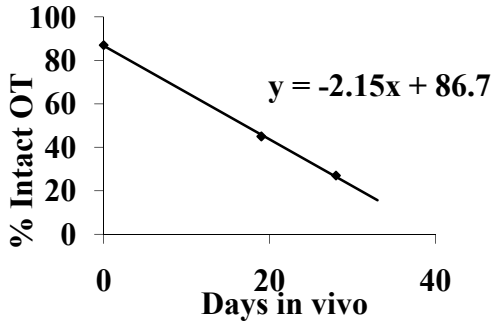
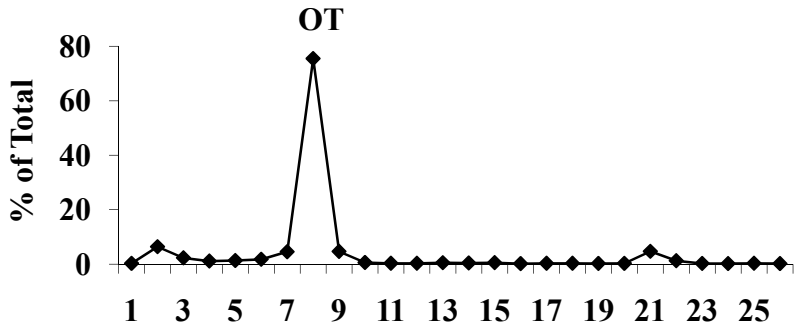
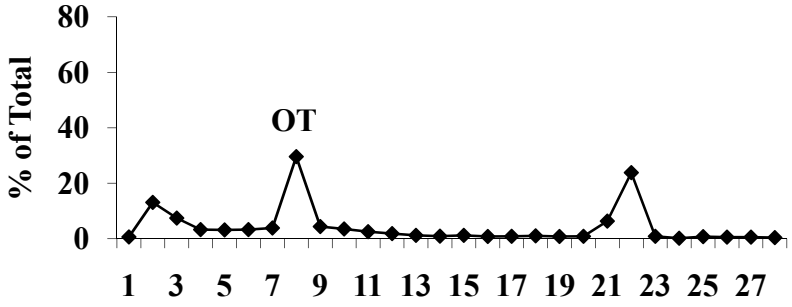

y = -2.15x + 86.7
% Intact OT
100
80
60
40
20
0
0
20
40
Days in vivo

Figure 4.6 HPLC analysis of pump contents at (A) 0 and (B) 4 weeks of incubation at 37°C *in vitro* revealed that OT concentrations diminished over time within the pumps and that presumed metabolite concentations increased accordingly.

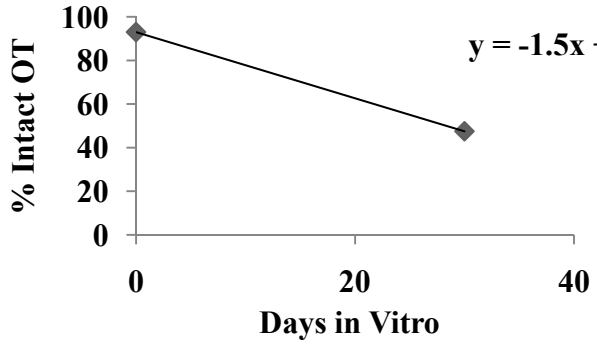
A



B



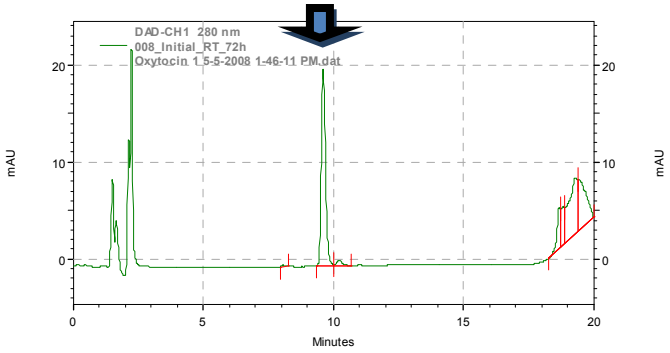
C



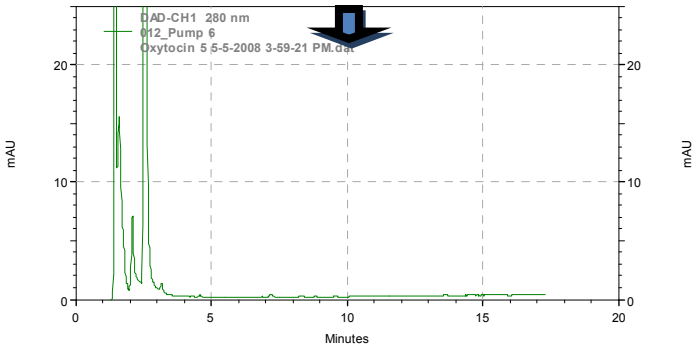
Infusion of OT into apoE$^{-/-}$ Mice

Figure 4.7 Comparison of OT concentrations from HPLC analyses of the initial pump solution (A), a pump from the vehicle control group (B), and a subset of pumps from the OT infusion group (C) indicated that OT in the pump remained approximately 80% intact after 6 weeks *in vivo*.

A


DAD-CH1 280 nm
008_Initial_RT_72h
Oxytocin 1 5-5-2008 1-46-11 PM.dat
mAU
Minutes

B


DAD-CH1 280 nm
012_Pump 6
Oxytocin 5 5-5-2008 3-59-21 PM.dat
mAU
Minutes

C

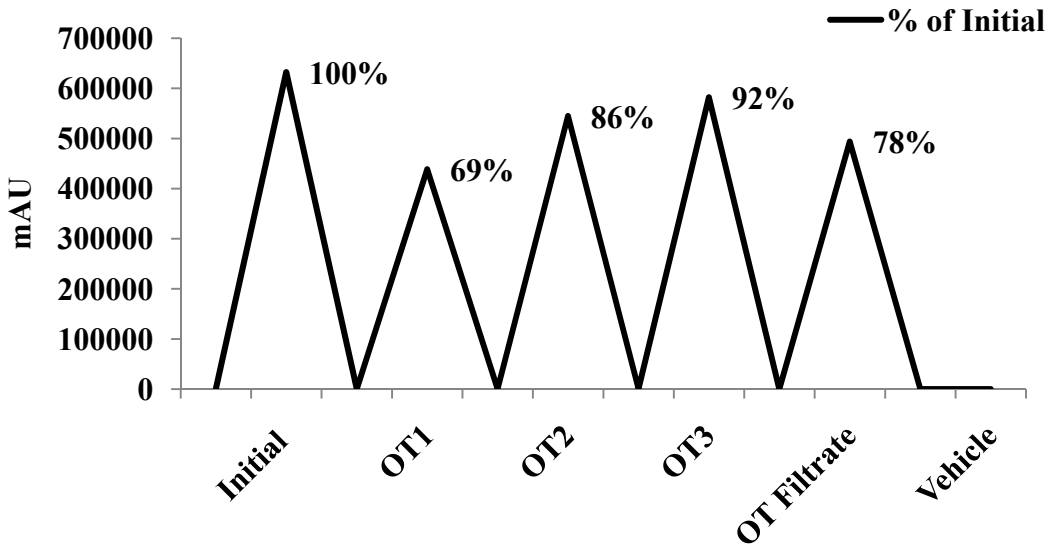

% of Initial
mAU
700000
600000
500000
400000
300000
200000
100000
0
100%
69%
86%
92%
78%
Initial
OT1
OT2
OT3
OT Filtrate
Vehicle

Figure 4.8 Animals chronically infused with OT showed significantly lower CRP (A) and adiponectin (B) levels after 6 weeks of treatment, but were not different at 12 weeks.

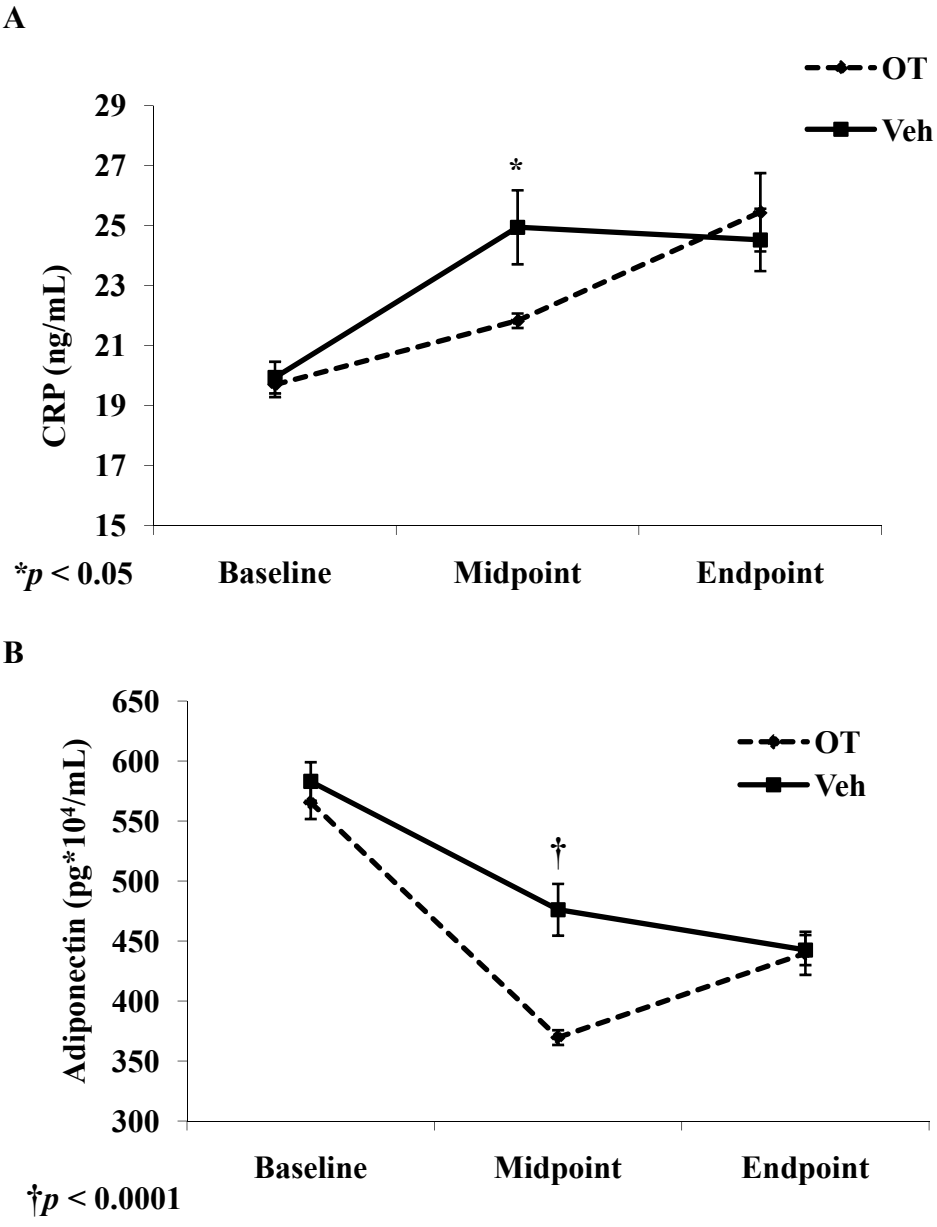


Figure 4.9 *Ex vivo* IL-6 secretion from adipose tissue was significantly lower in animals treated with OT for 12 weeks. Analysis of IL-6 secretion after stimulation with epinephrine or LPS revealed no significant group differences.

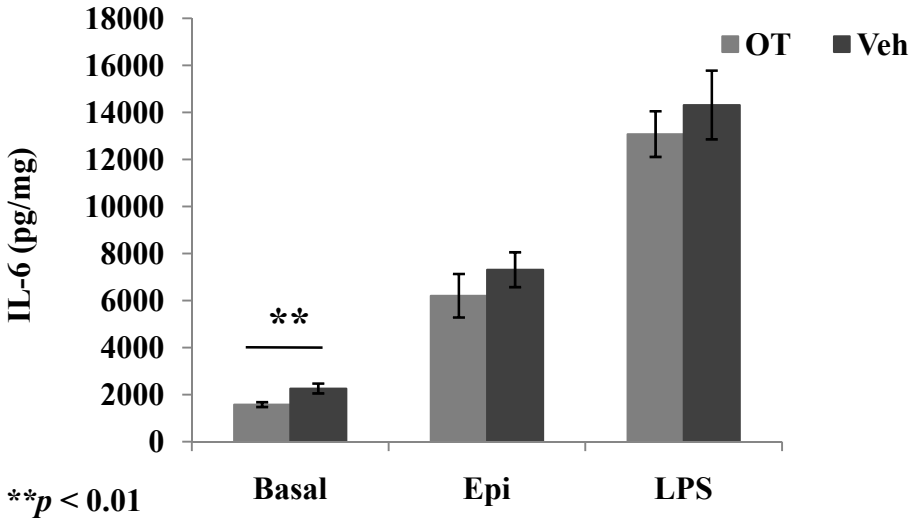
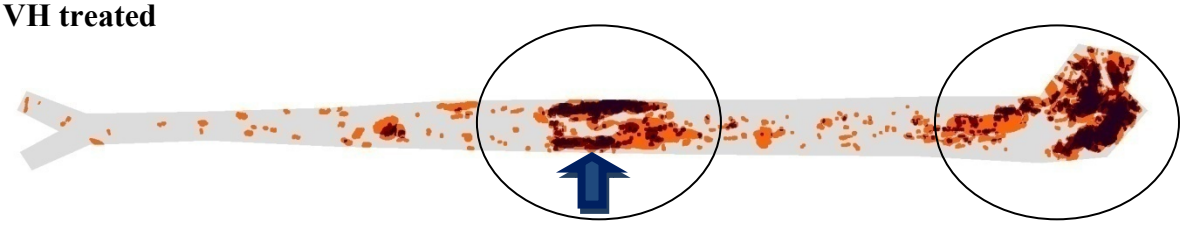


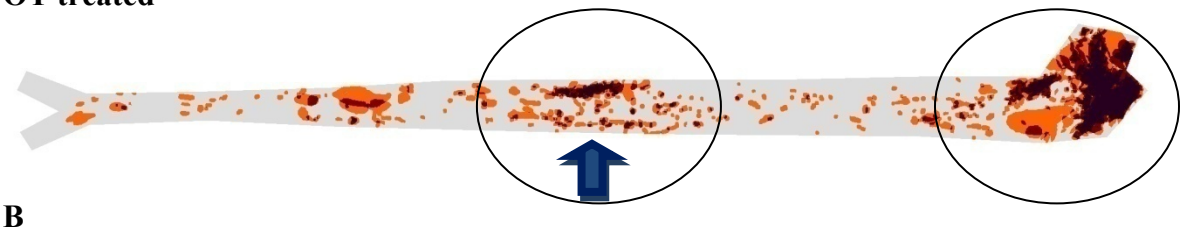
Figure 4.10 (A) Maps of lesion prevalence indicate that atherosclerosis displays a bimodal distribution along the aorta, with the majority of disease occuring at the aortic arch and a secondary region of increased lesion area near the renal bifurcation. Visual inspection of lesion prevalence maps suggests diminished atherosclerosis at the renal bifurcation in the OT treatment group. (B) Quantification of percent lesion area in these regions was achieved by overlaying a template aorta divided into 26 pixel counting regions and selecting the two principal areas of lesion prevalence.

A

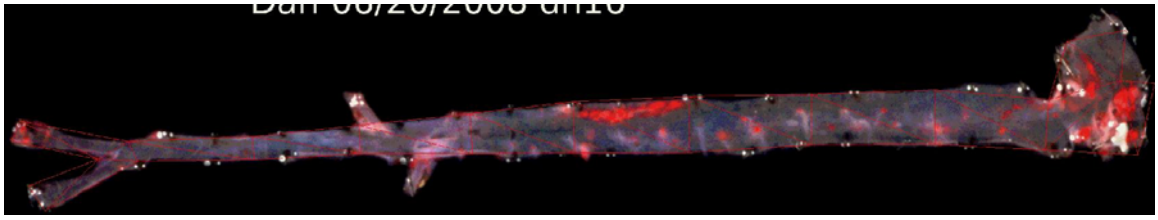
VH treated

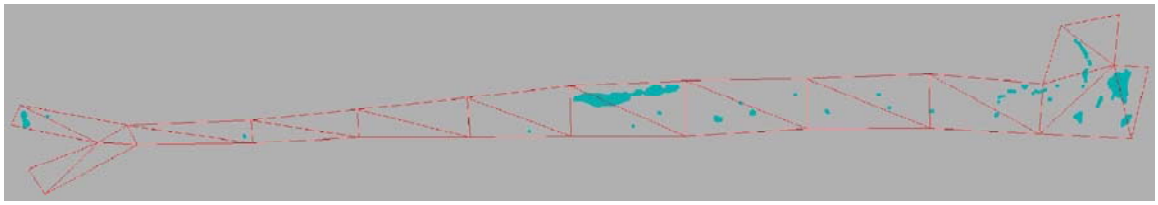


OT treated



B





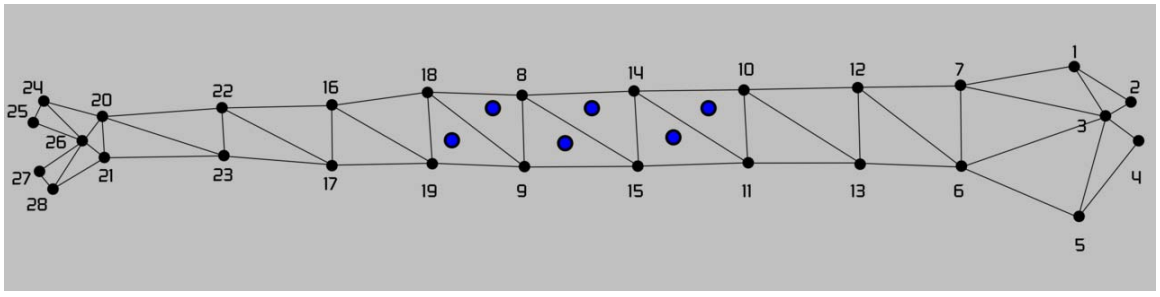


Figure 4.11 Comparison of treatment groups on percent lesion area in the aortic arch and thoracic aorta revealed statistically significant group differences in lesion area within the thoracic aorta.

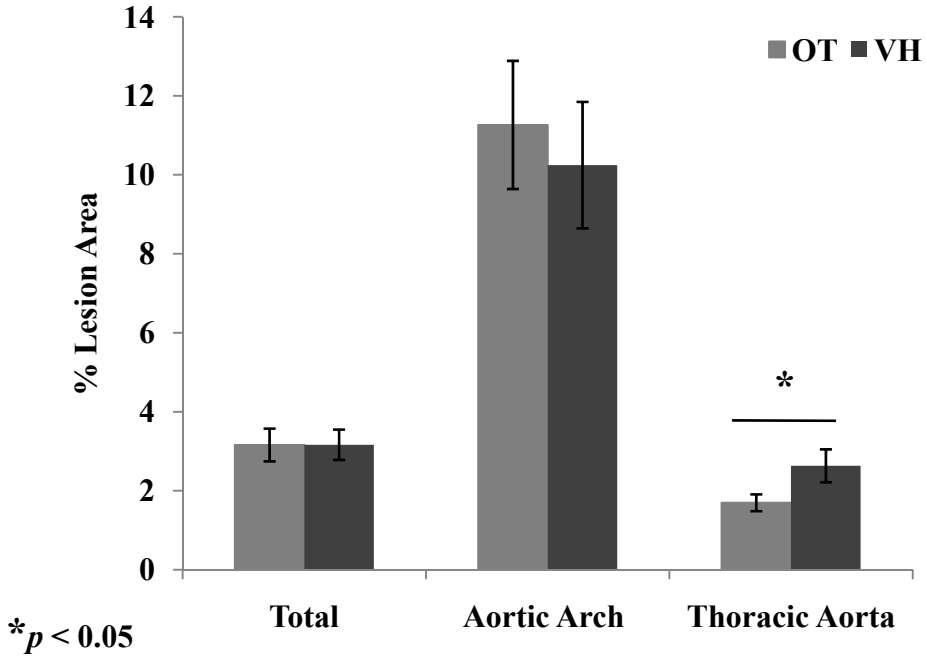


Figure 4.12 Baseline levels of plasma adiponectin predict total proportional lesion area.

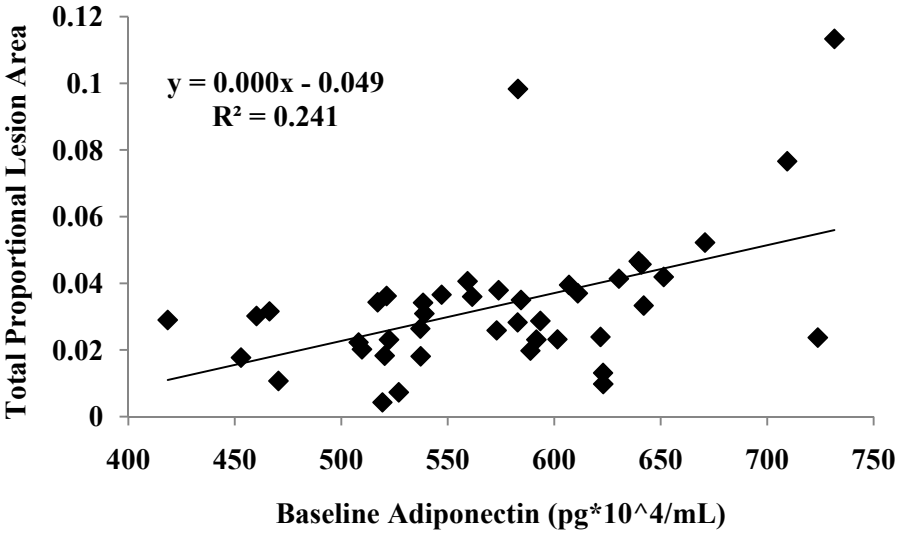


Table 4.1 Comparison of groups on potential risk factors over the 12 weeks of treatment.

	Vehicle	**Oxytocin**
Weight	g	
baseline	26.2 (±0.4)	26.0 (±0.4)
6 wks	29.7 (±0.4)	29.8 (±0.3)
12 wks	32.6 (±0.3)	32.0 (±0.3)
Cholesterol	mg/dL	
baseline	533.4 (±26.1)	520.2 (±25.5)
6 wks	455.7 (±19.6)	482.3 (±20.5)
12 wks	539.2 (±27.6)	514.1 (±23.8)
Triglycerides	mg/dL	
baseline	80.2 (±4.2)	75.8 (±4.5)
6 wks	86.0 (±8.4)	101.4 (±7.1)
12 wks	59.0 (±5.3)	51.7 (±3.2)
Insulin	pmol/L	
baseline	139.9 (±5.1)	138.9 (±3.5)
6 wks	166.3 (±10.8)	172.0 (±4.3)
12 wks	117.7 (±8.3)	99.3 (±10.9)
CRP	ng/mL	
baseline	20.2 (±1.6)	19.6 (±1.3)
6 wks	25.5 (±2.9)	22.3 (±1.9)
12 wks	24.4 (±1.6)	25.5 (±1.1)

Mean (±SD)