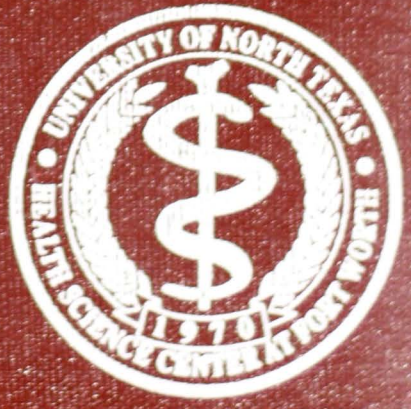
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Hypertension, diabetes mellitus and their presumed precursor the metabolic syndrome are part of a complex disease process associated with insulin resistance. Neurovascular complications in diabetics commonly involve the lower limbs resulting in a vicious cycle of autonomic neuropathy, painful occlusive claudication and resulting immobility that precipitates inactivity and progressive disability. The fixed neural and vascular diseases evolve slowly and the early events in this progressive decline in function are poorly understood. Sympathetic vasoconstriction is a major component of blood flow regulation in muscle. Active vasoconstriction in the lower limbs depends on continued transmission of efferent vasomotor signals through the lumbar sympathetic chain ganglia. Opioid receptors actively reduce normal ganglionic transmission presumably by lowering acetylcholine release. In the heart, the subtypes of delta-opioid receptors (DORs) facilitate (DOR-1, vagotonic) and inhibit (DOR-2, vagolytic) cholinergic transmission in the heart. The DOR-2 mediated inhibitory effects in heart are alterable and can change rapidly. Diabetes impairs vascular control. Ganglionic transmission is metabolically vulnerable during high fat feeding and insulin resistance. We hypothesized that the DOR-2 stimulation significantly facilitates vasodilation by reducing cholinergic transmission within the sympathetic chain ganglion. The ability to activate DOR-1 stimulation facilitates to cause further vasoconstriction in the anesthetized and surgically instrumented state of the dog did not show dose dependent activation. The DOR-1 activity in the insulin resistant dogs appears to be decreased as the DOR-1 blockade had no effect on the dose responses in the heart or the hindlimb.

Enhanced sympathetic tone through BCO by increasing and reducing cholinergic transmission in the lumbar sympathetic ganglion shows an enhanced pro-constrictor phenotype under stresses of severe hypotension possibly through a DOR-1 mediated activation.

DELTA OPIOID RECEPTOR PHENOTYPE MODULATION OF HINDLIMB VASCULAR CONDUCTION

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Forth Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Matthew A. Barlow, M.S.

Fort Worth, Texas

September 2008

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Hypertension, diabetes mellitus and their presumed antecedent the metabolic syndrome are part of a complex disease process. The American Diabetes Association estimates more than 20 million Americans have type II diabetes including an alarming increase in children and young adults. The devastating complications (renal, visual, neural and vascular) of diabetes are clearly disabling and prevalent. Neurovascular complications in diabetics commonly involve a decrease in blood flow to the lower limbs resulting in a vicious cycle of painful ischemic claudication, tissue damage and immobility that precipitates inactivity and progressive disability. The severity of the neurovascular pathology provides a clear rationale to determine the underlying mechanisms. Currently, surgical re-vascularization of the leg typically increases oxygen supply and nerve traffic to the affected limb but not the contralateral limb that is also likely to be compromised (63). The functional improvement following increased perfusion in the operated limb suggests that studies of non-surgical strategies that increase blood flow to both limbs simultaneously might be clinically important.

The sympathetic vasomotor ganglia which regulate peripheral vascular tone are obvious targets for control and possible adverse effects associated with hypertension and metabolic syndrome. Obesity and hyperinsulinemia are positively associated with a hyperadrenergic state. In a recent study, renal and cardiac NE spillover rates were

demonstrated to be significantly higher with obesity (15). Increased peripheral sympathetic nervous system activity is positively associated with body weight, BMI, and percentage of body fat as measured by muscle sympathetic nerve activity of the peroneal nerve (24, 25, and 58). Recent studies examining heart rate variability (HRV) in obese subjects suggest that obese people have a reduced overall power of HRV (36, 52). Power Spectral Analysis of HRV is a noninvasive tool for evaluating the cardiac autonomic nervous system activity which evaluates sympathetic and parasympathetic influences on heart rate. The high-frequency (HF) peak is a measure of the parasympathetic activity and the low-frequency (LF) component reflects a combination of the sympathetic and parasympathetic activity. In most studies the low to high frequency ratio (LF/HF) is then calculated as an index of sympathetic and vagal balance at the heart level. In obese individuals an increased LF/HF ratio indicated a predominance of SNS activity. This decrease in HRV was also observed in hyperinsulinemia in normal and obese subjects (56). Thus, we propose to better understand the mechanisms in the sympathetic vasomotor ganglion for maintaining normal vascular tone. Resting HR and the determination of HRV will verify a substantial decline in HF power commonly associated with the decreased vagal control of HR and the pathological changes that may occur during the progression of cardiometabolic disease.

The content of endogenous opioid peptides is decreased in diabetes and hypertension: The significance of decreasing the endogenous opioids in the hypertensive and diabetic states diminishes the ability to regulate the cardiovascular system. In animal studies using obese diabetic db/db mice, Timmers et al. reported alterations in the contents of opioid peptides in both the pituitary and the pancreas (53). In particular, met-

enkephalin content was inversely related to plasma insulin and pancreatic insulin content. This suggests there is a functional relationship of the insulin secretion and local enkephalins, which need to be further investigated. Additionally, met-enkephalin content was decreased in the hypothalamus and the pituitary in alloxan-diabetic rats and reversed based upon insulin treatment (51). Similar decreases in content were also reported in the small intestine of the diabetic rats. (26). Although, a direct met-enkephalin content measurement of the heart in a diabetic model is not reported in the literature, a recent article has shown an increased myocardial met-enkephalin in transgenic hypertensive mice (55). The changing content of the opioids in these animal models of disease may reflect maladaptions based upon the progression of the disease states.

The delta opioid receptors in obese diabetic mice increase glucose uptake into skeletal muscle: Skeletal muscle depends critically on its supply of energy from glucose metabolism and oxygen supplied by blood flow. However, in an insulin-resistant diabetic patient the uptake of glucose of skeletal muscle would be limited as insulin dependent Glut 4 receptors would be inactive. In a study of diabetic monkeys, the insulin-stimulated glucose disposal rates during euglycemic-hyperinsulinermic clamp studies were decreased. They attribute this pathological change to an insulin-dependent pathway with diminished levels insulin stimulated glucose disposal rates and activation of IRS-1-dependent PI3-kinase (49). Contradictory reports in the ob/ob obese diabetic mice indicated the glucose uptake is increased in leg skeletal muscle by activation of delta opioid receptors on the muscle surface (17). Evans et al. reported high numbers of opioid receptors present on the muscle cell surface by autoradiography. The radiolabeled

ligands bound to the surface receptors indicated a large population of DORs. When glucose uptake was tested, with selective DOR subtype agonists, only the DOR-1 agonist DPDPE increased the uptake of the 2-deoxy glucose (2-DOG) solution in to the leg skeletal muscle cells. Neither the DOR-2 selective agonist, Delt II, nor Kappa or Mu associated agonists showed any significant increase in 2-DOG. The contradictory evidence of glucose uptake in leg skeletal muscle of the diabetic animal may be species dependent. Interestingly, it is not mentioned if the DOR-1 receptors are functionally associated with the activity of Glut4 transporters in the skeletal muscle. Thus, the dysfunctional IRS-1-dependent PI3-kinase pathway may be inhibited or the cell surface DOR-1 activation increases during the progression of the cardiometabolic disease as a compensatory mechanism. The potential activation of DOR receptors during the initial stages of diabetes in other areas of the body needs to be addressed. In this regard, changes in DORs in the sympathetic chain ganglion may be early indicators of neurovascular changes.

Opioids and the Cardiovascular System: The endogenous opioids are effective regulators of cardiovascular function with significant influences in normal physiology and pathological conditions. The active opioid peptides MEAP, ME and LE modulate the vagus nerve producing a decrease in vagal transmission when introduced directly in the sinoatrial node tissue by microdialysis (19, 30, and 31). Opioids can increase or decrease the vagal transmission by binding to specific subtypes of the opioid receptors. Farias et al demonstrated that the DORs modulated vagal transmission bimodally in canine SA node (Fig 1). Lower concentrations (5×10^{-15} mol/min) of enkephalin

increased the stimulated vagal transmission and higher concentrations (5 X 10^{-12} mol/min) reduced the stimulated vagal transmission (18). The two opposing neural transmission effects were blocked by the selective subtype antagonists for each receptor implicating the two different DOR subtypes exist with opposing responses. The data in the canine model support the suggestion that the opioid receptors are on the prejunctional, postganglionic vagal nerve terminals of the sinoatrial node (6). In a recent study of myocardial ischemic tolerance, both dynorphins and enkephalins reduced subcellular and molecular indices of myocardial ischemic damage (43). An additional group investigating ischemic preconditioning in the heart concluded that preconditioning increased the bioactive forms of the enkephalins (62). In the heart, the enkaphalins modulate cardiac function in normal and pathological conditions.

Figure 1

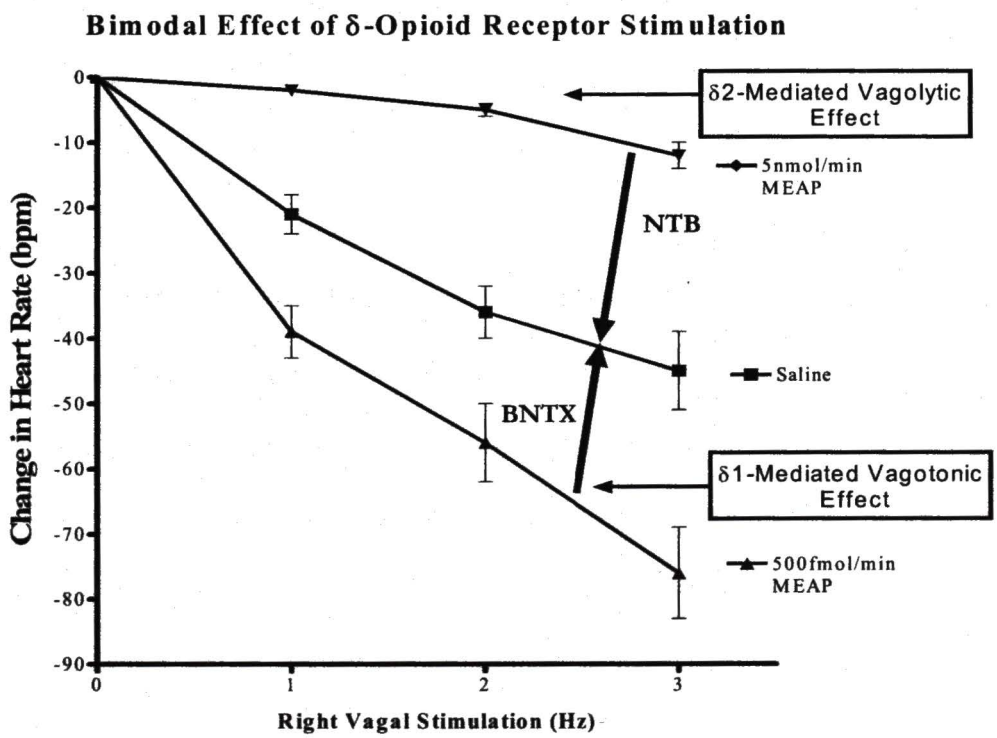


Figure 1. Vagotonic effect of low doses of methionine-enkephalin-arginine-phenylalanine (MEAP; *bottom curve*) on heart rate/frequency response relationships during right vagal stimulation and their reversal when combined with low doses of the DOR2-antagonist naltrindole (NTB) and the DOR1-antagonist BNTX. In this example, the dose rates for MEAP, naltrindole and BNTX were 500 fmol/min. The vagolytic effect (top curve) of higher dose rates (5nmol/min) of MEAP is also shown in the same animals. The Individual values represent means $\pm$ SE; $n = 5$. The *top* and *bottom curves* are significantly different from control. bpm, Beats per minute.

Ultra-low doses of MEAP (500 fmol/min) in to the SA node by microdialysis improves the stimulated vagal transmission. The selective DOR-1 antagonist, BNTX, blocked the DOR-1 mediated vagal enhancement. Infusion of higher doses (5nmol/min) of MEAP reduced vagal transmission. The reduced vagal transmission effect was blocked by a selective DOR-2 antagonist, naltriben. Thus, enkephalins act on the two phenotypes of δ-opioid receptor in a concentration dependent manner in the SA node.

In the heart the two opposing responses are consistent with observations made in dorsal root ganglion cells. Crain and Shen demonstrated both excitatory and inhibitory pathways in dorsal root ganglion (DRG) cells and suggested that opiate receptor polarity might shift between these modes. They suggested that the excitatory pathway increases the activity of the adenylyl cyclase-cAMP-PKA pathway by coupling with the stimulatory G-protein, $G_{s\alpha}$. The $G_{s\alpha}$ stimulates the activity of adenylyl cyclase converting ATP to cAMP and then activation of protein kinase A. Thus, stimulation of the Gs may

then lead to the prolongation of the action potential duration of the DRG cells followed by a subsequent increase in the neurotransmitter release (9, 43).

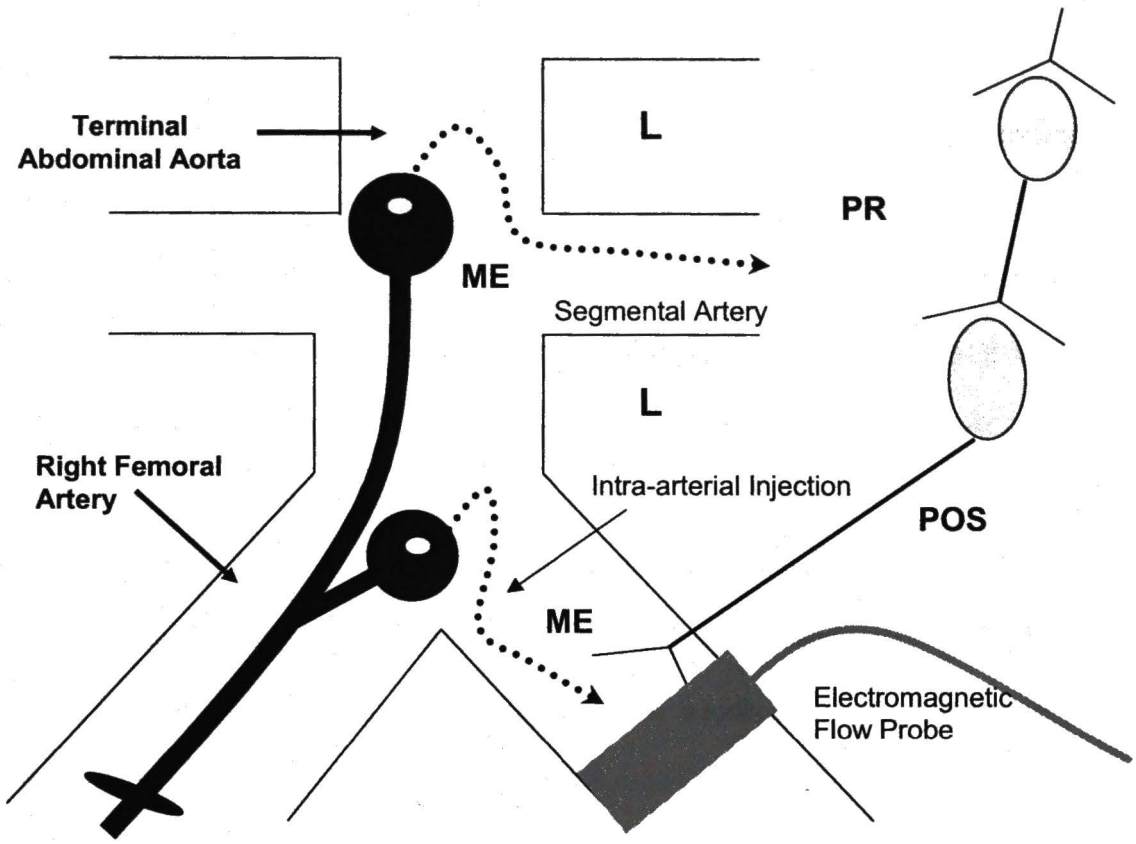
Opioids decrease blood pressure and increase vascular blood flow: Opioids have been associated with increased blood flow and decreased blood pressure. Holaday and Faden, suggested that endogenous opioid contributed to cardiovascular depression in circulatory shock. They demonstrated that animals treated with the non-selective opiate antagonist, naloxone, had improved circulatory function and prolonged survival time following endotoxin (28). Additional studies indicated similar results in other forms of hypotensive shock (2, 7, 10, 11, 44, and 57). The use of the non-selective opioid antagonist, naloxone only indicates that the hemodynamic response to the opioids involves an opioid receptor.

The mechanism through which the enkephalins and the delta opioid receptors reduce blood pressure and increase skeletal blood flow is not well defined. Caffrey et al. reported comparisons of the cardiovascular responses following administration of various opiate peptides. In general, the enkephalins commonly associated with the DORs, produced sharp hypotensive effects which were less evident with mu and kappa agonists (4). Enkephalin involvement in changing blood flow to the skeletal muscle was again confirmed in similar findings in rabbits (16, 59). There are conflicting results when comparing conscious animals and anesthetized animals. In a conscious animal the parasympathetic activity dominates and infusion of leu-enkephalin increased heart rate and blood pressure. However, in the anesthetized animals when the cardiovascular

function is dominated by the sympathetic nervous system, opiates produce hypotension and a decrease in heart rate (23, 46). The divergent hemodynamic responses may depend upon the current condition of the autonomic nervous system. In an open chest dog, the sympathetic outflow is elevated and thus the sympathetically mediated hypotensive effect of met-enkephalin injection is more prominent (40). As mentioned previously, a similar shift favoring the elevated sympathetic or lowered parasympathetic activity is also observed in diabetes and hypertension. Thus the DORs may have a higher responsiveness to enkephalins depending upon which limb of the autonomic nervous system is most prominent at that time of enkephalin exposure.

The location of the enkephalin receptors responsible for the proposed bidirectional responses in the skeletal muscle vasculature is important for understanding the normal regulation of vascular blood flow. Wightman et al. strongly argued that the mechanisms of the DOR's in question are based in the peripheral nervous system as opposed to a central nervous system location (59). Intravenous met-enkaphalin administrated during elevated sympathetic activity produced two to three times the greater hypotensive effect.

Figure 2



The peripheral component of the hypotensive response to intravenous met-enkephalin infusion was further addressed by our laboratory in the canine model (5). As illustrated in Figure 2, the previous investigation injected met-enkephalin (5 ug/kg) iv bolus into the jugular vein caused a decline in arterial pressure and a dramatic rise in hindlimb arterial blood flow. This increase in flow was not evident when met-enkaphalin was administered directly into the inter-arterial catherter of the internal iliac. The hyperemia was thus not a direct effect on the local vasculature of the skeletal muscle. The infusion of saline in the both areas had no effect. Injections of enkephalin produced the greatest decrease in resistance in the terminal abdominal aorta upstream from the

external iliac which in turn produced the least change in resistance. The intravenous infusion into the jugular vein was not as effective as that in the abdominal aorta indicating site of action was in the region of the terminal abdominal aorta above the branches to the iliac. Occusion of the segmental arteries branching off of the terminal abdominal aorta below the diaphragm blocked the ME mediated decrease in vascular resistance. To further test the injection pathway, a tracer dye was injected into the segmental arteries and tissue immediately dorsal to the aorta including the sympathetic chain ganglion at the L5 region was stained. Finally, blockade of the nicotinic receptors in the sympathetic chain ganglion was tested by injection of mecamylamine prior to met-enkephalin injection and the met-enkephalin response was significantly attenuated. Thus, the cholinergic synapse of the L5 sympathetic ganglion appears to by a major control site for the modification of ACh transmission which is modified influence of enkephalin.

Membrane associative proteins determine the pharmacological sybtypes of DOR: Crain and Shen proposed that the quality and sensitivity of the DOR response was governed by the ganglioside content of the neural cell membrane surrounding the opiate receptor. Membranes rich in the monosialosyl-ganglioside, GM1 favored excitatory opioid responses at very low doses. The excitatory response was further proposed to activate a positive feedback loop that increased its own excitatory activity by stimulating the synthesis of more GM1. Ultra-low opioid concentrations stimulate DOR-1 coupled through G_s to activate adenylyl cyclase. The hypothesis suggested that the resulting increase in the cyclic-AMP dependent protein kinase, phosphorylated glycosyl

transferase enzyme, and increased the synthesis of GM1. This increase in GM1 theoretically improved the efficiency of excitatory opioid receptor coupling and counteracted the inhibitory opioid receptor effects. In the absence of GM1 the same opioids reduced adenylyl cyclase activity through G_i/G_o-coupling (9). Thus, they suggested that the excitatory stimulation and the resulting changes in the environment around the receptor modified the response in isolated systems (61). A representation of the Crain and Shen model in the sympathetic chain ganglion is presented in Figure 3.

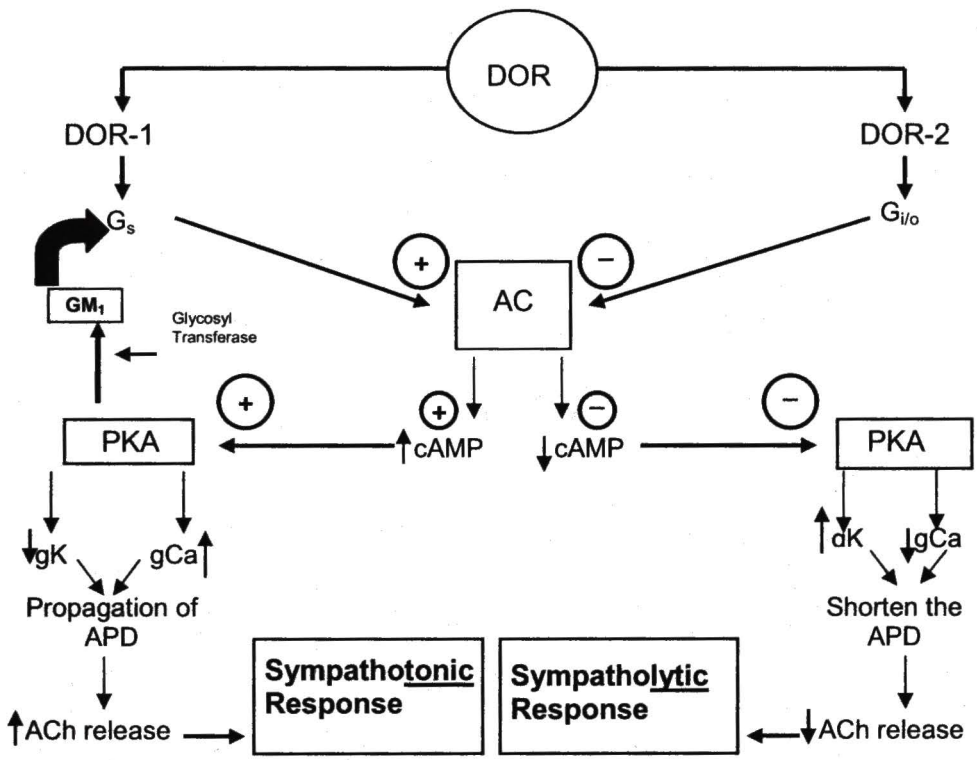


Figure 3: The figure illustrates how the opposing DOR interactions might translate into changes in neurotransmitter release. The DOR-1 subtype couples with the $G_{s\alpha}$ protein to activate the adenylyl cyclase enzyme (AC) and increase cyclic adenosine monophosphate (cAMP) concentration. cAMP in turn activates protein kinase A (PKA). PKA phosphorylates the voltage gated K^+ and Ca^{++} channels to increase Ca++ and decrease K+ conductance (gK and gCa). PKA also activates glycosyl transferase to increase the

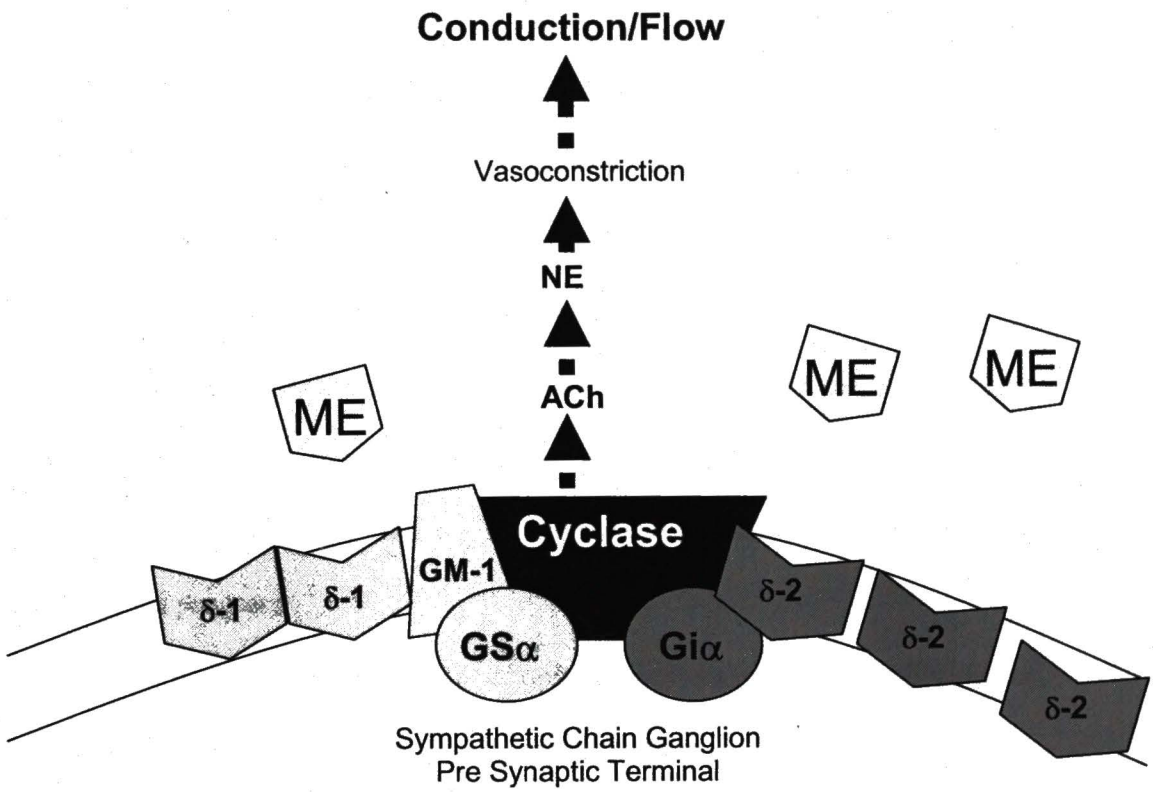
synthesis of neural membrane ganglioside (GM1). GM1 enhances DOR1 /$G_{s\alpha}$-coupling to complete a positive-feedback loop to prolong the action potential duration (APD) of the preganglionic sympathetic chain ganglion nerve membrane. Thus acetylcholine (ACh) secretion increases and sympathetic chain ganglion transmission increases. Reciprocal effects occur at the DOR-2 phenotype as it couples with the $G_{i/o}$ protein to cause inhibition of the AC. This leads to further reduction in all the components of the pathway shown in the figure to reduce the sympathetic transmission.

Disruption of the DOR associated proteins in the cellular membrane: A subtype inter-conversion during changes in the neural membrane environment might shift the balance of nerve transmission responses in favor of DOR-1 cardioprotective phenotype in the heart or a vasoconstrictive phenotype in the leg vasculature. Figure 4. illustrates the hypothesized functionally opposing DOR and the associated proteins that change the pharmacological subtype of the receptors in the cholinergic sympathetic chain ganglion. A single DOR transcript and two functionally opposing responses in the sympathetic chain ganglion indicate that an inter-conversion between the two subtype populations might be physiologically important. Cvejic et al. investigated that by increasing agoninst concentration on the DOR receptor in Dorsal root ganglion cells that there is an increase in the number of monomer forms of these receptors (12). In extracted atrial synaptosomes, Deo et al, confirmed through western blot analysis that there were two separate bands at the expected molecular mass indicating both monomer and dimers (14). These two studies have indicated that the dimmers and monomers might represent the functional DOR-1 and DOR-2 respecitvely. By using divalent ligands to target the

dimmer formation of the DOR there is a decrease in the monomer formation and internalization as monomerization precedes internalization. (42) The upregulation and internalization of the DOR can drastically change the nerve receptor balance and reduce the functional expression of one phenotype of the DOR and unmask or increase the expression of the opposing DOR phenotype. In a study of human aortic epithelial cells and atherosclerosis, it was observed that those cells with high exposure to oxidative low density lipoproteins (OxLDL) had a significantly greater stiffness, and higher force generation (3). Byfield et al. have shown that the exposure of the endothelial cells to the OxLDL induced changes in the endothelial cholesterol rich membrane domains. The cholesterol rich membranes make up the lipid rafts that contain the ganglioside GM1. Using cholera toxin to stain the membrane bound GM1 there is an indication in the endothelial cells that the lipid rafts have dispersed or that the OxLDL has induced internalization of the GM1. Although not reported in the current literature, the decease in GM1 in the sympathetic nervous system controlling vascular conduction could occur in insulin resistant and hyperglycemic animals for this same reason. This association has been reported in an animal model of diabetic peripheral neuropathy (33). Nerve growth factor in the sympathetic nerves is decreased in the streptozotocin induced diabetic rats (33). It is noteworthy that GM1 has been reported to enhance the activity of NGF and might have potential neuroprotective properties (50). Local infusion of exogenous GM1 by microdialysis into the SA node significantly increases vagal mediated vagotonic influence of femtomolar concentrations. The influence of the ganglioside also decreases the vagolytic namomolar concentrations through DOR influence modification of vagal transmission (13). Thus, the literature suggests that the membrane environment

associated with the DORs could dictate a shift from one subtype to the other. The resulting change in the balance between the two subtypes may prove to be pathologically important to the progression or recovery of peripheral vascular disease.

Figure 4



Conclusions:

1. Experimental shock improved when animals were treated with large doses of the opiate antagonist, naloxone.
2. IV administration of various opiate peptides acutely decreased blood pressure and they did so by altering sympathetic tone.
3. Sympathetic vasomotor ganglia of the lumbar region are major target sites for regulating flow in the lower limbs.
4. Changing the neuronal membrane environment in which the opioid receptors reside can modify the receptor associations including GM1 and the G-protein-coupled proteins (Gsα or Gi/Goα) to change the phenotype they express.
5. Cardiometabolic disease (insulin resistance syndrome, diabetes and hypertension) very likely changes how the opioid regulate neural traffic within the peripheral autonomic nervous system and could aggravate expression of the disease or accelerate its progress.
6. On the other hand, if the complement of opioid receptors can be favorably shifted, the disease may be responsive to positive modification

Clinical Significance:

In human diabetic patients, the occurrence of peripheral neuropathy and ischemic claudication are rising. The progressive peripheral artery disease often requires invasive surgery. Medical interventions have reduced the ischemic intermittent claudication (IC) in the general population however mortality rate of approximately 40 percent with in 10 years still persist (22). The proposed DOR pathway of increasing lower limb conduction

thus enhancing leg oxygen return and would decrease the leg discomfort in IC patients allowing them some pain relief. Thus by providing increased limb perfusion the patient would also increase mobility and reverse the downward spiraling effects toward disability.

HYPOTHESES

Specific Aim # 1 will test whether higher dose methionine-enkephalin (ME) stimulates DOR-2 receptors to increase femoral vascular conductance following intra-arterial ME.

Hypothesis 1A: Selective DOR-2 blockade with naltriben will eliminate higher dose ME mediated increases in femoral vascular conductance while selective DOR-1 blockade with BNTX is ineffective.

Hypothesis 1B: The selective DOR-2-agonist, deltorphin will duplicate the ME mediated increases in femoral vascular conductance while the selective DOR-1-agonist TAN-67 is ineffective.

Specific Aim#2 will test whether lower dose ME stimulates DOR-1 receptors to decrease vascular conductance in skeletal muscle following intra-arterial ME.

Hypothesis 2A: Selective DOR-1 blockade with BNTX will increase ME mediated femoral vascular conductance by eliminating opposing DOR-1-mediated vasoconstriction.

Hypothesis 2B: The selective DOR-1-agonist, TAN-67 will duplicate the ME mediated increases in femoral vascular conductance while the selective DOR-1-agonist BNTX is ineffective.

Specific Aim # 3: To test whether high fat feeding and insulin resistance lower DOR-2-mediated responses and raise DOR-1 mediated responses so as to express a more vasoconstrictive phenotype.

Peripheral vascular disease is an important component of diabetes and cardiometabolic disease. We know little about the early deviations from normal or when the early changes become fixed pathology. The novelty of this thesis arises from the unique evaluation of opioids and the little studied sympathetic ganglionic control of blood flow. The study of compensatory maladaptions during the early stages of the disease processes when correction or reversal may still be an option may be equally important.

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CHAPTER II

Femoral Artery Vascular Conductance Changes in the Presence of Enkephalin

Matthew A. Barlow, Shekhar Deo, and James L. Caffrey

Department of Integrative Physiology

Cardiovascular Research Institute

University of North Texas Health Science Center

3500 Camp Bowie Boulevard

Fort Worth, TX 76107

Corresponding author: James L. Caffrey

University of North Texas Health Science Center

Department of Integrative Physiology

3500 Camp Bowie Boulevard

Fort Worth, TX 76107

Tel. No. 817-735-2085

caffreyj@hsc.unt.edu

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ABSTRACT

Obstructive vascular disease and disabling leg pain in aging, diabetic, and smoking populations produce a progressive spiral of immobility, declining perfusion and muscle weakness. Sympathetic chain ganglia transmit vasomotor signals that regulate normal muscle blood flow. Enkephalins lower arterial blood pressure and increase regional blood flow through opioid receptors that appear to interrupt cholinergic transmission within these ganglia. Enkephalins in the sinoatrial node increase heart rate by interrupting vagal transmission through activation of the delta-2 subtype of the opioid receptor (DOR-2). The current investigation tested the hypothesis that enkephalin mediated increases in femoral conductance were mediated by similar ganglionic DOR-2 receptors. Graded pulses of met-enkephalin (ME) were administered (0.03ug/kg-10ug/kg) into the terminal aorta of anesthetized dogs just proximal to final segmental arteries, which perfuse the vasomotor ganglia that regulate femoral blood flow. Femoral vascular conductance increased immediately with an ED_{50} of 2.6×10^{-9} moles/kg. The selective DOR-2 antagonist, naltriben abrogated the hyperemic effect of ME with an ID_{50} of 1.4×10^{-9} moles/kg. DOR-1 blockade with 7-benzylidenenaltrexone (BNTX) was five fold less effective. The DOR-2 agonist, deltorphin II produced exaggerated increases in conductance and was also blocked by naltriben. DOR-1 blockade shifted the ME threshold left by one dose from 0.3 to 0.1ug/kg. ($p = 0.05$) suggesting a competing DOR-1 mediated constriction. Extended exposure to low dose BNTX gradually lowered the maximal ME mediated conductance by 30% suggesting that BNTX reduces the available pool of DOR receptors. Thus, enkephalin mediates its hyperemic effect through ganglionolytic DOR-2 receptors.

INTRODUCTION

Neurovascular complications in diabetic patients commonly involve the lower limbs resulting in a vicious cycle of autonomic neuropathy, painful occlusive claudication and eventual immobility that precipitates inactivity and progressive disability. The fixed neural and vascular diseases evolve slowly and the early events in this progressive decline in function are unclear. Sympathetic vasoconstriction is a major component of blood flow regulation in skeletal muscle. Active vasoconstriction in the lower limbs depends on continued cholinergic transmission of efferent vasomotor signals through the lumbar sympathetic chain ganglia. A number of potential neuromodulators including opioids and their respective receptors (14) populate the vasomotor ganglia responsible for the control the skeletal muscle blood flow. Opioid receptors actively reduce normal ganglionic transmission (3) presumably by lowering acetylcholine release. The specific character of these opioid receptors is unknown.

The literature suggests that opioids contribute to the peripheral vascular collapse associated with circulatory shock. Opiate receptor blockade with naloxone, improved arterial pressure and reversed shock like conditions in a number of experimental models of circulatory shock (1, 5, 6, 7, 15, 21, and 23). A series of elegant studies in dogs demonstrated that leucine-enkephalin produced hypertensive responses in conscious dogs and hypotensive responses in the same animals following anesthesia (20). A peripherally restricted opioid antagonist blocked these effects indicating the target opioid receptor was within reach of the peripheral circulation. The systemic administration of a variety of opioid peptides including enkephalins and dynorphins

produced similar sharp declines in blood pressure in anesthetized dogs (2). Much like anesthesia, reflexly increased sympathetic activity reversibly increased the magnitude of the hypotensive response suggesting that the enkephalins lowered blood pressure by opposing sympathetic activity.

Wightman et al. demonstrated that enkephalin produced a biphasic change in skeletal muscle blood flow in conscious rabbits (24). They suggested a direct effect on the vasculature or an indirect effect through the elimination of reflex sympathetic tone. Later work in dogs suggested that the hindlimb vasculature was unresponsive to enkephalin administered directly and that enkephalin interrupted transmission through the local lumbar sympathetic ganglia that innervated the hindlimb vasculature (3). The lumbar sympathetic chain ganglion receives blood through segmental arteries branching off the terminal aorta. Injection of enkephalin into the abdominal aorta just proximal to these segmental branches produced an immediate decrease in skeletal muscle vascular resistance. Injection directly into the femoral vasculature eliminated the response and as with the rabbit, ganglionic blockade eliminated the enkephalin mediated increase in blood flow (3, 24). Enkephalins modify parasympathetic control of heart rate from targets within the sinoatrial (SA) node where they produce a similar biphasic effect (11, 13, 16, and 17). Introduction of enkephalin by microdialysis into the sinoatrial (SA) node increased cholinergic transmission at femtomolar dose rates and inhibited cholinergic transmission at nanomolar dose rates. Local DOR-1 and DOR-2 receptors mediate respectively the opposing vagotonic and vagolytic effects (8, 11, and 13). The nature of the ganglionic opioid receptor is currently undefined but the influence of enkephalin on ganglionic transmission and the biphasic character of reported responses

suggested that subtypes of the DOR might also modify cholinergic transmission within the sympathetic ganglion (2, 20, and 24). The following studies evaluated the role of the DOR in femoral vascular control and tested the hypothesis that DOR-2 receptors increase femoral vascular conductance by interrupting ganglionic transmission and sympathetic vasomotor tone.

METHODS

The Institutional Animal Care and Use Committee approved all protocols in compliance with the NIH guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 85-23, revised 1996).

Surgical Preparation

Mongrel dogs of either gender weighing 15-30 kg were assigned at random to the experimental protocols. The animals were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and initially ventilated mechanically at 225 ml/min/kg with room air. A combination infusion catheter/pressure transducer (Millar Mikro-Tip) was introduced into the right femoral artery and positioned immediately upstream of the L5-L6 region. The catheter provided an arterial infusion port just proximal to origin of the L5 segmental arteries. The transducer provided a continuous measure of arterial pressure and heart rate (PowerLab; ADI Instruments, Colorado Springs, CO). A fluid filled catheter was inserted into the right femoral vein and advanced into the inferior vena cava to monitor central venous pressure. An electromagnetic flow probe (10 mm) placed around the isolated left femoral artery provided a continuous measure of femoral blood flow. The femoral arterial-venous pressure difference and femoral blood flow provided a continuous on-line recording of both femoral vascular resistance and conductance. Surface electrodes recorded a continuous electrocardiogram. The surgeons evaluated the anesthesia regularly and administered supplemental anesthetic as required. The acid-base balance and blood gases were determined with an Instrumentation Laboratories blood gas analyzer (Lexington, MA). The pO2 (90-120

mmHg), the pH (7.35-7.45), and the pCO2 (30-40 mmHg) were adjusted to normal by administering supplemental oxygen or bicarbonate or by modifying the minute volume.

The carotid arteries were isolated through a ventral midline incision and fitted with adjustable plastic snares to evaluate opioid interactions during sympathetic activation via bilateral carotid occlusion (BCO).

Materials

American Peptide (Sunnyvale, CA) supplied methionine-enkephalin and TAN-67 (2-methyl 4aa-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12a-octahydroquinolino[2,3,3-g]isoquinoline). Tocris Bioscience (Ellisville, MO) supplied the BNTX (7-benzylidenaltrexone), naltriben and deltorphin II.

Statistical Methods

All data were summarized as mean ± SEM. Within-group comparisons were made with a one way repeated measures analysis of variance (ANOVA). When the ANOVA indicated differences, multiple comparisons were made post-hoc, with Tukey's LSD or Dunnett's test. The student T-test analysis was used to compare between groups values. Differences were considered statistically significant when the probability they occurred by chance was $p < 0.05$ or less.

Protocol 1: ME Dose-Response. An initial ME dose response was conducted in each of 12 mongrel dogs. All peptides were introduced into the terminal aorta as bolus injections immediately upstream from the final (L5) pair of segmental arteries. A

standard 5 ml saline flush immediately followed each 1 ml vehicle or peptide injection. A seven step ME dose response (0.01μg/kg-10 μg/kg) was constructed. The hemodynamic effect of each dose was recorded and allowed to recover fully (5-10 min) before exposure to the next higher dose. On completing the first dose response, the system was permitted to re-equilibrate for 30 min. An identical second dose response was then conducted in a subset of four dogs to access reproducibility and/or the development of tolerance.

Protocol 2: DOR-1 and DOR-2 selectivity: Ten animals were assigned to this protocol. In each case, submaximal dose of ME (3.0μg/kg) was pulsed into the terminal aorta at approximately 10 min intervals while recording the femoral conductance. A cumulative dose response to either the DOR-1 antagonist, BNTX or the DOR-2 antagonist, naltriben (NTB) was then constructed in five animals each by administering increasing doses of antagonist into the aorta five min prior to each subsequent ME challenge. The relative ability of each antagonist to prevent the hyperemic effect of ME was then determined. The sequential ME challenges were repeated in three animals without the addition of antagonist to verify the temporal consistency of the ME response. ME was specifically selected relative to other potential DOR agonists because of its very rapid clearance from and/or degradation within the vascular space (2, 4).

Protocol 3: Evidence for DOR-1 participation in ME dose response: Two sequential seven step ME dose responses (0.01 ug/kg-10 ug/kg) were conducted in eight animals

as described in protocol 1. A dose of BNTX (0.3ug/kg) selective for the DOR-1 receptor was administered 5 min prior to beginning the second ME dose response. This protocol attempted to remove competing DOR-1 activity in order to demonstrate a shift in the second dose response curve to the left. A BNTX mediated leftward shift in the curve would indicate the removal of competition from a difficult to discern decrease in vascular conductance mediated by low doses of ME. The dose of BNTX was based on prior work indicating that the canine DOR-1 is $1X10^{10}$ times more sensitive to BNTX than the DOR-2 is to naltriben (11).

Protocol 4 Selective DOR-1 and DOR-2 agonist: After making control measurements in four animals, the native agonist ME (3.0μg/kg), the selective DOR-1 agonist, TAN-67 (3.0μg/kg), and the selective DOR-2 agonist, deltorphin II (0.3μg/kg) were tested in sequence at 10-20 min intervals. The doses of each were selected from prior experience and preliminary experiments. Then the DOR-2 character of the deltorphin response was tested by administering the selective DOR-2 antagonist, NTB (10.0μg/kg) and then re-administering the same dose of deltorphin.

RESULTS

Baseline Cardiovascular Indices: Table 1 summarizes the resting cardiovascular indices for each protocol. No differences were observed when comparing initial values among the four protocols. There were also no significant changes in baseline function during the course of each individual protocol.

Study 1 ME-ME Repeated Dose Response: Figure 1 illustrates a typical tracing following the injection of 1μg/kg ME into the terminal aorta. The vertical dashed line marks the point of injection followed by the abrupt increase in flow and conductance. The injection of ME gradually increased femoral vascular conductance as the dose increased with an apparent threshold between 0.3-1.0μg/kg and a maximum near 10μg/kg (Figure 2, filled circles). The last four doses were different from vehicle. Figures 3a-3d illustrate temporal changes in the potential contributory factors, heart rate, mean arterial pressure, femoral arterial blood flow, and femoral arterial resistance. Heart rate was largely unchanged at any dose (Figure 3a). Arterial pressure followed a biphasic pattern rising initially and then declining later in the response (Figure 3b). The dose responses for increasing femoral blood flow and declining femoral vascular resistance followed a similar dose relationship to conductance (Figure 3c and 3d). Flow increased rapidly and peaked at 40 seconds. Dose dependent declines in arterial pressure consistently trailed the changes in peripheral vascular hemodynamics by 10-20 seconds reinforcing the primacy of the effect on arterial resistance. Thus, the subsequent declines in arterial perfusion pressure presumably muted in part the magnitude and

duration of the change in flow. The injection artifact produced a consistent 3-5 mmHg increase in arterial pressure at 10 seconds that returned to baseline at 20 seconds. A dose related exaggeration and prolongation of the that increase in arterial pressure for an additional 20-30 seconds suggests the presence of a small sympathotonic effect of ME in advance of the more prominent sympatholytic effect. After 30 min washout and equilibrium, the dose response was repeated in four animals (open circles) to evaluate for tachyphylaxis. The two dose responses were comparable and not statistically different (Figure 2). The very short half-life of the peptide and the widely spaced injections may have obviated the rapid down regulation of opioid responses commonly observed with morphine. The carotid arteries were occluded bilaterally (BCO) just below the carotid sinuses to reversibly increase sympathetic activity. This produces a reversible increase in femoral vascular resistance and arterial blood pressure. When ME is administered during the BCO, the increase in femoral conductance was increased by approximately one third reinforcing the sympathetic dependence and sympatholytic character of the response (Figure 4).

Study 2 DOR-1 and DOR-2 selectivity: Study 2 was designed to test the hypothesis that ganglionic DOR-2 receptors are responsible for the enkephalin mediated increases in conductance. This was accomplished by comparing repeated ME mediated changes in conductance in the presence of increasing DOR-1 or DOR-2 blockade respectively with BNTX or NTB. A submaximal dose of ME (3.0μg/kg) selected from the dose response curve was administered into the terminal aorta at 10-15 min intervals. In each case, the response returned to baseline prior to the administration of the next dose. In

three animals, the ME was administered every 10 min without any other intervention as an injection/time control. The effect of ME was unchanged through five sequential injections. In the remaining animals, increasing doses of antagonist were administered into the aorta and equilibrated for 5 min prior to each subsequent ME challenge. The effects of DOR blockade on the integrated changes in conductance during each sequential ME injection are illustrated in Figures 5a. The DOR-2 antagonist, NTB (filled circles) quickly attenuated the increase in conductance with a threshold of 0.3μg/kg and an ID_{50} of 0.7μg/kg (1.4×10^{-9}moles/kg). The threshold and ID_{50} respectively for the DOR-1 antagonist, BNTX (open circles) were 3-6 times higher at 1.0 and 4.5μg/kg (8.6×10^{-9}moles/kg). The lowest dose of BNTX produced a non-significant but consistent small increase in conductance during ME injection suggesting DOR-1 mediated constrictor activity. BNTX has a much higher affinity for the canine DOR-1 receptor than its counterpart NTB has for the DOR-2 receptor reinforcing the DOR-2 character of the ganglionic effect. Based on prior studies, BNTX would saturate all DOR-1 receptors at the lowest dose employed (11). Naltriben alone had no effect on baseline femoral conductance but the lowest two doses of BNTX (0.3μg/kg) produced significant increases in conductance (figure 6) suggesting an immediate decrease in DOR-1 constriction. Other data that follow suggest that the BNTX mediated reduction in conduction at higher doses is not competitive inhibition of either DOR-1 or DOR-2 receptors but a time dependent sequestration of DOR-1 receptors at the expense of the total DOR pool.

The effect of enkephalin and selective DOR blockade were evaluated again during reflex sympathetic activation. A BCO was conducted after each dose of antagonist and ME was retested at the midpoint of the 90 second BCO. Enkephalin produced a 30% greater increase in conductance when administered during sympathetic activation. Naltriben quickly blocked the change in conductance with an ID_{50} near 0.7ug/kg and thus similar to that observed in the absence of increased sympathetic activation. The effect of BNTX was also very similar to that observed under resting conditions. Once again, the initial dose of BNTX (0.3ug/kg) produced a mild increase in conductance (figure 5b) during the injection of enkephalin suggesting that the activation of DOR-1 receptors may facilitate ganglionic transmission.

Study 3 Evidence for DOR-1 participation in ME dose response: This study systematically evaluated the presence of opposing DOR-1 mediated sympathetic facilitation during the DOR-2 conductance dose response curve. By eliminating baseline DOR-1 activity, BNTX should shift the ME conductance curve to the left. The dose of BNTX (0.3μg/kg) was selected since it did not reduce the ME response in study 2. The initial ME dose response curve (Figure 7) was comparable to the dose responses presented in Figure 2. The three highest doses of ME were all significantly different from the baseline saline injection. After 30 min equilibration, a single dose of BNTX was administered and the ME dose response was repeated. When the dose response reached 0.1μg/kg, a consistent though very modest increase in conductance was apparent compared to the prior dose. Thus, the threshold for increasing conductance was one dose earlier after DOR-1 blockade. The initial leftward shift was however, not

maintained, and the rising curve crossed over and finished significantly below the initial dose response. This dose of BNTX did not reduce conductance in study two when the ME was tested shortly after BNTX. However, when given sufficient time in this second experiment, BNTX shifted the late part of the ME dose response curve to the right and reduced the maximum response. This effect suggests that DOR-1 blockade reduces the number of DOR-2 receptors. Furthermore, the decline in the DOR-2 response after extended exposure to BNTX does not appear to be competitive inhibition since its negative influence is most effective at higher doses of agonist and less effective at lower doses when the competitive advantage is greatest. BNTX also eliminated the initial increase in arterial pressure during the first 30 seconds of the ME response (Figure 8). These data support the existence of DOR-1 mediated sympathotonic activity at low agonist concentrations and the likelihood that BNTX reduces available DORs through their sequestration in the as DOR-1 phenotype.

Study 4 Selective DOR-1 and DOR-2 agonists: Study 4 used subtype selective agents to verify the DOR-2 character of the ME mediated increase in femoral vascular conductance. The longer half-lives of the selective agonists, made full dose responses impractical. A submaximal dose of ME (3μg/kg) produced a consistent reference increase in femoral vascular conductance. The subsequent injection of an equimolar dose of the DOR-1 agonist, TAN-67 produced a small increase in conductance that was not different from control. Based on preliminary data, the dose of the DOR-2 agonist, deltorphin II was reduced ten fold to 0.3μg/kg. Even at this lower dose, deltorphin increased femoral conductance to nearly double that of ME and significantly higher that

the maximal response observed for ME in study one. The response to deltorphin recovered as quickly as that for ME suggesting that the difference in response was not the result slower metabolism or clearance from the target compartment. Following recovery to baseline, an ID90 (versus ME) dose (3 μg/kg) of the DOR-2 antagonist NTB was administered. NTB had no effect of its own but reduced the response to deltorphin by 60% reinforcing the DOR-2 character of the sympatholytic effect. Increasing the dose of NTB three fold reduced the response to deltorphin another 5% reinforcing the much higher efficacy of deltorphin versus ME. Thus, the hyperemic effects of the ME were reproduced by the DOR-2 selective agonist deltorphin and subsequently reduced by the DOR-2 antagonist, NTB. A ten fold higher dose of the DOR-1 agonist TAN-67 was largely ineffective.

DISCUSSION

The elegant central control of arterial pressure via reflex activation of efferent sympathetic vasomotor pathways is well documented. The endothelial and local neuroendocrine mechanisms that regulate the distribution of blood flow within the target vasculature have also been investigated in significant detail. By comparison, the regulation of blood pressure and/or the distribution of blood flow by moderating ganglionic transmission within the sympathetic ganglion are relatively unexplored. The hemodynamic consequences of ganglionic blockade are well described in any basic pharmacology text. The utility of these drugs however, is severely limited because of the non-specific character of the resulting interruption in both sympathetic and parasympathetic regulation. However, the very existence of the ganglia suggests that they are more than mere way stations in route to assorted peripheral sympathetic targets. The synaptic organization of the ganglia is complex and contains terminal fibers that are positive for a variety of neuromodulators including enkephalins (19). Some of these fibers form characteristic terminal baskets around the cell bodies of post-junctional motor nerves but many others do not. The enkephalin positive nerve endings are widely distributed within the lumbar ganglia and are seldom associated with the innervations of specific cell soma. This suggests that their actions may be largely pre-junctional. Another hierarchy of potential control results from the fact that the complement of neuromodulators differs among ganglia from different anatomical locations. For example, enkephalins are more abundant in the lumbar sympathetic chain ganglia (18, 22). The lumbar concentration of enkephalin suggests that

ganglionic opioid receptors may be particularly important for regulating the buffering capability of the large reserve of vascular capacitance in the lower limbs.

Opioid receptors are clearly capable of making significant changes in regulation of ganglionic transmission and the subsequent distribution of blood flow in the large muscle mass comprising the lower body. The rapid time course of the responses to intravascular enkephalin suggests that the ganglionolytic effect is faster yet since the disposal of norepinephrine at the vascular target is presumably slower than the hydrolysis of acetylcholine within the ganglion. Thus, DOR receptor activation provides the ability to make rapid high volume changes in the distribution of muscle blood flow. As such, the clinical implications for moderating leg blood flow are potentially important. DORs may be important for overriding central constrictor signals and reinforcing the regional demand for blood flow. Sensory afferent signals from areas of functional ischemia may orchestrate the regional flow of blood by short circuiting sympathetic signals at the ganglion. The pharmacologic manipulation of this system might be clinically helpful to those with sympathetically mediated hypertension or those with restricted flow or claudication in the lower extremities. The plasticity of opioid receptors further suggests that a shift in DOR phenotypes within the ganglion to favor DOR-1 or DOR-2 could contribute respectively to the development of hypertension or orthostatic hypotension. Pathologically, excess stimulation of the sympatholytic DOR-2 pathway might explain in part the role of endogenous opioids in circulatory shock. Understanding DOR dynamics may be particularly important in

determining the character of the opioid or opioid antagonist best used when circulatory collapse is in progress.

Exogenous enkephalins and the associated DOR are capable of dramatically modifying sympathetic ganglionic transmission and increasing femoral conductance in dogs and rabbits (3, 24). The present study provides evidence that higher doses of ME produce a robust DOR-2 mediated increase in femoral conduction most likely through the intra-ganglionic inhibition of acetylcholine secretion (3). This effect would be very similar to the vagolytic effect of DOR-2 activation in the SA node (11). The sympathotonic effect of lower enkephalin is more difficult to demonstrate perhaps due to existing sympathetic activation in the anesthetized animal or the prior activation of the DOR-1 pathway by easily achieved local endogenous enkephalin secretion. Nonetheless, a small progressive increase baseline vascular resistance and subsequent arterial pressure occurred as the dose of ME was increased. DOR-1 blockade also produced a clear increase in baseline femoral vascular conduction and small though not significant increase in enkephalin mediated vascular conduction. The concentration of endogenous enkephalin present in anesthetized animals might be sufficient to produce a DOR-1 mediated constriction and mask the DOR-1 response to exogenous enkephalin. The pharmacological evaluation of DORs in the canine SA Node supports the existence of both DOR-1 and DOR-2 phenotypes in the dog (11). The receptor protein was co-localized with cholinergic nerve terminals and isolated cardiac cholinergic synaptosomes (9). The DOR-1 responses were 10,000 fold more sensitive to enkephalin than their companion DOR-2 responses in the same tissue. BNTX and NTB

blocked the DOR-1 and DOR-2 responses respectively. The differences in sensitivities to antagonist were remarkable with DOR-1 receptors being several orders of magnitude more sensitive to BNTX than DOR-2 receptors were to NTB. The parallels between the nodal and ganglionic findings suggest that similar DOR interactions may be at work at cholinergic junctions within the sympathetic ganglion. In each case, the DOR-2 receptor interrupts cholinergic transmission and is sensitive to blockade by nanomolar doses of NTB. DOR-1 activation in both tissues appears to do the opposite and facilitate cholinergic transmission. Like the vagotonic effect in heart, the sympathotonic effect occurs at lower enkephalin doses, is less robust and is sensitive to sub-nanomolar doses of BNTX. The higher sensitivity of the DOR-1 responses may indicate that basal opioid activity within the ganglion normally supports vascular resistance and the ability of higher concentrations to increase flow represents an acute adjustment to sudden changes in demand. An increase in basal opioid concentrations and/or sympathetic tone in the anesthetized animal may contribute to the practical difficulty in demonstrating the sympathotonic response in that model.

The third study was designed to demonstrate the existing constrictive phenotype of DOR-1 activation as stimulated by low dose ME. The studies discussed above clearly demonstrated the DOR-2 sympatholytic phenotype. However, the hypothetical DOR-1 constrictor response was difficult to demonstrate directly. The experiment proposed to demonstrate the constrictor response indirectly by selective blockade that would be evident in greater vasodilation and a shift in the ME dose response up and to the left. The lowest dose of BNTX appeared to improve ME mediated increases in

conductance. The inhibition of competing constrictor activity presumably would shift the conductive dose response curve to the left, particularly at the low end of the ME dose response. BNTX eliminated the initial ME mediated increase in arterial pressure in support of a DOR-1 mediated increase in vasomotor transmission. As predicted, BNTX shifted the ME threshold dose, one dose lower (0.1 vs 0.3μg/kg), however, contrary to expectation the curve then shifted right and downward. The reduction in the maximal response strongly suggests that BNTX reduces the number of available DOR-2 receptors. If simple competitive inhibition of the DOR-2 receptor was at work, BNTX should have been more effective when the agonist dose was lower. Thus, BNTX appears to foster the depletion of DOR-2 receptors as the time of exposure increases. The same dose of BNTX produced no loss of conduction after 5 min (Figure 4) but produced a substantive change after 60 min (Figure 6). This supports the thesis proposed by Deo (10) that the two DOR phenotypes are in dynamic equilibrium and that BNTX sequesters the DOR-1 receptors. As DOR-2 receptors gradually reestablish the subtype equilibrium, the high affinity BNTX continues to divert the repleted DOR-1 receptors and slowly depletes the DOR-2 ranks until their numbers are insufficient to produce the maximal response.

The current findings and prior work in the SA Node supports the hypothesis that DOR receptor trafficking and inter-conversion is physiologically important. The loss of the DOR-2 response in the sympathetic ganglion following extended exposure to BNTX suggests that plasticity among subtypes may be an important determinant of muscle blood flow.

The forth protocol used selective DOR agonists to verify the DOR-2 character of the ME mediated increase in femoral vascular conductance. In this regard the DOR-2 agonist, deltorphin was very effective and the DOR-1 agonist, TAN-67 was ineffective. Deltorphin much like ME, was blocked by the DOR-2 antagonist, naltriben. The failure of TAN-67 to produce a constrictor response does not rule out a DOR-1 mediated constriction. The dose of TAN-67 was selected for comparison with ME to rule DOR-1 participation in the ganglionolytic effect. However, the vagotonic effect of enkephalin in the heart wanes with increasing doses (11) and thus additional studies including complete dose responses with assorted DOR-1 agonists like TAN-67 and/or DPDPE will be required to verify the sympathotonic DOR-1 pathway..

In conclusion, ganglionic DOR-2 stimulation increases femoral vascular conductance. Support for an opposing DOR-1 mediated increase in ganglionic transmission though present was less convincing. If as in heart, the DOR-1 receptors are far more sensitive to agonist, endogenous enkephalin concentration within the ganglion may mask the effect of exogenously administered enkephalin. Clinically, the ability to target and increase the DOR-2 or inhibit the DOR-1 in the ganglion to increase femoral blood flow may provide an alternate therapeutic target to increase femoral perfusion, reduce leg pain and improve mobility in subject populations with occlusive or spastic peripheral vascular disease.

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LEGENDS

Figure 1: Figure 1 illustrates a typical tracing following the injection of 1μg/kg ME into the terminal aorta.

Figure 2: The figure illustrates integrated changes in femoral conductance mediated by increasing bolus injections of ME (0.01-10.0 μg/kg) into the terminal aorta in two sequential dose responses. The symbol (++) indicates the change in the conductance was significantly different from control at $P<0.01$. Values are means and standard error of the mean for 12 and 4 subjects in the first and second dose responses.

Figure 3: Figures 3a-3d illustrate dose dependent changes in heart rate, mean arterial pressure, femoral resistance, and femoral flow for 90 seconds after ME injection into the terminal aorta. The symbol (+) indicates that the response was significantly different from saline injection at $P<0.05$. Values are means and standard error of the mean for 12 subjects.

Figure 4: The figure compares ME (3.0μg/kg) mediated increases in femoral vascular conductance during resting hemodynamic conditions and during reflex sympathetic activation mediated by 90 seconds bilateral carotid occlusion. Values are means and standard error of the mean for 12 subjects; (*) indicates $p < 0.05$.

Figure 5: Figures 5a and 5b illustrate the effects of the DOR-1 and DOR-2 blockade on ME mediated increases in femoral vascular conductance, respectively under resting

hemodynamic conditions (5a) and during reflex sympathetic activation (5b). Values are means and standard error of the mean for 7 subjects in each group. The symbol (*) indicates significant differences from ME alone. The symbol (**) indicates the significant differences between DOR antagonists at each dose.

Figure 6: This figure illustrates the effects of BNTX and NTB alone on the integrated changes in femoral conductance. Values are means and standard error of the mean. The symbol (#) indicates a significant increase in femoral conductance at 0.3μg/kg of BNTX compared to all other doses of BNTX. The symbol (*) indicates a significant increase from the conductance changes of the control/saline. Values are means and standard error of the mean for 7 subjects in each group.

Figure 7: The figure illustrates the effect of DOR-1 blockade with BNTX on changes in femoral conductance during the ME dose response. The symbol (*) indicates the change in the femoral conductance was significantly different from control at $P<0.05$. Values are means and standard error of the mean for five subjects.

Figure 8: This figure illustrates ME mediated changes in arterial pressure following DOR-1 blockade with BNTX. The symbol (+) in the legend box indicates the change in MAP was significantly different from the changes in MAP from BNTX exposure. Note the absence of the early hypertensive response at 20 and 30 seconds.Values are means and standard error of the mean for 7 subjects in each group.

Figure 9: The figure compares the effect of selective DOR-1(Tan-67) and DOR-2 (Delt II) agonists with ME on femoral conductance. Also illustrated is the ability of DOR-2 (NTB) blockade to reduce the hyperemic effects of the DOR-2 agonist deltorphin II. The symbol (**) indicates the change in the femoral conductance was significantly different from all other treatments. The symbol (*) indicates the change in the femoral conductance was significantly different from the saline. Values are means and standard error of the mean for four subjects.

TABLE 1: Resting Cardiovascular Indices:

Protocol 1: ME-ME dose response (n=4)									
DOSE (μg/kg)	**Saline/ Saline**	**ME 0.01**	**ME 0.03**	**ME 0.1**	**ME 0.3**	**ME 1.0**	**ME 3.0**	**ME 10.0**	**ME 30.0**
MAP (mmHg)	110±7 102±1	107±4 101±1	107±5 100±2	108±4 102+2	108±4 103+2	110±3 103±1	109±5 104+1	109±4 105±1	110±4 106+2
HR (bpm)	113±6 96±4	110±7 95±4	110±7 95+4.5	109±6 95±5	107±5 94±4.5	108±5 94±4	109±5 93±3	109±6 93±3	109±6 93±4

Protocol 2a: NTB (DOR-2 antagonst) Dose Response (n=5)								
DOSE (μg/kg)	**Saline**	**ME 3.0**	**NTB 0.3**	**ME 3.0**	**NTB 1.0**	**ME 3.0**	**NTB 10.0**	**ME 3.0**
MAP (mmHg)	97±3	97±4	98±3	99±3	99±4	98±4	104±3	102±4
HR (bpm)	118±10	121±12	123±11	123±15	121±14	118±11	124±12	120±13

Protocol 2b: BNTX (DOR-1 Antagonist) Dose Response (n=5)								
DOSE (μg/kg)	**Saline**	**ME 3.0**	**BNTX 0.3**	**ME 3.0**	**BNTX 1.0**	**ME 3.0**	**BNTX 10.0**	**ME 3.0**
MAP (mmHg)	96±2.5	94±2	97.5±2	98±3	98±2	98±2.5	98.5±3	99±3
HR (bpm)	133±6	132±7	130±6	130±6	129±6	129±7	129±6	127±7

Protocol 3: ME-ME dose response (n=4)									
DOSE (μg/kg)	**Saline/ Saline**	**ME 0.01**	**ME 0.03**	**ME 0.1**	**ME 0.3**	**ME 1.0**	**ME 3.0**	**ME 10.0**	**ME 30.0**
MAP (mmHg)	110±7 102±1	110+7 101+1	107+5 100+2	108+4 101+3	108+4 103+2	108+5 103+1	109+5 104+2	109+6 105+1	109+6 106+1
HR (bpm)	113±6 96±4	110±7 95±4	110±7 95±5	109±6 95±5	108±5 94±5	108±5 95±4	109±5 93±3	109±6 93±3	109±6 93±3

Protocol 4: ME-ME+BNTX (n=5)									
DOSE (μg/kg)	**Saline/ BNTX .3**	**ME 0.01**	**ME 0.03**	**ME 0.1**	**ME 0.3**	**ME 1.0**	**ME 3.0**	**ME 10.0**	**ME 30.0**
MAP (mmHg)	102±3 103±2	101±2 105±4	102±1 105±3	103±1 105±4	104±1 106±3	104±1 105±4	105±1 106±3	106±2 105±4	106±3 105±2
HR (bpm)	112±7 100±6	111±6 99±6	111±6 99±6	111±5 98±7	110±5 98±6	109±5 97±7	109±6 96±6	112±7 97±7	107±7 91±2

Protocol 5: Sal-ME-Tan-67-Delt II-NTB-Delt II (n=4)						
DOSE (μg/kg)	**Saline**	**ME 3.0**	**Tan-67 3.0**	**Delt II 0.3**	**NTB 3.0**	**Delt II 0.3**
MAP (mmHg)	107±5	107±6	109±6	110±6	104±5	103±5
HR (bpm)	117±16	115±15	114±14	116±14	113±13	114±13

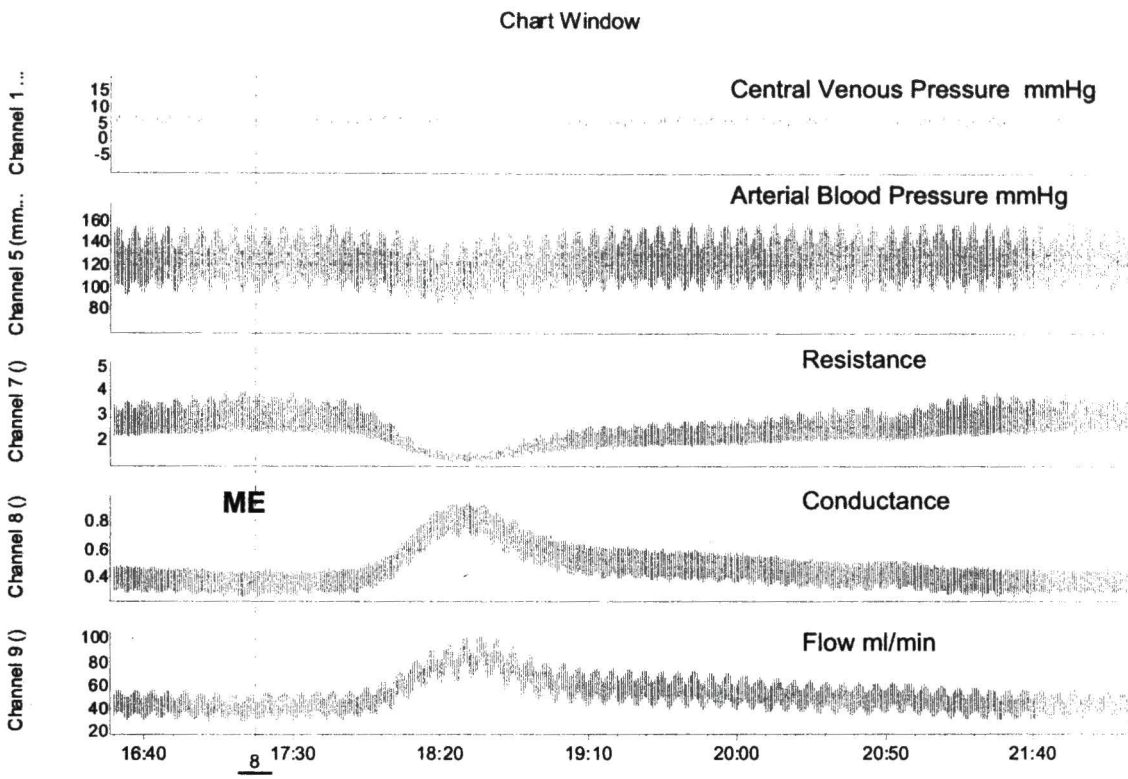
Chart Window
Channel 1 ...
Channel 5 (mm...
Channel 7 ()
Channel 8 ()
Channel 9 ()
Central Venous Pressure mmHg
Arterial Blood Pressure mmHg
Resistance
ME
Conductance
Flow ml/min
16:40
17:30
18:20
19:10
20:00
20:50
21:40

Figure 1

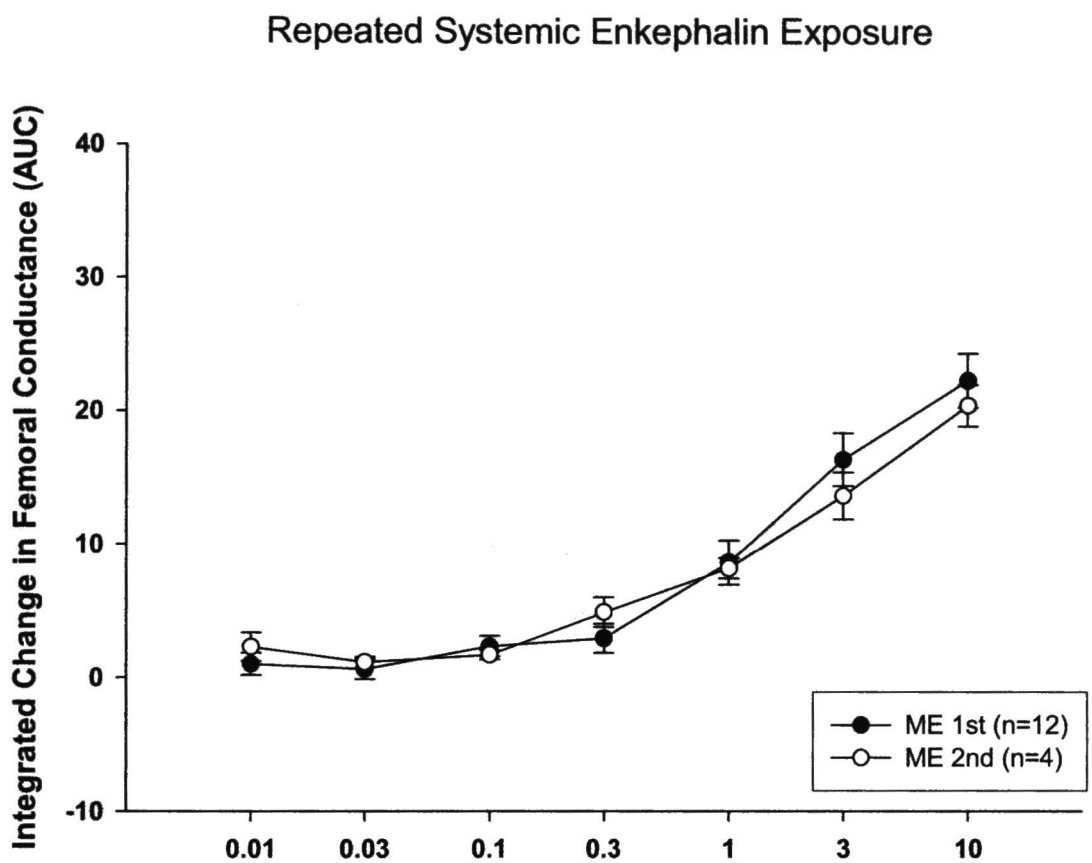
Repeated Systemic Enkephalin Exposure
Integrated Change in Femoral Conductance (AUC)
40
30
20
10
0
-10
0.01
0.03
0.1
0.3
1
3
10
ME 1st (n=12)
ME 2nd (n=4)

Figure 2

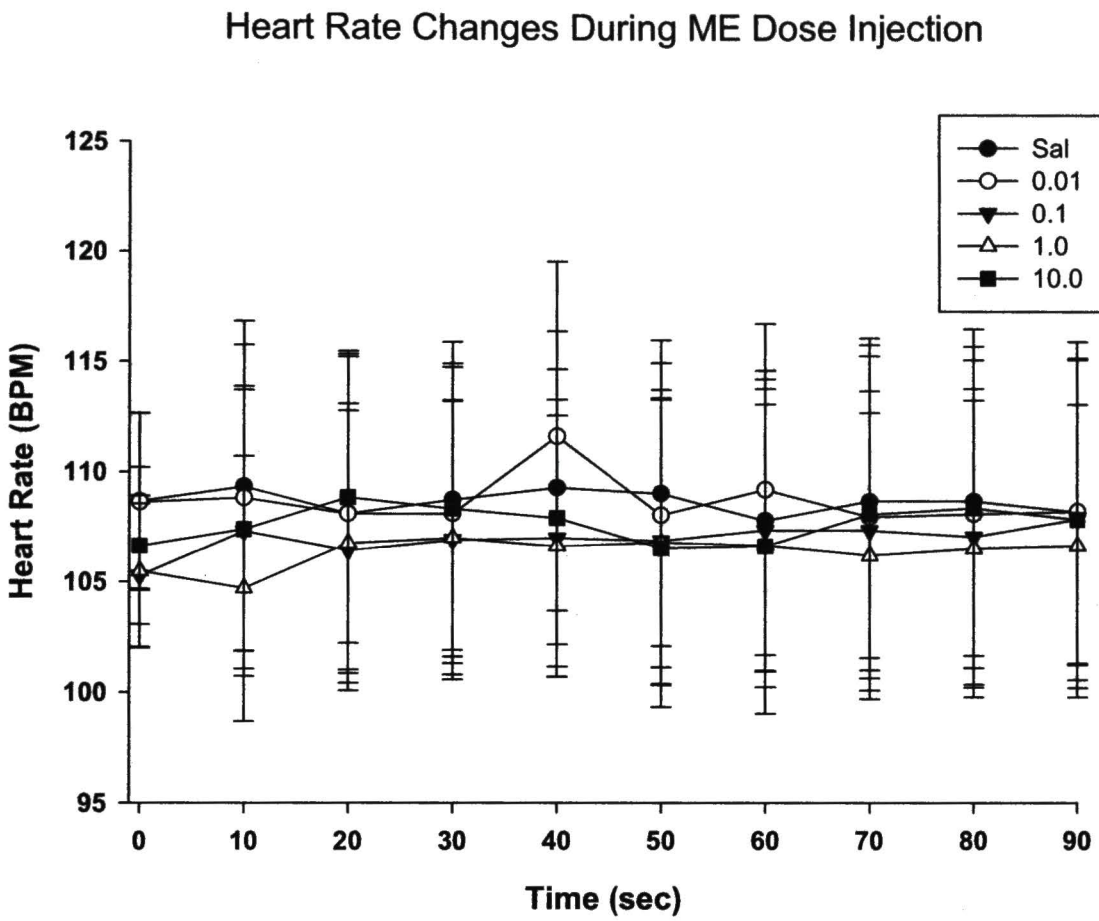
Heart Rate Changes During ME Dose Injection
Sal
0.01
0.1
1.0
10.0
Heart Rate (BPM)
125
120
115
110
105
100
95
0
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 3a

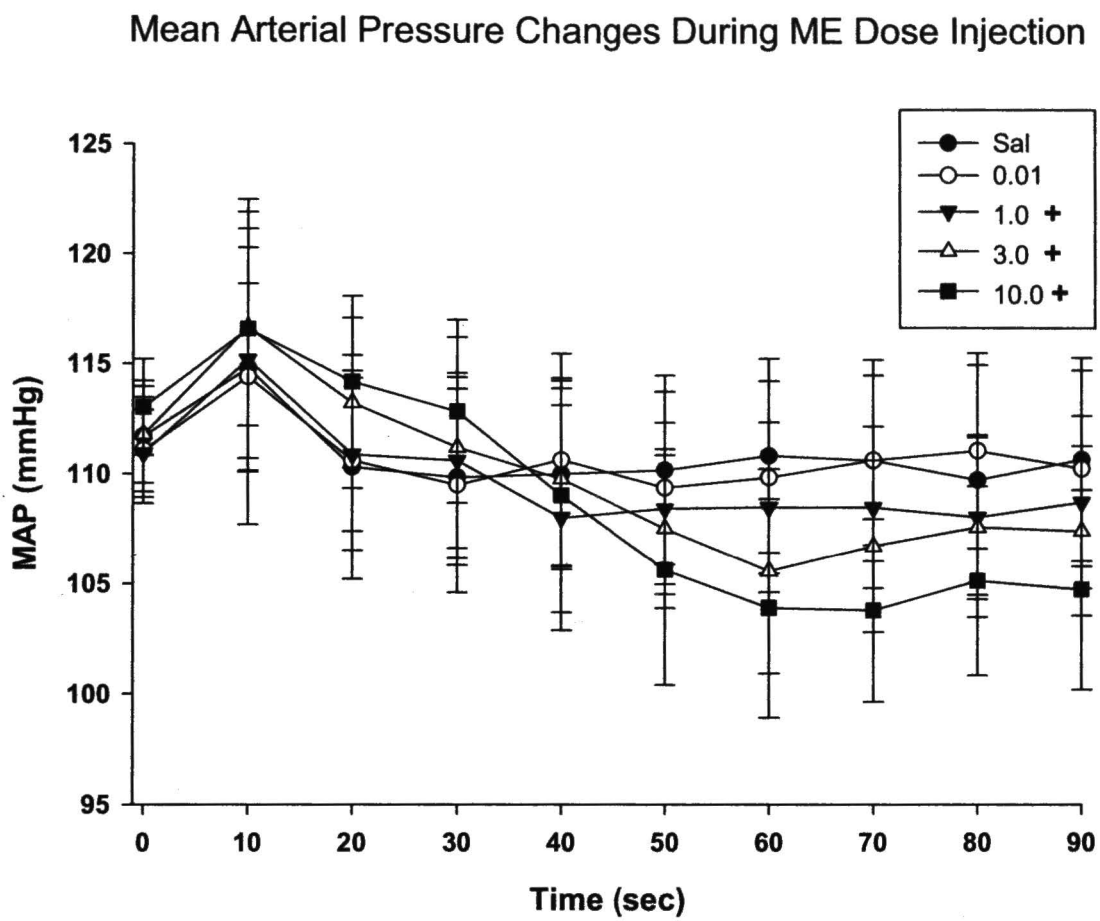
Mean Arterial Pressure Changes During ME Dose Injection
Sal
0.01
1.0 +
3.0 +
10.0 +
MAP (mmHg)
125
120
115
110
105
100
95
0
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 3b

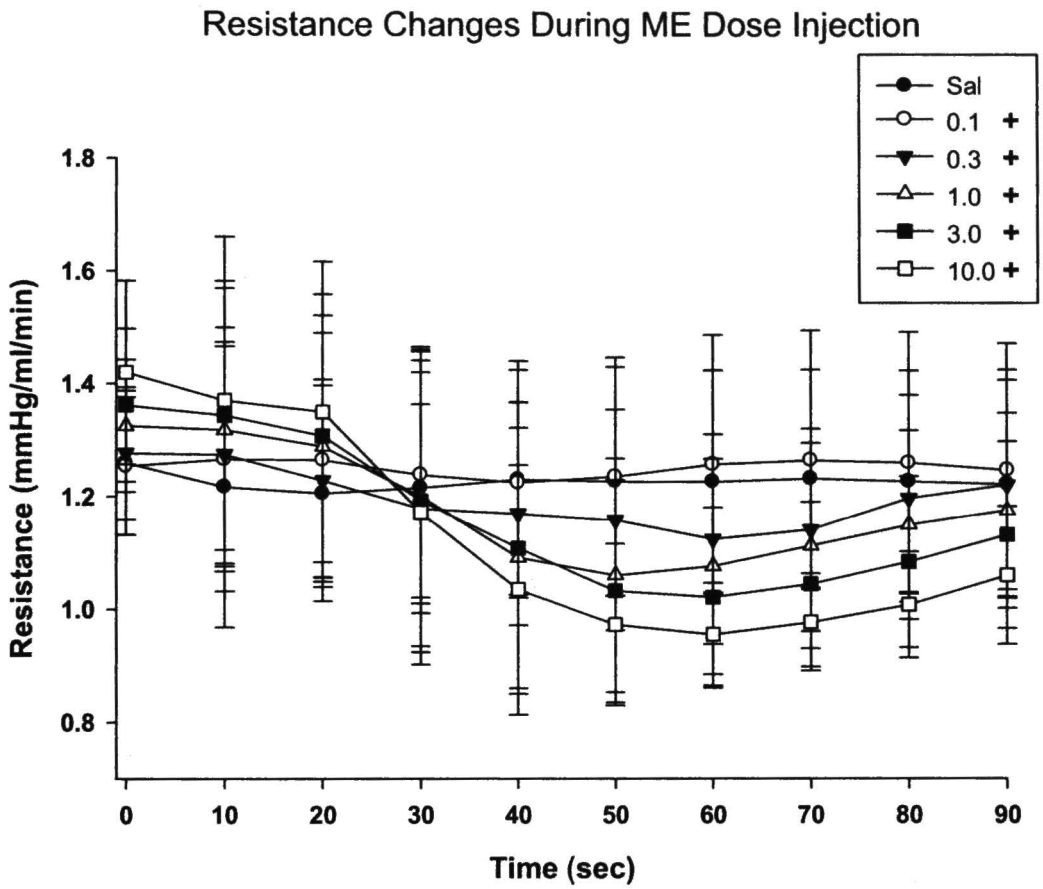
Resistance Changes During ME Dose Injection
Sal
0.1 +
0.3 +
1.0 +
3.0 +
10.0 +
Resistance (mmHg/ml/min)
1.8
1.6
1.4
1.2
1.0
0.8
0
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 3c

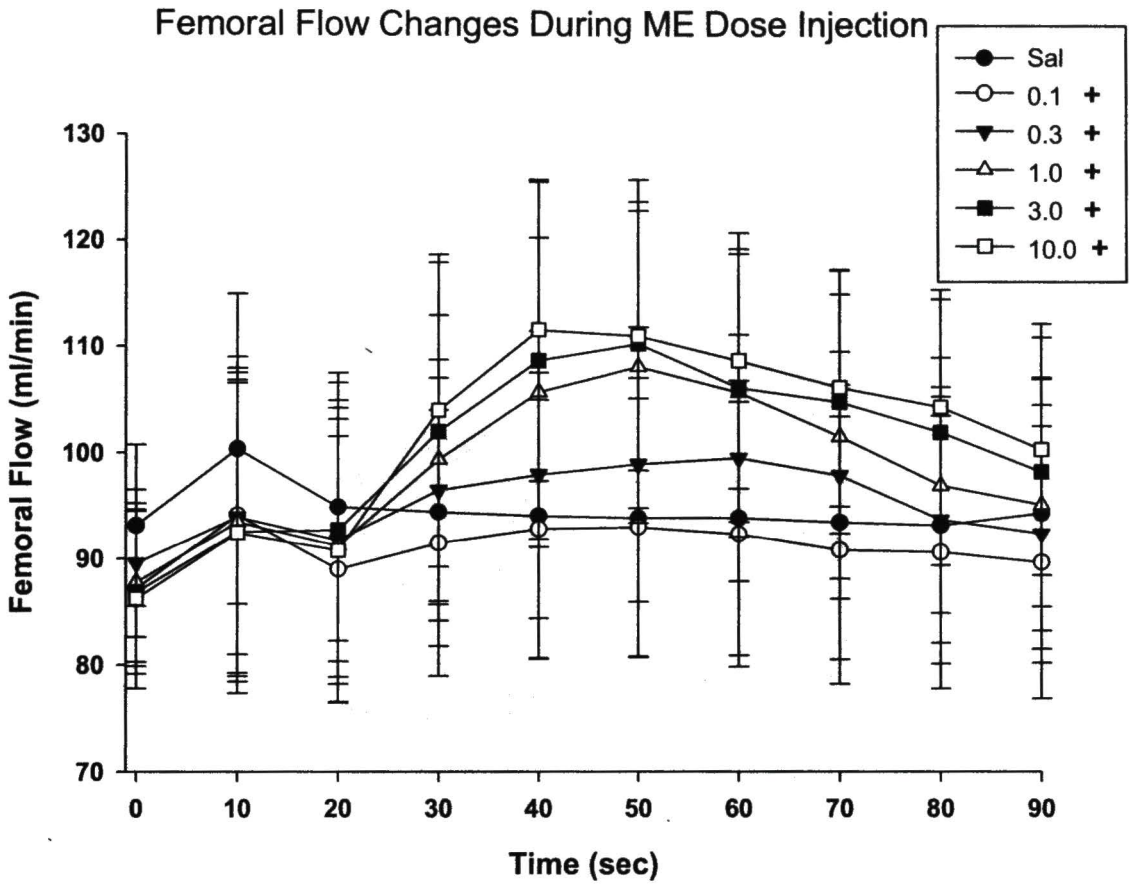
Femoral Flow Changes During ME Dose Injection
Sal
0.1 +
0.3 +
1.0 +
3.0 +
10.0 +
Femoral Flow (ml/min)
130
120
110
100
90
80
70
0
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 3d

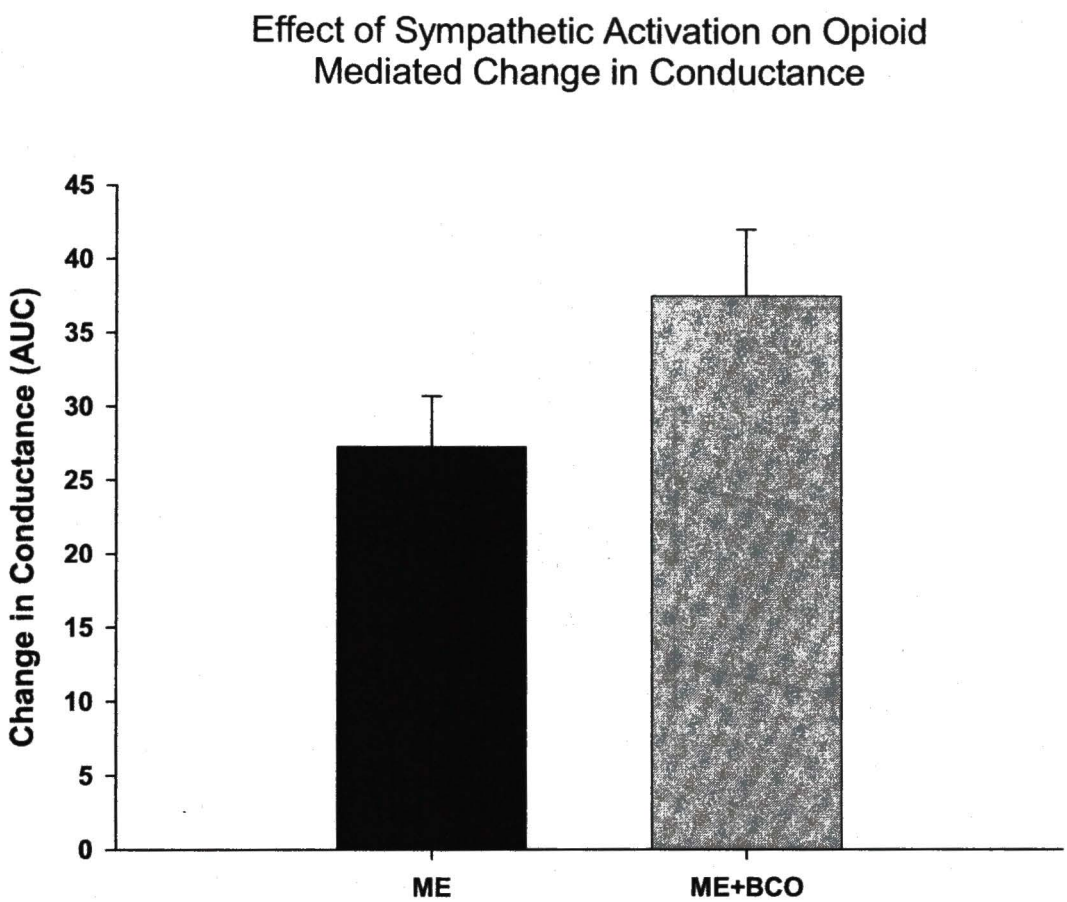
Effect of Sympathetic Activation on Opioid Mediated Change in Conductance
Change in Conductance (AUC)
45
40
35
30
25
20
15
10
5
0
ME
ME+BCO

Figure 4

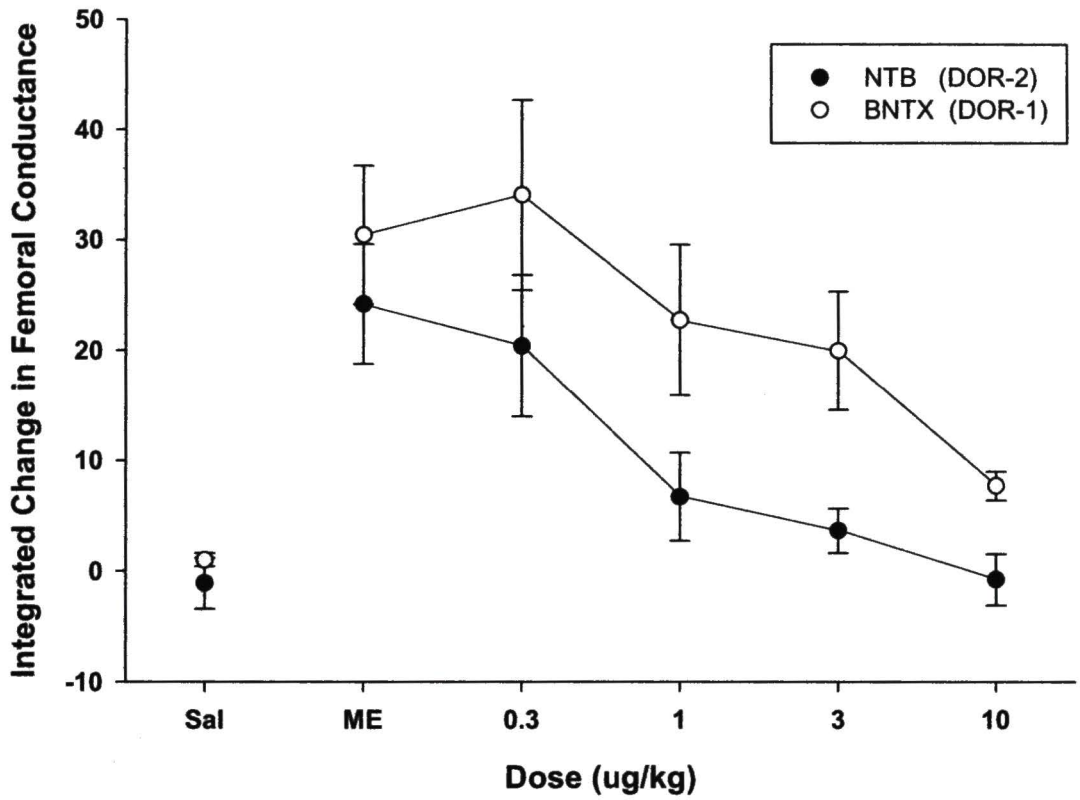
Integrated Change in Femoral Conductance
50
40
30
20
10
0
-10
NTB (DOR-2)
BNTX (DOR-1)
Sal
ME
0.3
1
3
10
Dose (ug/kg)

Figure 5a

Figure 5b: Effect of DOR Blockade of Met Enkephalin Mediated Change in Femoral Conducatance During BCO

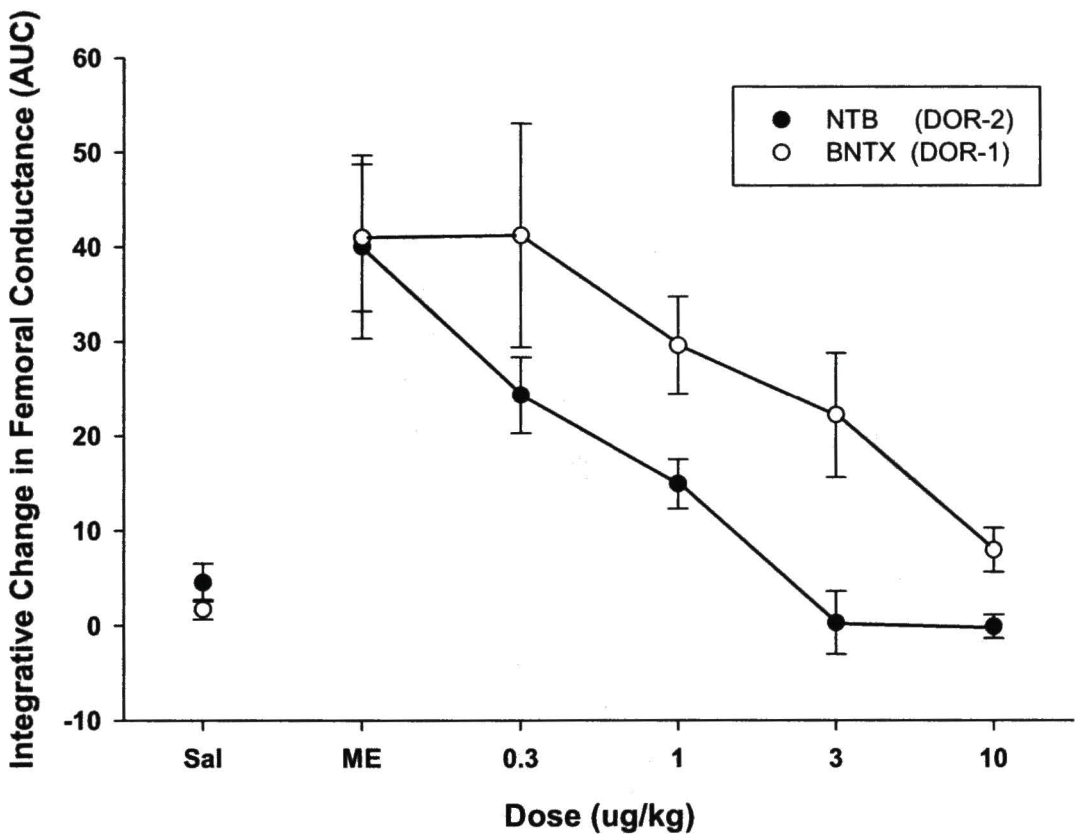

Figure 5b

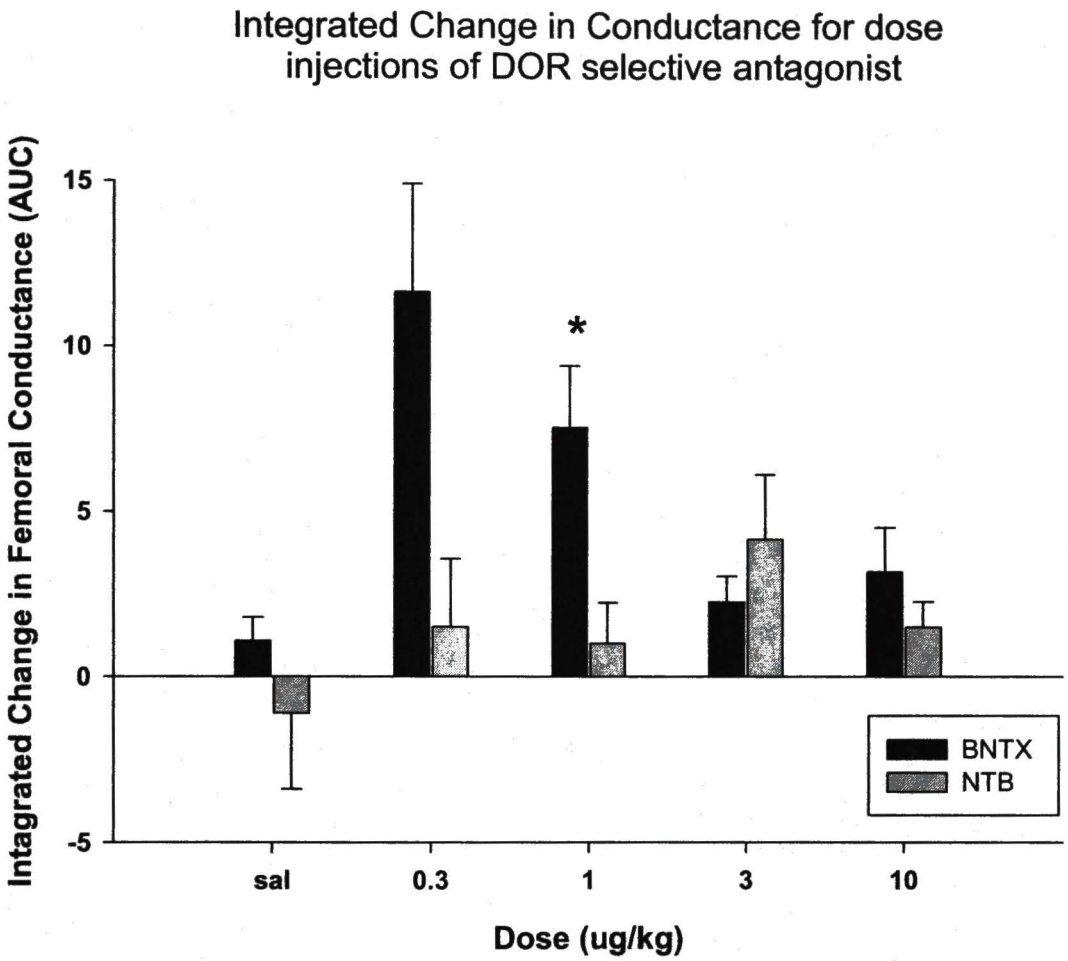
Integrated Change in Conductance for dose injections of DOR selective antagonist
Intagrated Change in Femoral Conductance (AUC)
15
10
5
0
-5
*
BNTX
NTB
sal
0.3
1
3
10
Dose (ug/kg)

Figure 6

Femoral Conductance Changes During ME Dose Injection
with DOR-1 Blockade

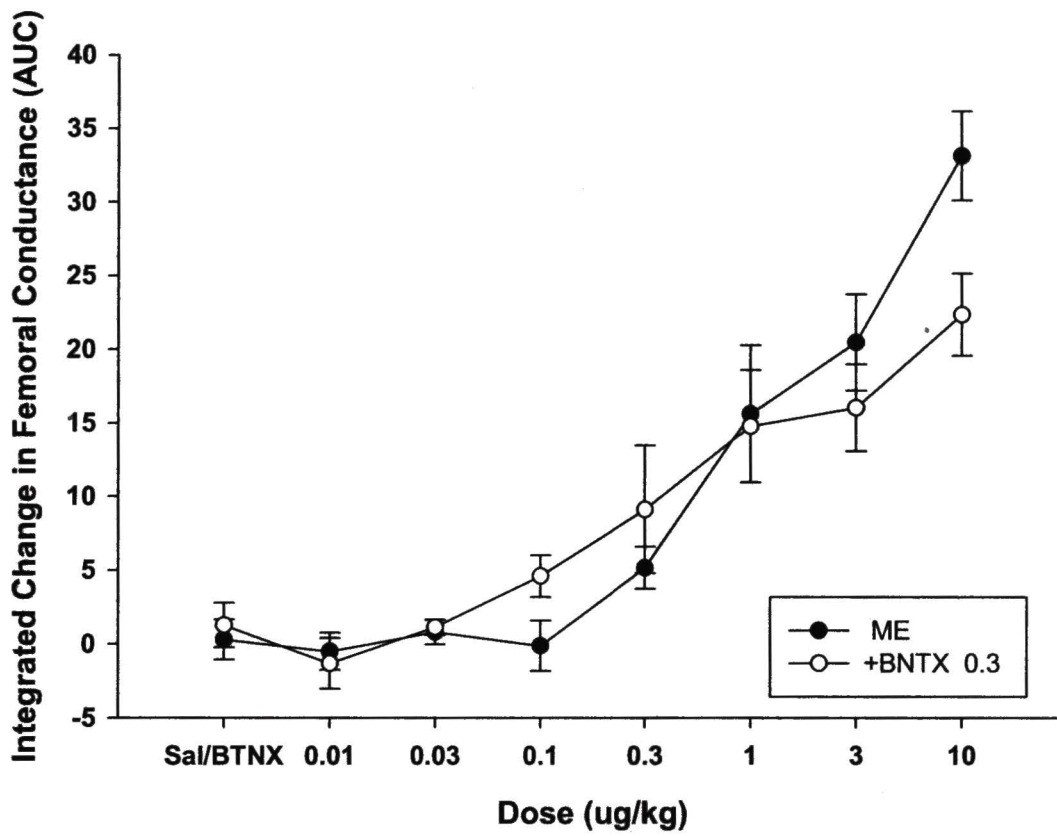

Figure 7

MAP Changes During ME Dose Injection with DOR-1 Blockade

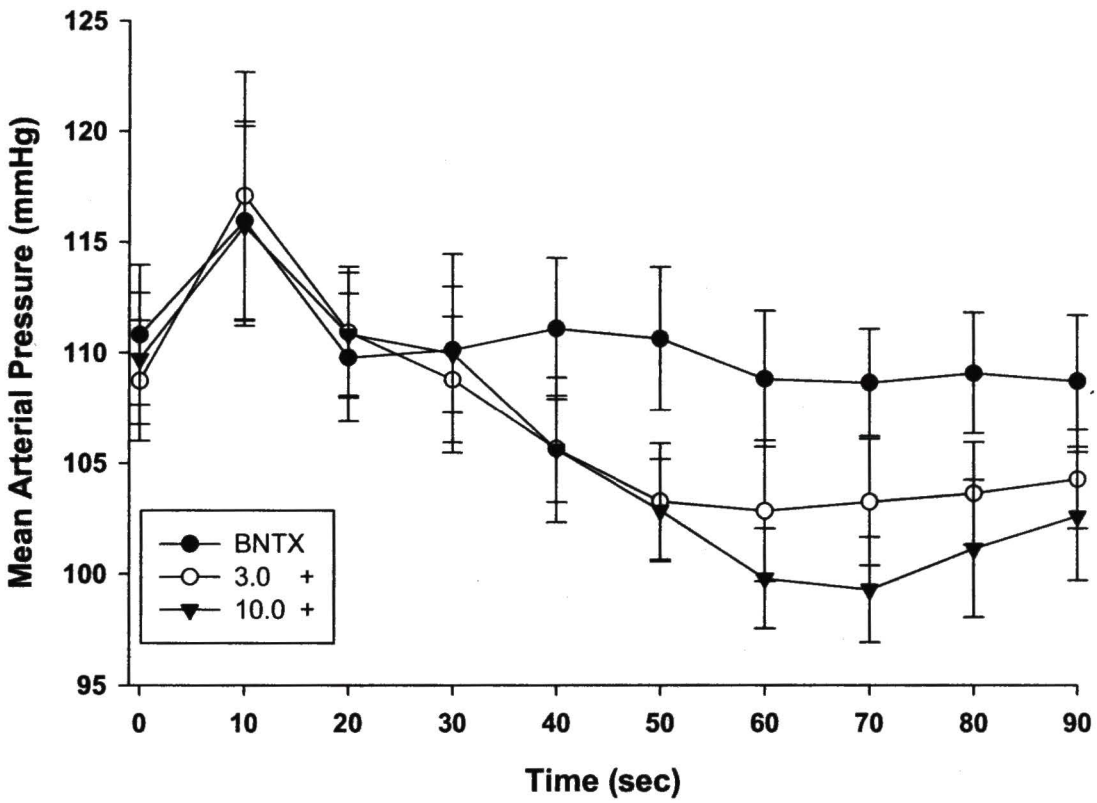

Figure 8

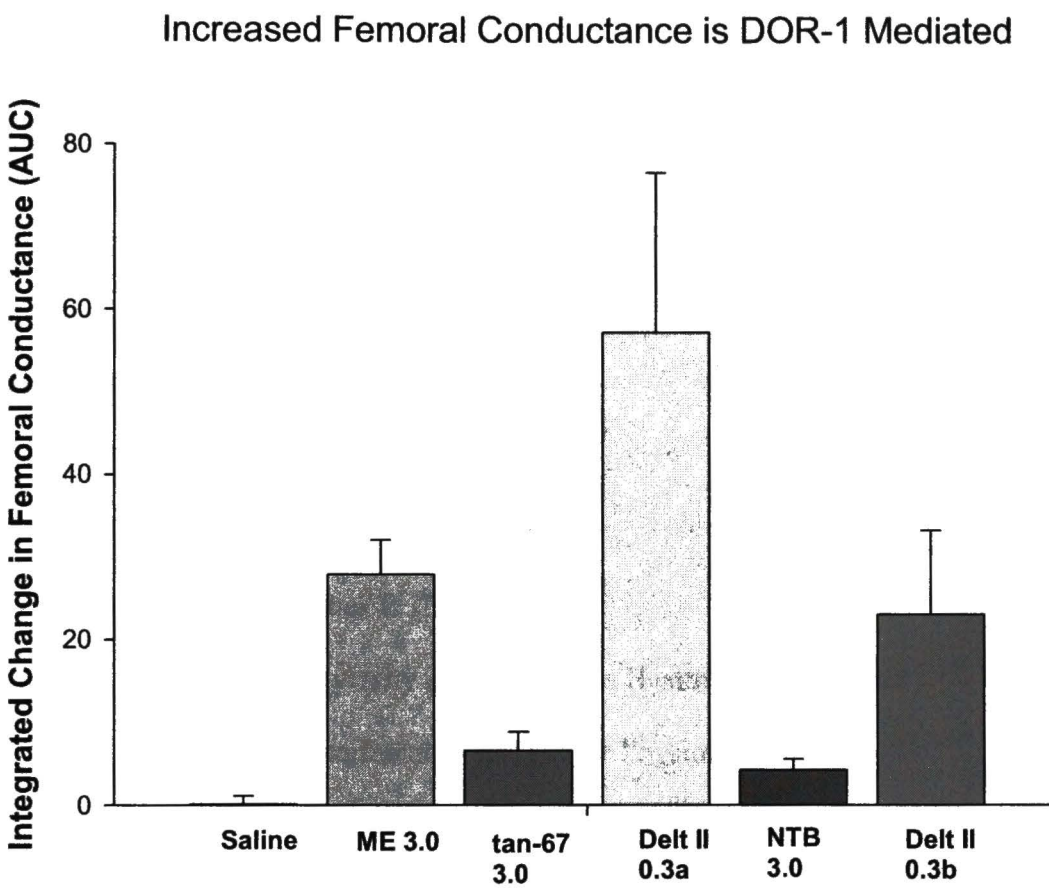
Increased Femoral Conductance is DOR-1 Mediated
Integrated Change in Femoral Conductance (AUC)
80
60
40
20
0
Saline
ME 3.0
tan-67 3.0
Delt II 0.3a
NTB 3.0
Delt II 0.3b

Figure 9

CHAPTER III

Delta opioid receptors (DORs) and the Autonomic Control of the Heart and Circulation in Fat Fed Canines.

Matthew A. Barlow, Shekhar H. Deo, Darice Yoshishige, Leticia Gonzalez, and James L. Caffrey

University of North Texas Health Science Center

Department of Integrative Physiology

3500 Camp Bowie Boulevard

Fort Worth, TX 76107

Corresponding author: James L. Caffrey

University of North Texas Health Science Center

Department of Integrative Physiology

3500 Camp Bowie Boulevard

Fort Worth, TX 76107

Tel. No. 817-735-2085

caffreyj@hsc.unt.edu

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Running Title: DORs and Cholinergic Transmission in Insulin Resistant Dogs

ABSTRACT

The neuroendocrine complications of diabetes involve increased heart and peripheral vascular disease. Reduced leg blood flow precipitates a vicious cycle of ischemic pain, immobility, inactivity and progressive disability. The neuroendocrine regulation of the heart and circulation in the early evolution of diet induced metabolic disease is unclear. The autonomic nerves and vasomotor ganglia that regulate muscle blood flow in particular are obvious potential targets during hyperglycemia associated with insulin resistance. Delta opioid receptors (DORs) on cholinergic nerve endings in the heart and within sympathetic ganglia actively regulate cholinergic transmission. The DOR-1 subtype facilitates and the DOR-2 subtype inhibits junctional neurotransmission. The functional expression of DOR phenotypes depends on gangliosides and lipid rafts in the local membrane lipid environment. Oxidized lipids disrupt that membrane organization and should alter the mix of receptors in favor of a decrease in the DOR-1 phenotype. The current studies test the hypothesis that high fat feeding produces a hyperactive adrenergic state through an increase in oxidized LDL that reduces the beneficial DOR-1 modulation of heart rate and peripheral vascular resistance. Body mass, insulin sensitivity, heart rate, heart rate variability, and femoral blood flow were determined in mongrel dogs fed high fat diets for six weeks. In week seven, DOR receptor mediated changes in vagal control of heart rate and sympathetic control of leg blood flow were determined. Indicative of the hyper-adrenergic state, high fat feeding increased heart rate and lowered muscle blood flow. Fat feeding reduced the DOR-2 vagolytic effect by half but left the companion sympatholytic effect unchanged. Consistent with the hypothesis, facilitory

DOR-1 vagotonic and sympathotonic responses to exogenous enkephalins were weak or absent. These data support the hypothesis that fat feeding reduces DOR mediated neuromodulation and triggers an unopposed sympathetic overcompensation.

INTRODUCTION

Changes in the opioid control of neurovascular transmission during the early stages of obesity and diabetes are not well defined. The presence of enkephalins in key locations like the sympathetic ganglia and cardiomyocytes has important implications for cardiovascular control (17). Though their content is often relatively low (22, 33), and the proteases that degrade them are abundant (19, 31), they are well positioned to interact locally with prejunctional opioid receptors on local cardiac nerves and with opioid receptors on preganglionic neurons within the sympathetic ganglia (2,4). Tissue opioid content is lower in hyperinsulinemic animal models (15, 26, 27, and 29) limiting perhaps the ability of endogenous opioids to regulate normal cardiovascular function.

Enkephalins modify parasympathetic control of heart rate from targets within the sinoatrial (SA) node where they produce a biphasic effect (10, 11, 13, 18, and 19) Cardiac cholinergic transmission via the vagus nerve depends on a mixture of DOR-subtypes to which the enkephalins bind. Introduction of enkephalin into the sinoatrial (SA) node by microdialysis increased cholinergic transmission at low dose rates and inhibited cholinergic transmission at higher dose rates. Prejunctional DOR-1 and DOR-2 receptors were responsible respectively for the opposing vagotonic and vagolytic effects (9, 11, and 20)

DORs also moderate cholinergic transmission within the sympathetic chain ganglion (1, 3, 24, and 32). The vasomotor components of the lumbar sympathetic chain

ganglion are particularly important for regulating flow distribution within the large skeletal muscle vasculature. Like the heart, both DOR receptor subtypes appear active in the ganglia and thus a bimodal interaction like that in the heart is an attractive hypothesis (1). The sympatholytic DOR-2 response is quite clear; enkephalin injected into the terminal aorta interrupts local ganglionic transmission, reduces femoral vascular resistance and sharply increases femoral blood flow (1, 4). The support for an opposing sympathotonic DOR-1 response is less complete and depends largely on enkephalin mediated increases in arterial pressure and the indirect evidence that DOR-1 blockade increased blood flow (1). Thus, the cholinergic transmission within sympathetic vasomotor ganglia may have similar biphasic effects that alternately facilitate and inhibit acetylcholine secretion.

Autonomic dysfunction is one of the diagnostic hallmarks of chronic diabetes. Though chronic hyperglycemia clearly damages nerves, the consequences of less severe forms of insulin resistance are poorly defined. Diabetes predisposes its victims to cardiovascular disease and though not necessarily related, DOR-1 receptor activation is cardioprotective and supportive of peripheral vascular stability. The functional expression of the DOR-1 receptor depends on its association with the membrane ganglioside, GM-1 in organized lipid rafts (7, 8). Dispersion of the lipid rafts may favor instead the DOR-2 phenotype along with tachycardia, hypertension, increased myocardial vulnerability, vascular instability, and an unopposed hyper-adrenergic state (30). Increased oxidized-LDL (oxLDL) production is proposed to accompany high fat diets and insulin resistance. When added to cultured cells oxLDL reduces the cholesterol rich

membrane domains and their associated GM-1 (2). These observations support the hypothesis that high fat feeding facilitates adrenergic hyperactivity by increasing oxLDL, and reducing the beneficial DOR moderation of heart rate and peripheral vascular resistance. To test the hypothesis, mongrel dogs consumed a high fat, hypercaloric diet for six weeks. Insulin sensitivity, heart rate variability, and Doppler blood flow documented their cardiometabolic status during the feeding protocol. In the seventh week, the DOR mediated regulation of heart rate and muscle blood flow was tested in anesthetized animals.

METHODS

All protocols were approved by the Institutional Animal Care and Use Committee in compliance with the NIH guide for the Care and Use of Laboratory Animals.

Animals

Twelve animals of either gender were randomly assigned to either a Control (n=4) or High Fat Diet Group (n=8) for a duration of 7 weeks. The animals were housed in the University of North Texas Health Science Center at Fort Worth's Lab Animal Care Facility. At week 0, baseline measurements were recorded of heart rate variability (HRV), femoral blood flow, and insulin sensitivity. These data were collected again after six weeks of control or high fat feeding. During the seventh week, DOR receptor function was evaluated in anesthetized animals.

Diet

For two weeks prior to the protocol, all animals ate a control diet of 650 g of the Harlan Teklad 2021 dry chow. That diet provides 2.2 Kcal/day comprised of 22% Protein, 20% Fat, and 56% Carbohydrate. Control animals remained on that diet for the duration of the subsequent seven-week protocol. The high fat diet consisted of 12.5oz Purina CV Canine Formula, 650g Harlan Teklad 2029 dry chow, and 2g/kg rendered bacon fat. The high fat diet provides ~3.9 kcal/day comprised of22.8% protein, ~44.3% fat, 32.9 % carbohydrate. Fat fed animals ate this diet for weeks 3-9. The high fat diet is

compatible with that reported elsewhere to produce a diet-induced insulin resistance and hyperinsulinemia in dogs (23).

Heart Rate Variability

Heart rate variability (HRV) and power spectral analysis (PSA) provided an index of autonomic nervous system activities in the resting conscious animals. Surface electrocardiograms (ECG) recorded heart rate signals online with a DI729 DATAQ data acquisition system and Windaq Pro+ software. The power spectral density was integrated for selected frequency ranges as follows: 1) high frequency (HF), 0.15-0.40 Hz; 2) low frequency (LF), 0.08-0.15 Hz; and 3) very low frequency (VLF), 0.01-0.08Hz.

Femoral Doppler Blood Flow

In each animal, resting Femoral Blood Flow (FBF_D) was determined prior to beginning the experimental diet and again after six weeks of the experimental feeding protocol. B-scan ultrasound was used estimate the femoral arterial diameter (FAD) and pulse Doppler then provided an estimate of the mean femoral velocity (Vm). The diameter and velocity were used to calculate FBF_D as follows $FBF_D = Vm\pi(FAD/2)^2$.

Insulin Sensitivity Index ($ISI_{0,30}$)

At weeks 0 and 6 oral glucose tolerance tests (OGTT) were conducted to calculate an Insulin Sensitivity Index at 30 min ($ISI_{0,30}$). In each subject, a fasting venous blood sample was drawn to measure fasting glucose (glucometer), and insulin (RIA). Glucose (50g in 250 ml distilled water flavored with non-fat chicken broth) was ingested orally

over 5 min. Plasma glucose and insulin were determined again 30 min later. The $ISI_{0,30}$ was calculated as reported elsewhere (6, 16):

$$\mathbf{ISI_{0,30}=MCR/log\ MSI=}\boldsymbol{m}\mathbf{/MPG/log\ MSI}$$

Glucose (G) uptake by the tissue (m) = (50,000 mg + (G_0-G_{30}) · 0.19 · kg body mass)/30 min

Mean plasma glucose (MPG) = G_0+G_{30}/2 and metabolic clearance rate MCR = mMPG.

Mean serum insulin (MSI, mU/l)

OxLDL Elisa

At weeks 0 and 6 a 1ml venous blood sample was drawn and the plasma stored in EDTA at -80 °C. At the end of the diet protocol the samples were thawed and 25ul was analyzed for oxidized LDL using a human oxidized LDL ELISA Kit (Alpco Diagnostics, Salem, NH).

Norepinephrine:

Myocardial tissue homogenized in 1N acetic acid, 0.02 N HCL and 0.1% beta-mercaptoethanol was preserved with sodium metabisulfite and stored at -90°C. Norepinephrine was extracted with alumina, eluted with perchloric acid, separated by HPLC and quantified coulometrically.

Surgical Preparation

The animals were anesthetized with sodium pentobarbital (32.5 mg/kg), incubated and initially ventilated mechanically at 225 ml/min/kg with room air. A Fluid filled catheter was inserted into the right femoral vein and advanced into the inferior vena cava. An infusion port Millar Mikro-Tip transducer was introduced into the right femoral

artery and advanced into the aorta just above the L5 region. The Millar transducer measured heart rate and blood pressure and served to perfuse the target L5 ganglion with experimental agents. The venous line was attached to a Statham P23ID pressure transducer to measure central venous pressure continuously online (PowerLab; ADI Instruments, Colorado Springs, CO). Surface electrodes were attached and the electrocardiogram (ECG) was continuously recorded. An electro-magnetic flow probe (10 mm) was placed around the isolated left femoral artery to measure hind limb blood flow. The depth of anesthesia was regularly evaluated and supplemental anesthetic was administered as required. The acid-base balance and blood gases were determined with an Instrumentation Laboratories blood gas analyzer (Lexington, MA). The pO2 (90-120 mmHg), the pH (7.35-7.45), and the pCO2 (30-40 mmHg) were adjusted to normal by administering supplemental oxygen or bicarbonate or by modifying the tidal volume.

The right and left cervical vagus nerves were isolated from the carotid arteries through a ventral midline incision. The carotid arteries were fitted with adjustable plastic snares for evaluation of reflex sympathetic responses during bilateral carotid occlusion (BCO). Anesthesia was carefully evaluated, and a single dose of succinylcholine (1 mg/kg) was administered intravenously to reduce muscle movement for the 10 min required for electrosurgical incision of the chest. A right side thoracotomy was performed to expose the heart, the pericardium was opened, and the pericardial margins were sutured to the body wall to support the heart.

Nodal Microdialysis

Dialysis probes were fabricated from 1 cm lengths of dialysis fiber (200 μm i.d. x 220 μm o.d.) from a Clirans TAF 08 (Asahi Medical, Northbrook, IL) artificial kidney. The probe was fitted with glass fiber inflow and outflow lines and inserted into the SA nodal tissue with the aid of a 25g needle. The probe restricts the transmural passage of molecules with masses greater than 36 kDa. The inflow line was then attached to a micro infusion pump and perfused with vehicle (saline) at 5ul/min for a one-hour equilibration period.

Materials

Methionine-enkephalin-arginine-phenylalanine (MEAP) and methionine-enkephalin (ME) were obtained from American Peptide (Sunnyvale, CA) and 7-benzylidenaltrexone (BNTX) was obtained from Tocris Bioscience (Ellisville, MO).

Statistical Methods

All data were presented as mean ± SEM. Conductance and blood flow were compared within groups using one way repeated measures analysis of variance (ANOVA). Differences were resolved further post-hoc with Tukey's and Dunnet's tests where appropriate. The Student's T-test was used to evaluate between group differences. Differences deemed to occur by chance with a probability of $P<0.05$ were accepted as significant.

Protocol 1: DOR mediated vascular responses in the high fat fed animals.

ME dose responses were conducted in lean and fat fed animals. ME was administered into the terminal aorta immediately upstream from the final (L5) pair of segmental arteries followed by a standard 5 ml saline flush. An initial seven step ME dose response (0.01μg/kg-10 μg/kg) was constructed. The hemodynamic effect of each dose was recorded and allowed to recover fully (5-10 min) before exposure to the next higher dose. After 30 min equilibration, BNTX (0.3μg/kg) was administered to remove competitive DOR-1 activity during a second ME dose response. A BNTX mediated leftward shift in the curve would indicate the removal of a difficult to discern decrease in vascular conductance mediated by low doses of ME. The dose of BNTX was based on prior work (11).

Protocol 2: High fat feeding, DOR-1 receptors and reflex sympathetic reactivity.

Reversiable 90 sec bilateral carotid occlusions were performed to evaluate reflex sympathetic reactivity. At 90 seconds the snare was released and the cardiovascular responses were allowed to return to normal. A BCO was performed after the initial saline injection and again after BNTX to evaluate the influence of DOR-1 blockade.

Protocol 3: High fat feeding, DOR subtypes and vagal bradycardia.

This protocol was conducted to determine whether the high fat feeding would produce shifts in DOR dose responses in the SA node during vagally induced bradycardia. After the initial 1 hr equilibration, the right cervical vagus nerve was

stimulated at a supra-maximal voltage (15 V) for 15 sec at 1 and 3 Hz. The frequencies were selected empirically in each animal to produce 10–20-bpm and 30–40-bpm decreases in heart rate, respectively. Two min of recovery was allowed between successive vagal stimuli. A cumulative seven-step MEAP dose-response (0.005–1500 pmol/min) was conducted. Each dose delivered by microdialysis for 5 min prior to the next two-step vagal stimulation. After completing the dose-responses, the enkephalin was discontinued and the dialysis line was flushed with saline for 45 min. The clearance of peptide and the restoration of baseline vagal function were evident after 10 min illustrating the absence of lasting time or treatment-dependent changes.

RESULTS

Resting heart rates increased (109±6 to 122±4 BPM) significantly in the high fat fed group (Figure 1) while rates in the lean controls were unchanged (118±10 to 100±7 BPM). Though not significantly different, the high frequency power spectral density in the fat fed animals was consistent with the resting tachycardia, trending lower (258±73 to 170±58) at six weeks compared to the lean controls (209±30 to 278±108). Low and very low frequency power measurement were far more variable and thus uninterpretable. Consistent with the suggestion that high fat diets produce a hyper-adrenergic state, resting femoral blood flow measured by ultasound imaging and pulse Doppler was lower after six weeks in the fat fed animals while that in the lean controls was unchanged (Figure 2). Myocardial norepinephrine was lower in fat fed animals (24.2 ±1.2 vs 31.4 ± 2.1 pmoles/mg protein $p < 0.01$) suggesting increased secretion and/or turnover. The estimated insulin sensitivity $ISI_{0,30}$, declined in the high fat fed group after six weeks (Figure 3). There was no change in the insulin sensitivity for the lean controls during the same interval. The initial circulating oxLDL was unexplainably higher in the fat fed animals compared to the lean controls. Contrary to expectations that insulin resistance and fat feeding would increase circulating oxLDL, there was no change in fat fed (196±16 to 209±11 ng/ml) or lean controls (157±13 to 151±15 ng/ml) following the feeding protocol (Figure 4). The initial difference in oxLDL was apparently random since the protocol was conducted in groups of three with one control and two fat fed animals in each group.

Evidence for altered DOR mediated vascular responses:

Figure 5 illustrates the arterial conductance dose response for intra aortic ME in lean controls. ME produced a dose dependent increase in femoral conductance with an apparent threshold between 0.1-0.3μg/kg. Thirty min after completing the first dose response, the restoration of baseline vehicle/saline responses were verified. DOR-1 blockade with BNTX (0.3μg/kg) was instituted and the dose response was repeated. BNTX did not alter the baseline conductance. As reported previously (1), the terminal portion of the subsequent dose response curve shifted down and to the right, reducing the effect of ME; particularly at higher doses. This shift did not occur in back to back dose responses without BNTX (1).

Figures 6a, b, and c illustrate temporal changes in mean arterial blood pressure, resistance and femoral flow during the ME dose response. For clarity, the figures include only dose effects that were significant. Heart rate was unchanged and is not presented. The fall in arterial pressure was less sensitive to ME than the increase in flow or the decline in resistance. ME altered both flow and resistance at significantly lower doses. The peak change in flow also preceded the decline in pressure by 10-20 seconds collectively suggesting that increasing arterial run-off was the primary cause of the hypotension. The change in arterial pressure was biphasic with a consistent dose dependent increase in arterial pressure that persisted for 10-20 seconds prior to the subsequent more dramatic hypotensive effect.

Figures 7a, b, and c illustrate the same data after DOR-1 blockade. The results are qualitatively similar to those obtained before DOR-1 blockade with one notable exception. BNTX eliminated the initial increase in blood pressure supporting the

potential DOR-1 character of this sympathonic effect. The persistence of the hypotensive and hyperemic responses reinforce the DOR-2 character of those effects. The cause of the gradual attrition of the conductance at the higher ME doses after BNTX is illustrated by a consistent decrease in peak and duration of the flow response.

Figure 8 illustrates the ME conductance dose response and the effect of DOR-1 blockade in the fat fed animals. The initial response is indistinguishable from the lean controls. The post-BNTX response is different in two respects. The DOR-1 antagonist, BNTX (0.3μg/kg) produced a significant increase in conductance by itself suggesting a modest constitutive DOR-1 mediated vasoconstriction in the fat fed animals consistent with the decline in femoral blood flow in the conscious animals. Secondly, unlike the lean controls here and in prior work (1), the dose response with DOR-1 blockade was not significantly different from the initial ME dose response curve. The between curve differences in conductance for controls and fat fed animals for the last two doses were significant between lean and fat fed animals ($p < 0.05$).

Figures 9 and 10 illustrate the changes in mean arterial blood pressure, resistance, and femoral flow in the high fat fed group before and after DOR-1 blockade. Once again, heart rate was unaltered. The plus signs represent doses that are significantly different from vehicle or BNTX alone. Figure 9(a) illustrates the resting (119±5 mmHg) and dynamic enkephalin induced changes in mean arterial pressure. In comparison to the lean group in figure 6(a) (108±2.3 mmHg) both the resting pressure was elevated though the ME mediated changes in pressure were similar. Additionally, the resting resistance (1.7±0.3 mmHg/ml/min) is also elevated in figure 9(b) compared to the resting resistance

(1.3±0.2 mmHg/ml/min) of the lean controls in figure 6(b). The responses after BNTX are comparable to the lean control group in their resting values except for the resistance response. Figure 7(c) illustrates a starting resistance at 1.3 ± 0.2 mmHg/ml/min compared to figure 10(c) where the high fat group starting resistance is 1.7±0.2 mmHg/ml/min. Thus, the high fat fed group had a greater resting resistance and pressure. Similar to the lean controls, the change in arterial pressure was biphasic with a consistent dose dependent increase in arterial pressure that persisted for 10-20 seconds prior to the subsequent more dramatic hypotensive effect. This initial hypertensive effect was again eliminated by DOR-1 blockade.

Figure 11 illustrates the change in femoral conductance during sympathetic activation mediated by a 90 second bilateral carotid occlusion (BCO). In controls, conductance increased sharply while the exact opposite occurred in the fat fed animals suggesting a far more intense reflex vasoconstrictor response in the fat fed animals. The increase in conductance in controls appears to have been mediated by reduced DOR-1 activity since it is eliminated by DOR-1 blockade. This same DOR-1 activity appears to be absent in the fatt fed animals.

Evidence for DOR mediated vagal heart rate responses

This part of the study was designed to evaluate the influence of fat feeding on DOR-vagal interactions in the SA node. Figures 12a and b illustrate a two-step frequency response to right vagus nerve stimulation during a dose response to the enkephalin, MEAP in lean controls and fat fed animals. This dose response was

constructed to evaluate DOR-1 mediated vagotonic effects at low doses and DOR-2 mediated vagolytic effects at higher doses (10). A very modest vagotonic effect in the lean group is not significant however a progressive decline in vagal transmission appears to begin at 5 femtomoles/min and reaches a maximum near 1.5 nanomoles/min. The 68% inhibition from peak observed here is very typical for this response.

Figure 12b illustrates the same vagal frequency responses during MEAP in the high fat fed dogs. Once again the mild vagotonic effect at 5 and 50 femtomoles/min was not significantly different from vehicle. The DOR-2 mediated vagolytic effect at the higher doses was both delayed in appearance and reduced in magnitude to approximately 30% inhibition in the fat fed animals. Figure 13 illustrates this as a direct comparison of percent inhibition. Thus, fat feeding shifts the vagolytic dose response curve to the right.

DISCUSSION

The present study demonstrates that opioid receptors regulating normal nerve traffic in both limbs of the ANS may be altered in the early stages of the cardiometabolic syndrome. As evident from previous work, opposing DOR phenotypes regulate cholinergic transmission both in the heart and in the lumbar sympathetic chain ganglia (1, 11,12 13, and 14). The expressed DOR phenotype is proposed to depend on the lipid raft environment of the nerve membranes in which the receptors reside. Thus, changes in diet might logically alter neurotransmission by changing the lipid environment surrounding the DORs.

The current model provides evidence for early cardiometabolic changes similar to irregualties observed in the human metabolic syndrome. The fat fed dogs in this investigation had higher resting heart rates, lower leg blood flow, and lower insulin sensitivity. Resting conscious blood pressure was not recorded though hypertension has been demonstrated in this model (30). Although not quanitified visceral adipose was visually increased in agreement with previous investigations using a simular duration high fat diet (23).

Enkephalins and the associated DORs are clearly able to modify vasomotor transmission through the lumbar sympathetic chain ganglia. Intra-aortic enkephalin decreases mean arterial pressure, vascular resistance and increases blood flow in the femoral artery (4, 32). Recent studies with selective antagonists demonstrated that the enkephalin mediated increase in femoral vascular conductance was DOR-2 mediated (1).

The hyperemic effect was duplicated by the DOR-2 agonist deltorphin II and blocked by the DOR-2 antagonist naltriben. Thus DOR-2 mediated inhibition of cholinergic transmission is similar in SA node and lumbar sympathetic chain ganglia and may represent important common mechanisms for the autonomic control of the heart and vasculature (13, 20).

This investigation proposed that a high fat diet would increase lipid oxidation and disrupt the local environment surrounding the DOR, deplete DOR numbers and reduce the fine control of muscle blood flow. Though blood flow declined in the conscious animals on the high fat diet, the blood flow response to enkephalins was remarkably similar between lean and fat fed animals. Thus the DOR-2 function appeared intact and undiminished. Contrary to both current and historical controls (1), DOR-1 blockade did not alter the conductance dose response in fat fed animals suggesting a reduction in the functional DOR-1 population. Prior work suggested the lowered response to ME in controls following BNTX resulted from a time dependent DOR depletion via a gradual shift into the DOR-1 phenotype and the progressive sequestration of these receptors by BNTX. Thus the absence of this effect in fat fed animals suggests that there are fewer DOR-1 receptors. This would be consistent with a reduction in the quality of their lipid environment.

These observations lead to the proposal that the sympathotonic DOR-1 acts as a regional ganglionic amplifier. This allows for a lower overall background sympathetic tone that can be enhanced as needed to assure the most appropriate distribution of muscle blood flow while maintaining control of the critical buffering capability of this large

vascular capacitance. The loss of DOR-1 function in the fat fed animal results in increased baseline activity as evident in increased heart rate, reduced leg blood flow and exaggerated vasoconstriction during reflex sympathetic activation. In the control animal the femoral capacitance buffers the reflex increase in arterial pressure by permitting a modest increase in conductance. DOR-1 blockade with BNTX elimninates that buffering capacity. In the fat fed animal both the buffer effect and the BNTX response are absent suggesting again the depletion of the DOR-1 pool.

Contrary to predictions, plasma oxLDL was not increased by the feeding protocol at six weeks. Whether circulating oxLDL concentrations are good measures of tissue concentrations is undetermined. However, the study proposed that increased oxLDL would disrupt lipid rafts in favor of the DOR-2 phenotype making the heart more susceptable to damage and require greater sympathetic activity to maintain vascular tone. This shift away from DOR-1 receptors was more evident in the ganglion than in the heart where the DOR-1 vagotonic response was weak and inconclusive in both groups. The results in heart suggest fewer DOR-2 receptors in the fat fed animals. Increased exposure to oxLDL in vascular studies was accompanied by a loss of the lipid raft marker GM1 and an increase in endothelial force generation (2). GM1 is also abundant in nerve terminals and is concentrated in lipid rafts where it is proposed to associate with DOR-1. GM1 added into the SA node increased DOR-1 (vagotonic) activity and inhibited the DOR-2 (vagolytic) activity (8) The apparent loss of ganglionic DOR-1 receptors might be explained if fat feeding lowers GM1 and/or disperses the lipid rafts in ganglionic nerve terminals. The loss of the lipid raft environment might then facilitate total DOR downregulation and the subsequent reduction in the nodal DOR-2 vagolytic response.

Further investigation measuring tissue oxLDL and GM-1 could indicate a more direct role for oxLDL in the loss of the GM1 and associated DOR function.

The third experiment was designed to demonstrate maladaptions in cardiac DOR activty during the early stages of the cardiometabolic syndrome. The cardiac enkephalins and the associated DOR receptors are capable of effecting significant changes in parasympathetic transmission in the SA node. The prior pharmacological studies suggest that opposing vagotonic DOR-1 and vagolytic DOR-2 phenotypes are present in heart. The mix of DOR receptors is fluid and the sum of their opposing actions may determine the efficacy of vagal transmission at any given time. The balance of these DOR phenotypes is of major importance since DOR-1 receptors facilitate ischemic preconditioning. Repeated coronary occlusions increase DOR-1 and reduced DOR-2 activity (9). DOR-1 blockade with BNTX abolished the vagotonic effects confirming DOR-1 participation. In the present study, the typical low dose vagotonic effects of intranodal MEAP were relatively weak in both lean and fat fed animals. At higher doses the vagolytic effects were easily demonstrated. However in the high fat fed group the vagolytic effect was significanlty reduced in comparison to lean and historical controls (11). The vagolytic effect of MEAP was observed earlier and was greater in magnitude in the lean controls.

In conclusion, the DOR-2 mediated increases femoral conduction are unaltered early in the adaptation to high fat feeding. In contrast the DOR-2 mediated vagolytic response is reduced by half. DOR-1 blockade appears to expose a decline in sympathotonic DOR-1

receptors in the high fat fed group necessitating perhaps an overall increase in baseline sympathetic activity. Thus, high fat feeding provokes significant alterations in DOR interactions with both the parasympathetic and sympathetic cholinergic nerve terminals after only six weeks. This investigation suggests that DOR receptors are common features of cholinergic transmission that adapt relatively quickly to short term changes in diet. Whether the changes are reversible or continue to evolve further during longer exposure to high fat feeding are important unanswered questions relative to the progression of cardiometabolic disease.

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LEGENDS

Figure 1 illustrates resting heart rates in conscious animals before and after the high fat (HF) diet protocol. Values are means and standard error of the mean. The symbol (**) indicates a significant difference from week zero at $P<0.01$.

Figure 2 illustrates resting femoral blood flow in conscious animals before and after the high fat (HF) diet protocol. Values are means and standard error of the mean. The symbol (*) indicates a significant difference from week zero at $P<0.05$.

Figure 3 illustrates the calculated Insulin Sensitivity Index ($ISI_{0,30}$) in fasted conscious animals before and after the high fat (HF) diet protocol. Values are means and standard error of the mean. The symbol (*) indicates a significant difference from week zero at $P<0.05$.

Figure 4 illustrates fasted plasma oxidative LDL in conscious animals before and after the high fat (HF) diet protocol. Values are means and standard error of the mean for six fat fed and three controls respectively.

Figure 5 illustrates the effect of increasing terminal aortic doses of ME on integrated femoral vascular conductance in control subjects before and after DOR-1 blockade with BNTX (0.3 μg/kg). The symbol (*) indicates the conductance at that dose was significantly different from vehicle ($P<0.05$). The symbol (**) indicates the conductance

at that dose was significantly different from the same dose after BNTX. Values are means and standard error of the mean.

Figures 6a, b, and c illustrate dose dependent changes in mean arterial pressure, femoral resistance, and femoral flow in control animals for 90 seconds after aortic ME administration. Some non-significant dose effects were omitted for clarity. The symbol (+) indicates that the dose effect was significantly different from vehicle ($P<0.05$).

Figure 7a, b and c illustrate dose dependent changes in mean arterial pressure, femoral resistance, and femoral flow in BNTX treated control animals for 90 seconds after aortic ME administration. Some non-significant dose effects were omitted for clarity. The symbol (+) indicates that the dose effect was significantly different from vehicle ($P<0.05$).

Figure 8: illustrates the effect of increasing terminal aortic doses of ME on integrated femoral vascular conductance in fat fed subjects before and after DOR-1 blockade with BNTX (0.3 μg/kg). The symbols * and ** indicate respectively that the initial dose or both doses were different from vehicle. The symbol + indicates that BNTX produced an initial increase in conductance ($P<0.05$). The absence of change after BNTX is significantly different from Figure 5 for the two highest doses ($P<0.05$).

Figures 9a, b, and c illustrate dose dependent changes in mean arterial pressure, femoral resistance, and femoral flow in fat fed animals for 90 seconds after aortic ME

administration. Some non-significant dose effects were omitted for clarity. The symbol (+) indicates that the dose effect was significantly different from vehicle ($P<0.05$).

Figure 10a, b and c illustrate dose dependent changes in mean arterial pressure, femoral resistance, and femoral flow in BNTX treated fat fed animals for 90 seconds after aortic ME administration. Some non-significant dose effects were omitted for clarity. The symbol (+) indicates that the dose effect was significantly different from vehicle ($P<0.05$).

Figure 11 compares the integrated changes in femoral conductance during sympathetic activation mediated by bilateral carotid occlusion (BCO) before and after DOR-1 blockade with BNTX. Values are means and standard error of the means. The symbol # indicates that the sympathetically mediated conductance in the lean controls and fat fed animals is different $P<0.05$. The symbol * indicates that the sympathetically mediated conductance in the lean animals is different from control after DOR-1 blockade $P<0.05$.

Figure 12 a and b illustrate changes in heart rate mediated by low (1 Hz) and high (3 Hz) frequency stimulations of the right vagus nerve during exposure to increasing MEAP doses introduced into the interstitium of the SA node by microdialysis in the lean controls and fat fed animals respectively. Values are means and standard error of the mean and the symbols * and ** indicate that the change in heart rate was different from vehicle respectively at the high frequency or both frequencies.

Figure 13 compares the vagolytic dose effect of MEAP as percent blockade in control and fat fed animals for the higher frequency stimulation. Values are means and standard error of the means. The symbols + and ++ indicate respectively that fat fed animals are different from controls at $P<0.05$ and $P<0.01$.

FIGURES

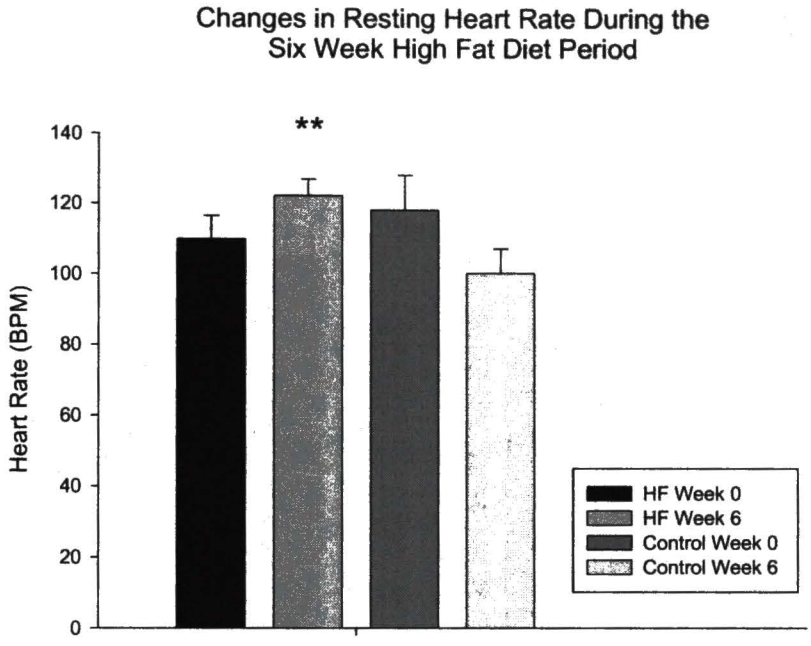

Figure 1

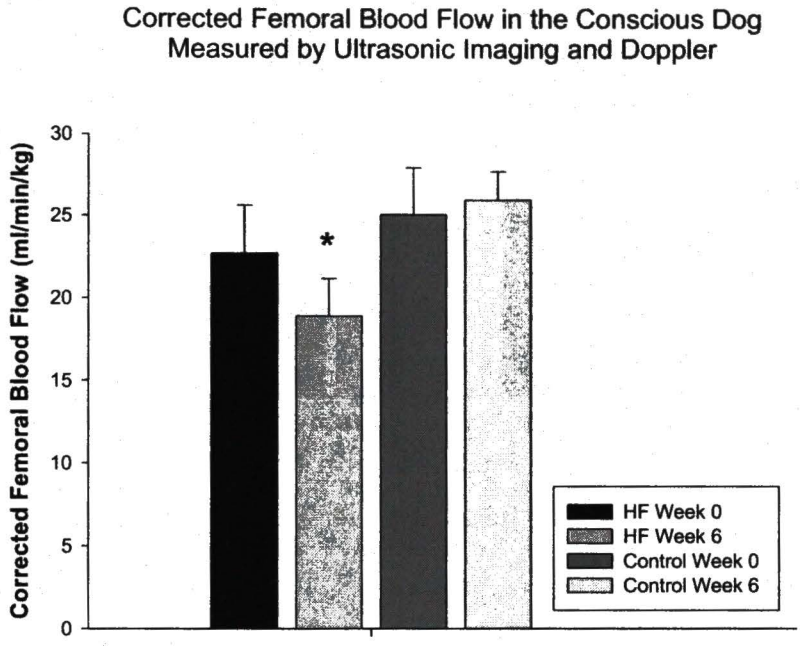
Corrected Femoral Blood Flow in the Conscious Dog
Measured by Ultrasonic Imaging and Doppler
Corrected Femoral Blood Flow (ml/min/kg)
30
25
20
15
10
5
0
*
HF Week 0
HF Week 6
Control Week 0
Control Week 6

Figure 2

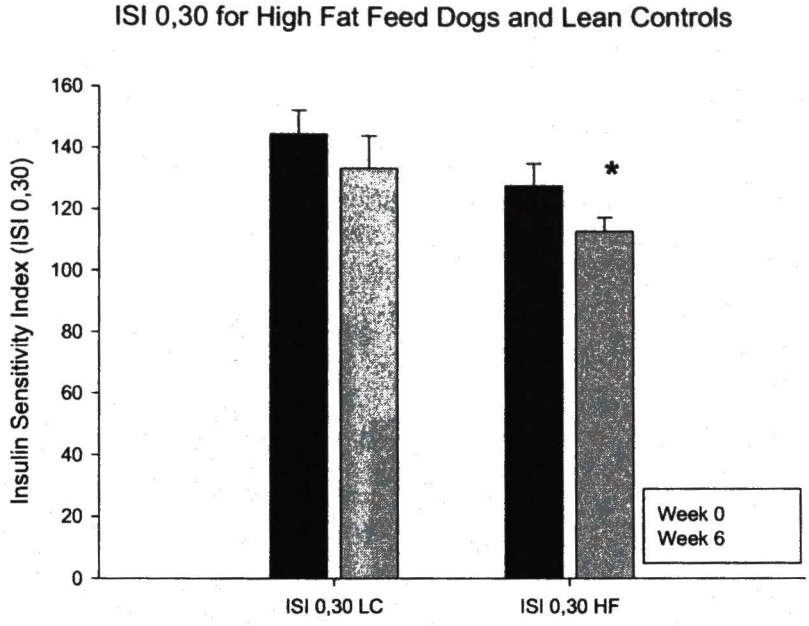
ISI 0,30 for High Fat Feed Dogs and Lean Controls
Insulin Sensitivity Index (ISI 0,30)
160
140
120
100
80
60
40
20
0
*
Week 0
Week 6
ISI 0,30 LC
ISI 0,30 HF

Figure 3

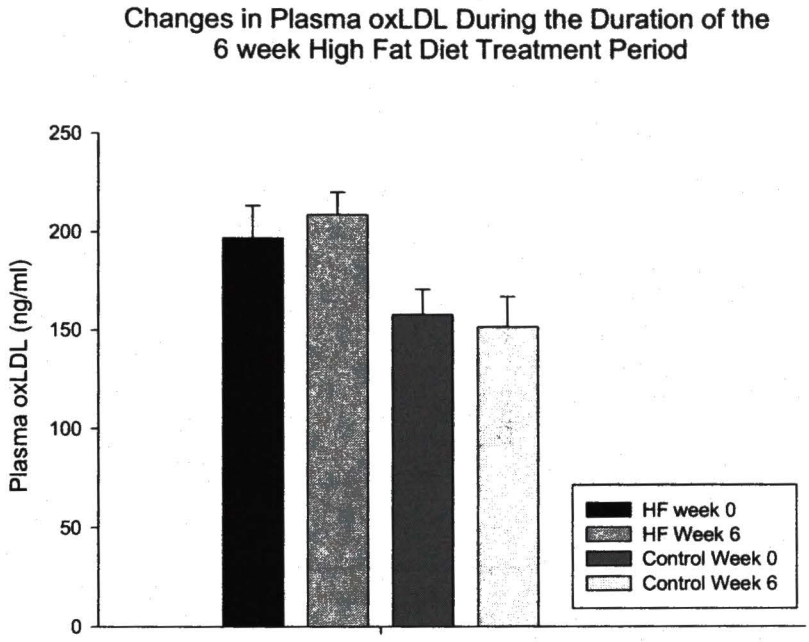
Changes in Plasma oxLDL During the Duration of the 6 week High Fat Diet Treatment Period
Plasma oxLDL (ng/ml)
250
200
150
100
50
0
HF week 0
HF Week 6
Control Week 0
Control Week 6

Figure 4

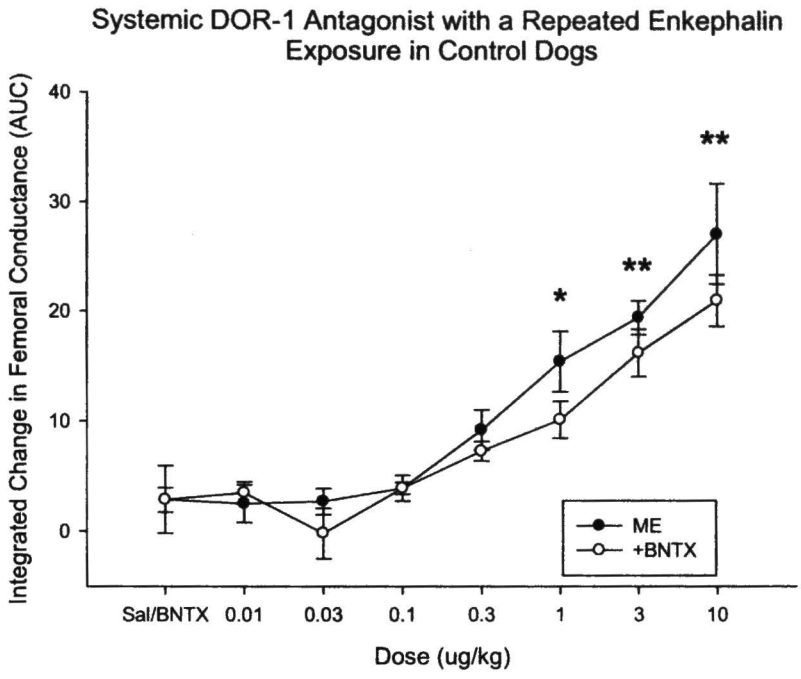
Systemic DOR-1 Antagonist with a Repeated Enkephalin Exposure in Control Dogs
Integrated Change in Femoral Conductance (AUC)
40
30
20
10
0
*
**
**
ME
+BNTX
Sal/BNTX
0.01
0.03
0.1
0.3
1
3
10
Dose (ug/kg)

Figure 5

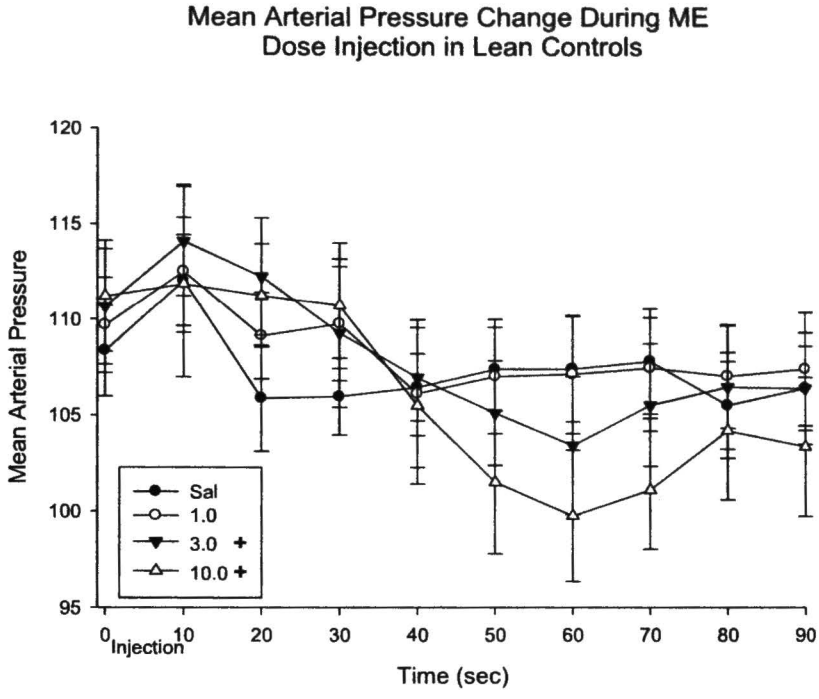
Mean Arterial Pressure Change During ME
Dose Injection in Lean Controls
Mean Arterial Pressure
120
115
110
105
100
95
Sal
1.0
3.0 +
10.0 +
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 6a

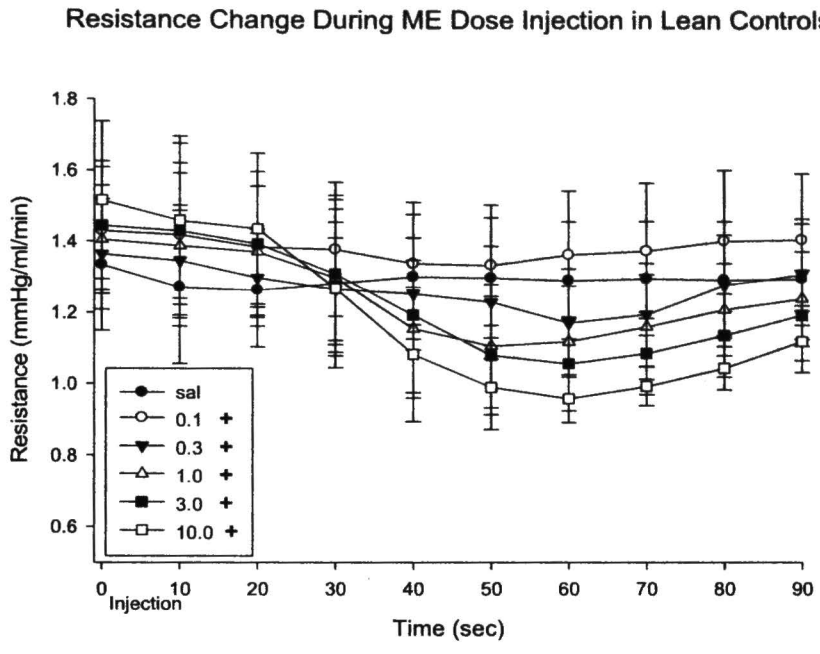
Resistance Change During ME Dose Injection in Lean Controls
Resistance (mmHg/ml/min)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
sal
0.1 +
0.3 +
1.0 +
3.0 +
10.0 +
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 6b

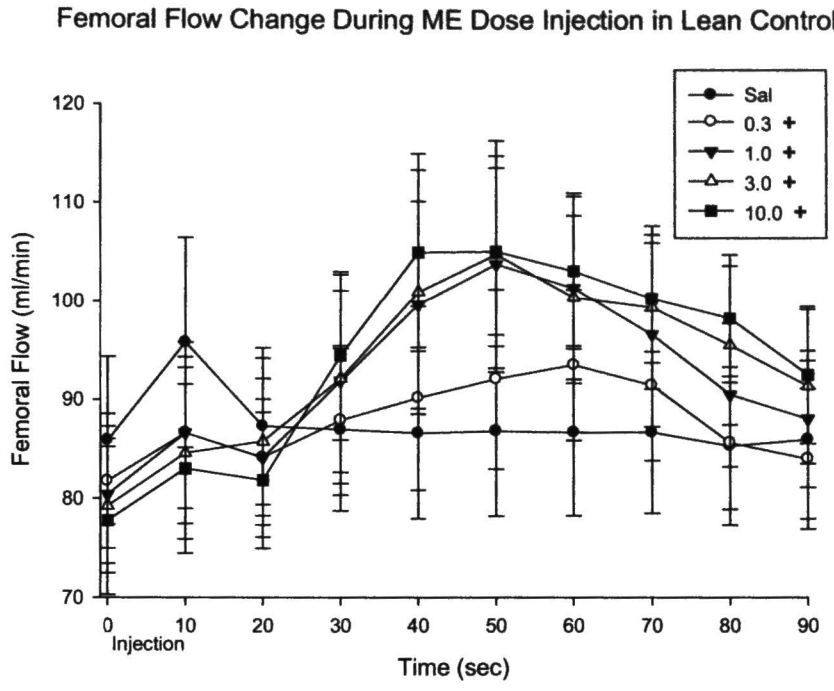
Femoral Flow Change During ME Dose Injection in Lean Controls
Sal
0.3 +
1.0 +
3.0 +
10.0 +
Femoral Flow (ml/min)
120
110
100
90
80
70
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 6c

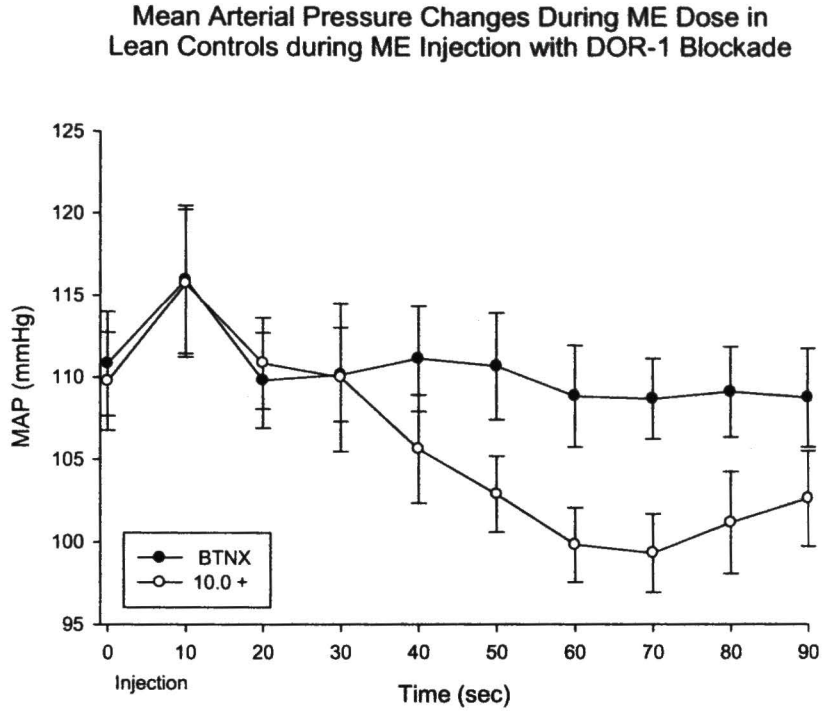
Mean Arterial Pressure Changes During ME Dose in
Lean Controls during ME Injection with DOR-1 Blockade
MAP (mmHg)
125
120
115
110
105
100
95
BTNX
10.0 +
0
10
20
30
40
50
60
70
80
90
Injection
Time (sec)

Figure 7a

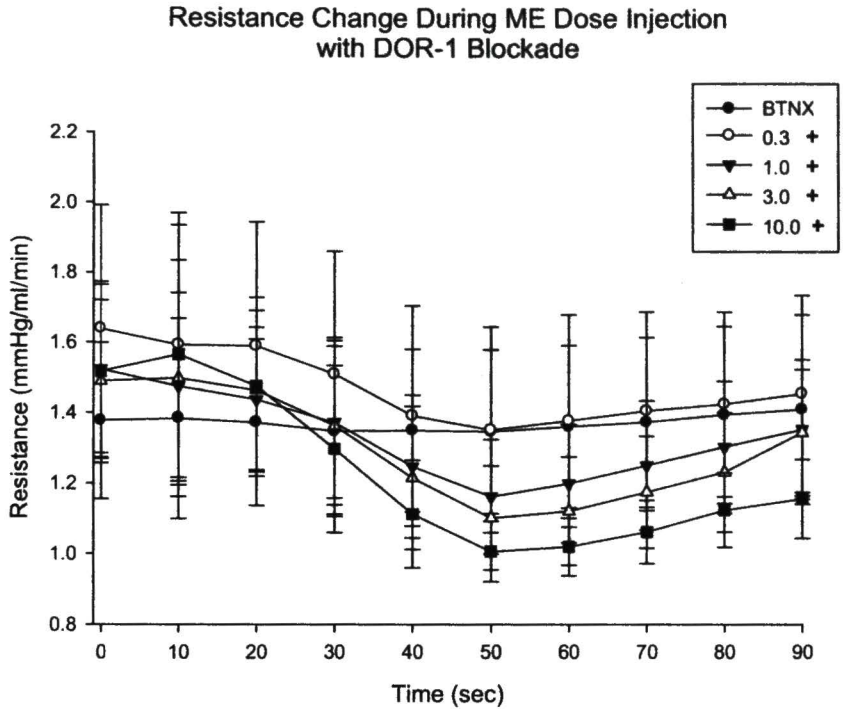
Resistance Change During ME Dose Injection
with DOR-1 Blockade
BTNX
0.3 +
1.0 +
3.0 +
10.0 +
Resistance (mmHg/ml/min)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 7b

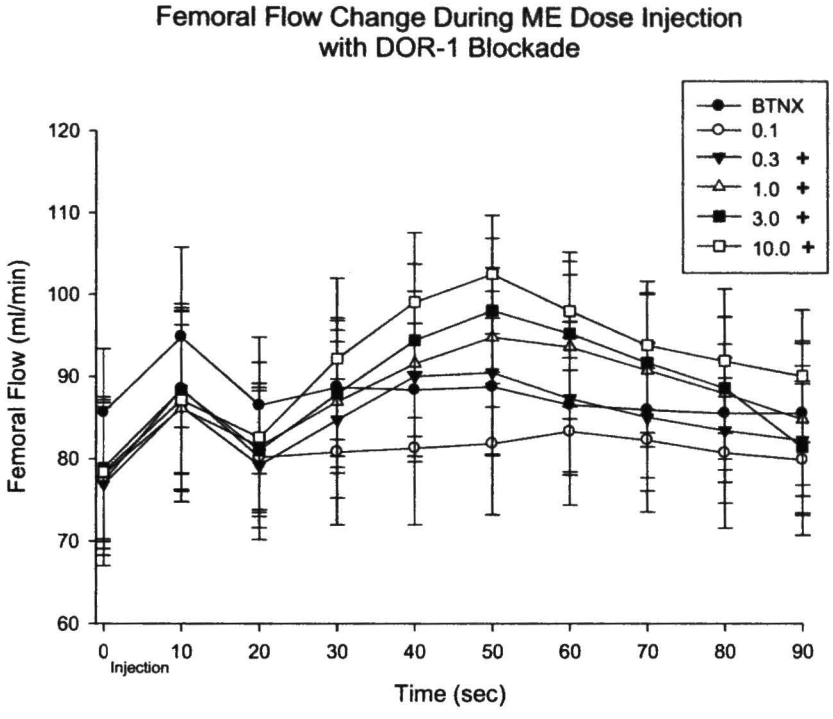
Femoral Flow Change During ME Dose Injection
with DOR-1 Blockade
BTNX
0.1
0.3 +
1.0 +
3.0 +
10.0 +
Femoral Flow (ml/min)
120
110
100
90
80
70
60
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 7c

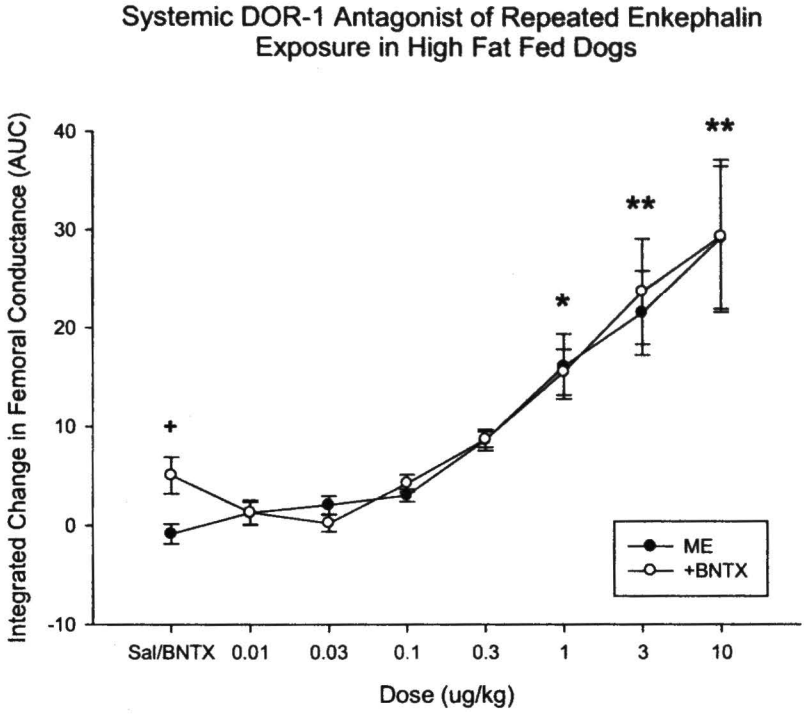
Systemic DOR-1 Antagonist of Repeated Enkephalin Exposure in High Fat Fed Dogs
Integrated Change in Femoral Conductance (AUC)
40
30
20
10
0
-10
+
*
**
**
ME
+BNTX
Sal/BNTX
0.01
0.03
0.1
0.3
1
3
10
Dose (ug/kg)

Figure 8

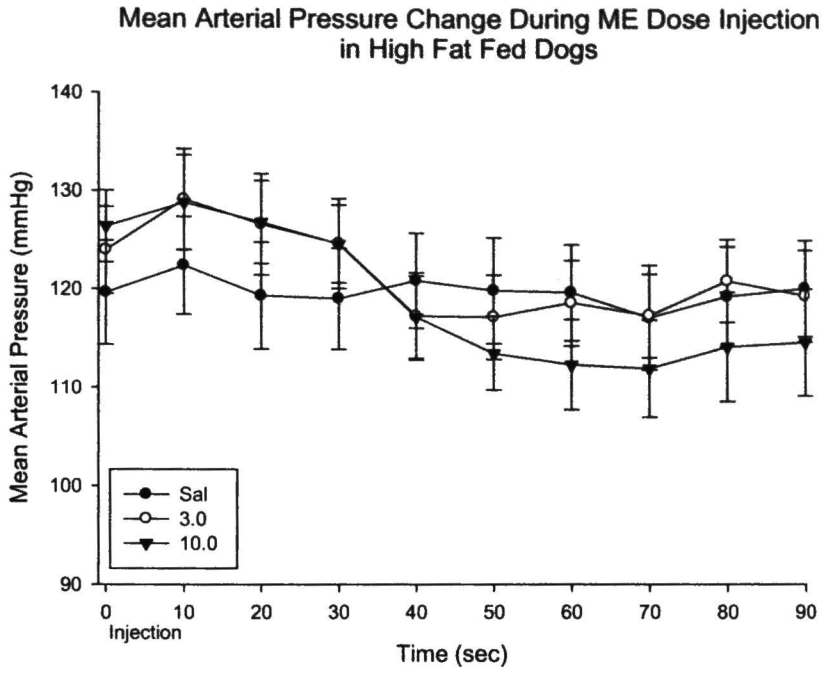
Mean Arterial Pressure Change During ME Dose Injection
in High Fat Fed Dogs
Mean Arterial Pressure (mmHg)
140
130
120
110
100
90
Sal
3.0
10.0
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 9a

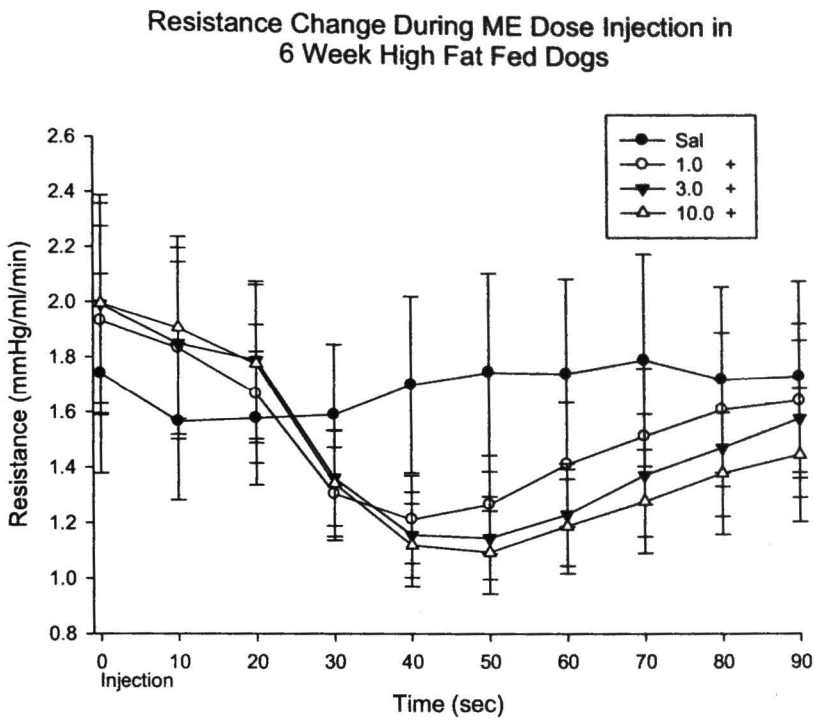
Resistance Change During ME Dose Injection in
6 Week High Fat Fed Dogs
Sal
1.0 +
3.0 +
10.0 +
Resistance (mmHg/ml/min)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 9b

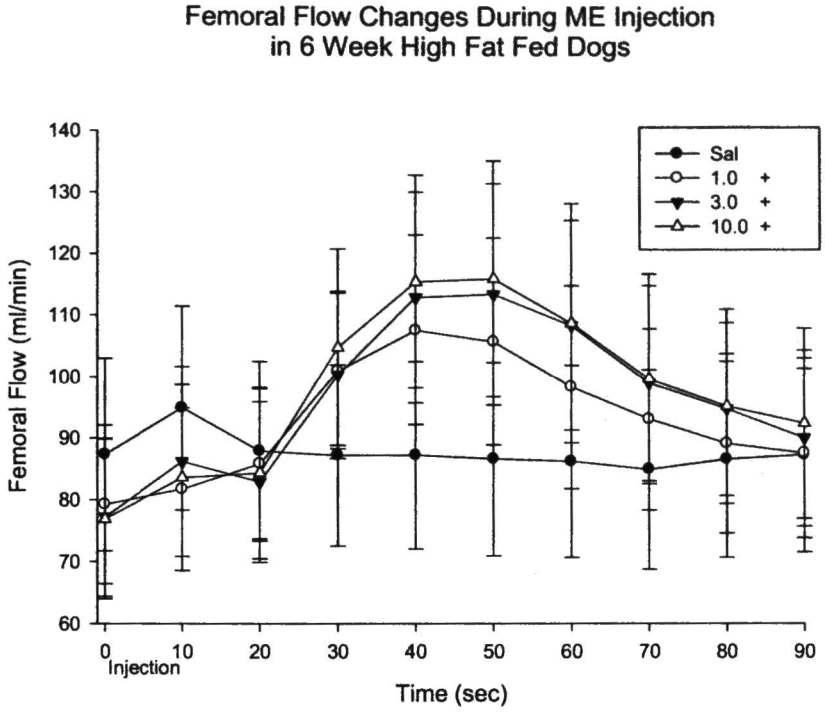
Femoral Flow Changes During ME Injection
in 6 Week High Fat Fed Dogs
Femoral Flow (ml/min)
140
130
120
110
100
90
80
70
60
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)
Sal
1.0 +
3.0 +
10.0 +

Figure 9c

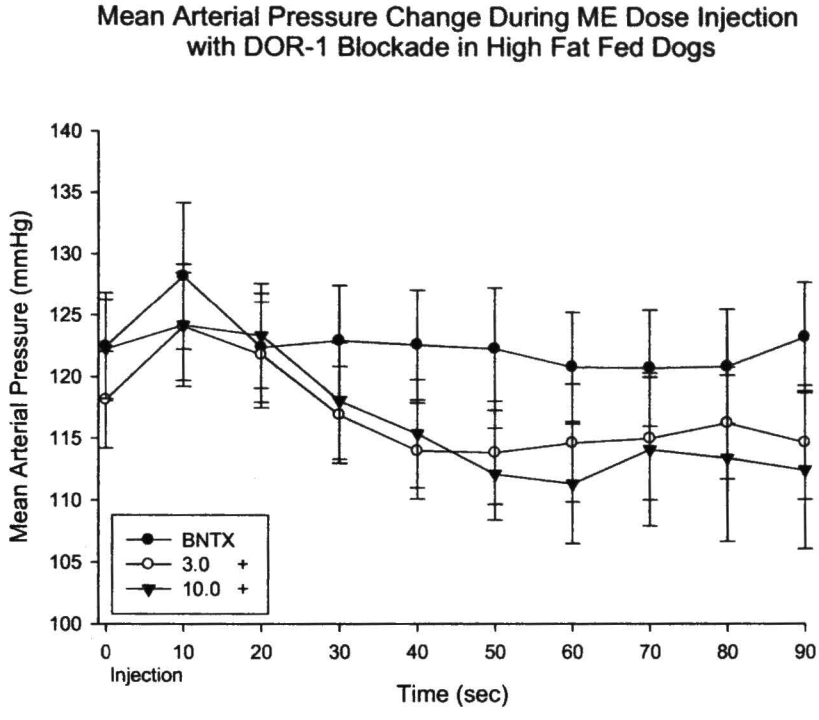
Mean Arterial Pressure Change During ME Dose Injection
with DOR-1 Blockade in High Fat Fed Dogs
Mean Arterial Pressure (mmHg)
140
135
130
125
120
115
110
105
100
BNTX
3.0 +
10.0 +
0
10
20
30
40
50
60
70
80
90
Injection
Time (sec)

Figure 10a

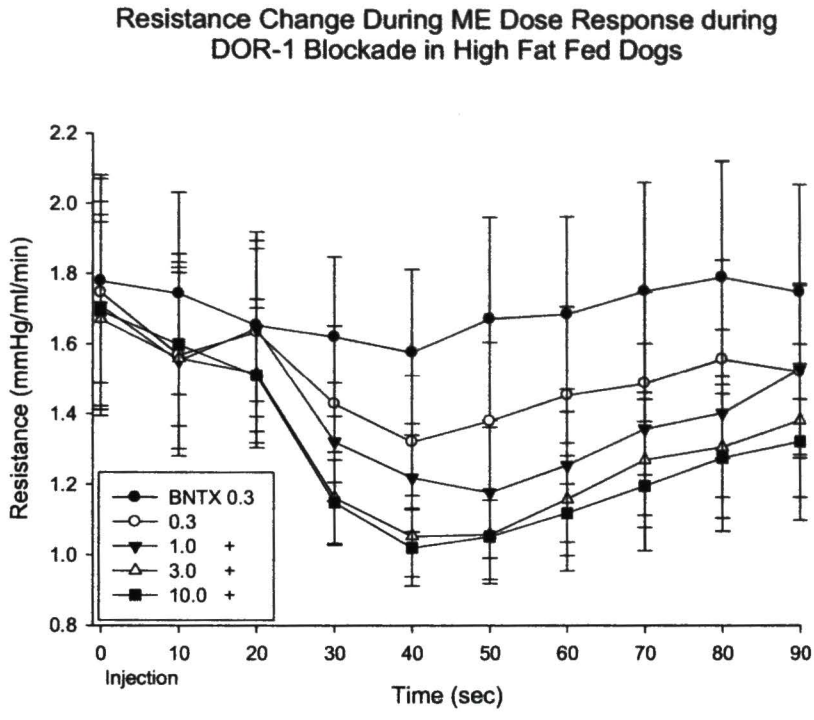
Resistance Change During ME Dose Response during
DOR-1 Blockade in High Fat Fed Dogs
Resistance (mmHg/ml/min)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
BNTX 0.3
0.3
1.0 +
3.0 +
10.0 +
0
Injection
10
20
30
40
50
60
70
80
90
Time (sec)

Figure 10b

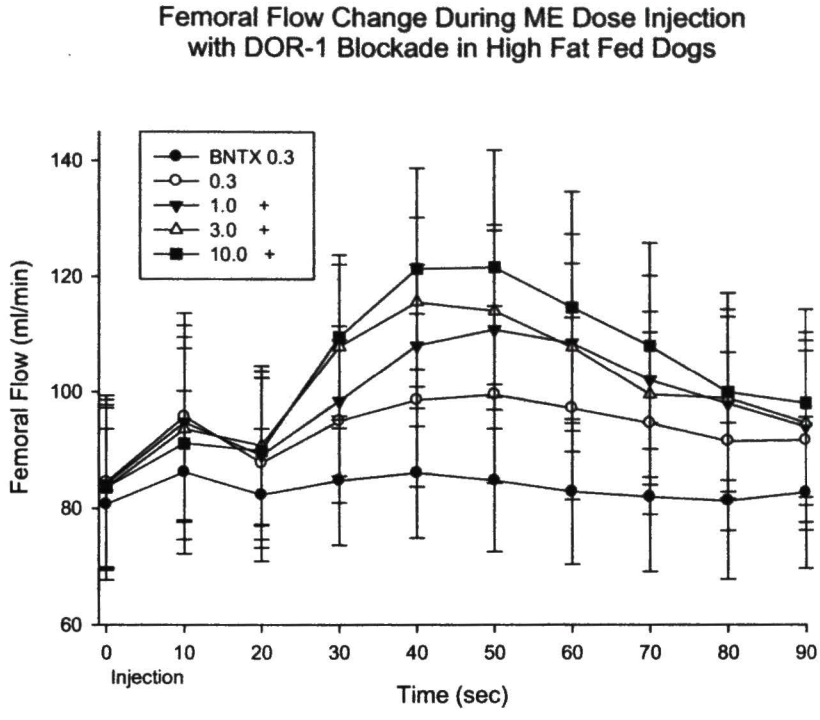
Femoral Flow Change During ME Dose Injection
with DOR-1 Blockade in High Fat Fed Dogs
BNTX 0.3
0.3
1.0 +
3.0 +
10.0 +
Femoral Flow (ml/min)
140
120
100
80
60
0
10
20
30
40
50
60
70
80
90
Injection
Time (sec)

Figure 10c

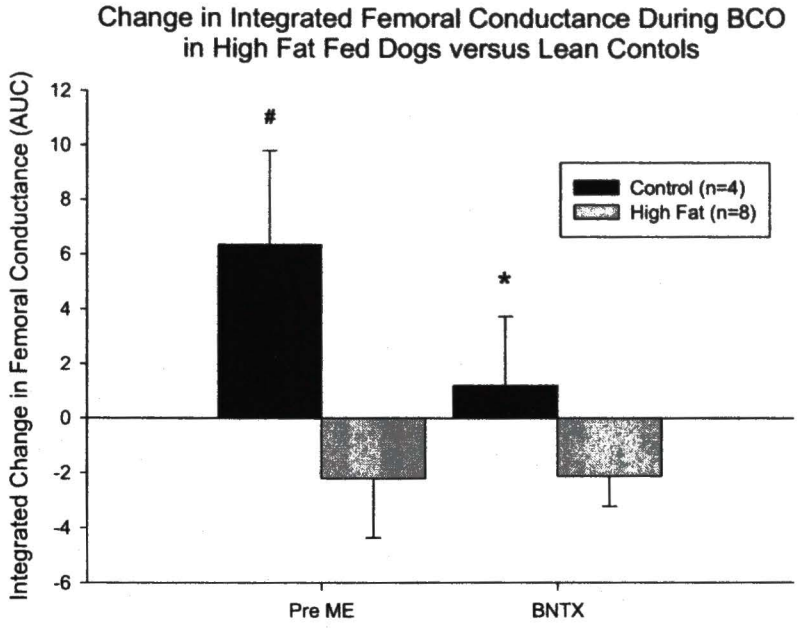
Change in Integrated Femoral Conductance During BCO
in High Fat Fed Dogs versus Lean Contols
Integrated Change in Femoral Conductance (AUC)
12
10
8
6
4
2
0
-2
-4
-6
#
*
Control (n=4)
High Fat (n=8)
Pre ME
BNTX

Figure 11

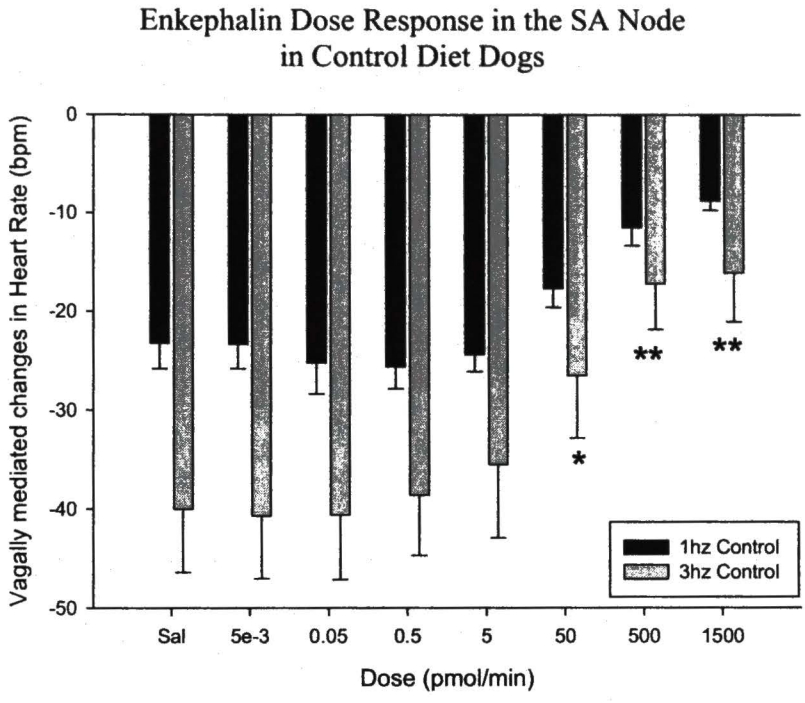
Enkephalin Dose Response in the SA Node
in Control Diet Dogs
Vagally mediated changes in Heart Rate (bpm)
0
-10
-20
-30
-40
-50
Sal
5e-3
0.05
0.5
5
50
500
1500
Dose (pmol/min)
*
**
**
1hz Control
3hz Control

Figure 12a

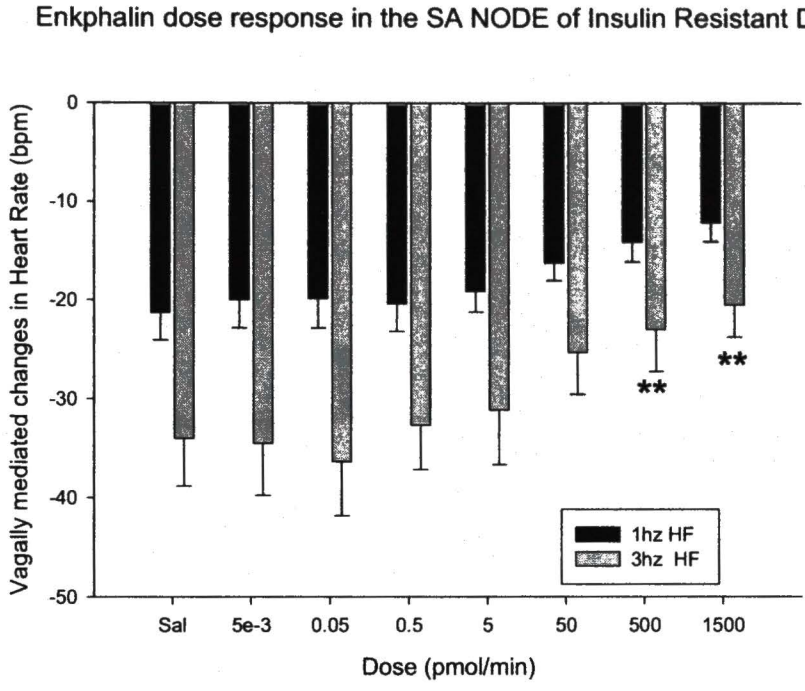
Enkphalin dose response in the SA NODE of Insulin Resistant Dogs
Vagally mediated changes in Heart Rate (bpm)
0
-10
-20
-30
-40
-50
Sal
5e-3
0.05
0.5
5
50
500
1500
**
**
1hz HF
3hz HF
Dose (pmol/min)

Figure 12b

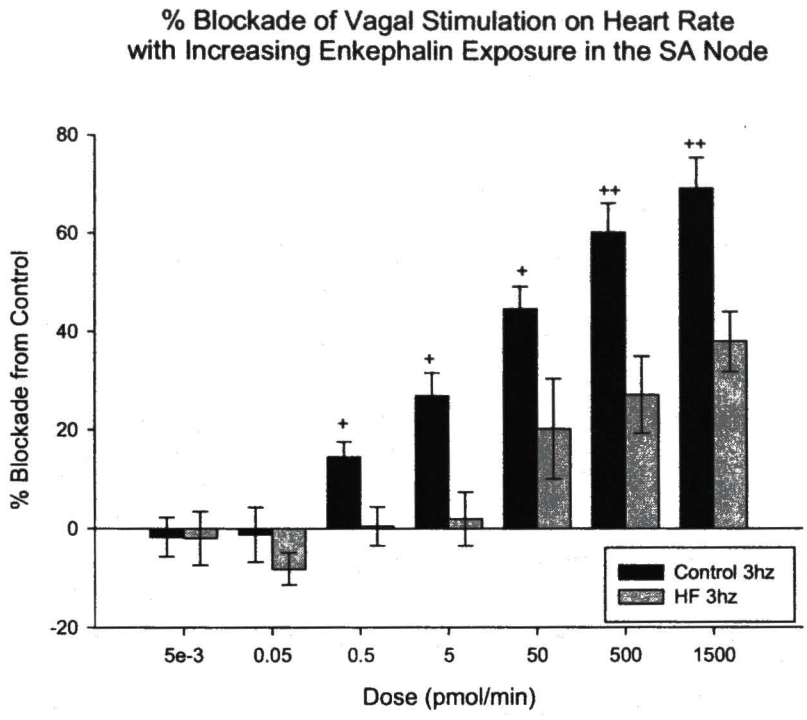
% Blockade of Vagal Stimulation on Heart Rate
with Increasing Enkephalin Exposure in the SA Node
% Blockade from Control
80
60
40
20
0
-20
+
+
+
++
++
5e-3
0.05
0.5
5
50
500
1500
Dose (pmol/min)
Control 3hz
HF 3hz

Figure 13

CHAPTER IV

SUMMARY AND CONCLUSIONS

- Enkephalins modulate femoral vascular conduction by interrupting vasomotor transmission through lumbar sympathetic chain ganglion.
- Enkephalins interrupt ganglionic transmission through interaction with ganglioninc DOR-2.
- Hyperinsulinemic canine model may shift the balance of the opposing ganglionic DORs. Whether this is pathology or compensation remains to be determined.
- Changing the neuronal membrane environment in which the opioid receptors reside can modify the receptor associations including GM1 and the G-protein-coupled proteins (Gsα or Gi/Goα to change the phenotype they express.
- Cardiometabolic disease (insulin resistance syndrome, diabetes and hypertension) very likely changes how the opioid regulate neural traffic within the peripheral autonomic nervous system and could aggravate expression of the disease or accelerate its progress.
- However, if the complement of opioid receptors can be favorably shifted, the disease may be responsive to positive modification.

CHAPTER V

FUTURE STUDIES

The following studies are proposed to further clarify the role of DOR's and their changing phenotypes in the cardiovascular system which may be prone to maladaption during cardiometabolic desease progression.

1. To measure the proportion of DOR on the sympathetic and parasympathetic prejunctional nerve terminal following hyperlipidemic diet treatments using immunocytochemistry and western blot and correlate these changes with function in *vivo.*
2. Using a prolonged hyperinsulinermic diet protocol of 12 weeks, determine the continued DOR function continued progression of dysfunction of the cardiometabilic disease in both the SA node and sympathetic chain ganglion.
3. Determine who endurance exercise training in the dog model may change the DOR phenotype expression in the SA node and sympathetic chain ganglion.
4. After prolonged 12 week diet, define the role of intermittent hypoxic conditioning and exercise training to possible reverse or protect from the maladaptation roles of the DOR in these peripheral ganglion.
5. Determine if treatments of hyperlipidemia and exersize run concurently would be able to counter any of the expected cardiometabolic maladaptations of the DOR control of nerve traffic control.