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Methionine enkephalin arginine phenylalanine (MERF) has been shown to be co-stored with catecholamines in vesicles. The catecholamines appear to decrease the degradation rate of ^{3}H-MERF in vitro. The aim of this study is to investigate the spillover and metabolism of MERF across the canine heart vascular bed. I hypothesize that ^{3}H-MERF is either degraded in the plasma or taken up and degraded by the heart. I further hypothesize that the exogenous catecholamine, isoproterenol, inhibits or reduces the rate of MERF degradation. Mongrel dogs were anesthetized and instrumented to record cardiovascular parameters, infuse ^{3}H-MERF, and obtain blood samples across the heart. Blood samples were taken before and after stopping ^{3}H-MERF infusion to evaluate kinetics, show steady state, and test the effect of treatments. Steady state concentration of ^{3}H-MERF was observed after 30 min of infusion. Chromatography separated intact from degraded ^{3}H-MERF. Three experimental groups were used: control, propranolol plus isoproterenol, and propranolol only. Blockade of β - receptors was necessary to prevent changes in coronary blood flow. Propranolol bolus (0.2 mg/kg) was administered IV at 50 min. 3 μg/min isoproterenol or 0.5 ml/min normal saline was infused starting at 70 min until the end of sample collection. The ^{3}H-MERF venous-arterial (V-A) difference prior to treatment was negative, indicating degradation in the plasma or uptake and degradation by the heart. The 75 min V-A difference was used to calculate the effect of

the infusions on the degradation or uptake of the ^{3}H-MERF; this value was unchanged by any treatment. Spillover of ^{3}H-MERF was significantly lower in the propranolol + isoproterenol dogs ($p < 0.05$) compared to propranolol only treatment at 75 min. Heart rate was significantly lower for the propranolol only group compared to control. Blood pressure and change in coronary flow were unchanged. In conclusion, isoproterenol does not affect the metabolism of ^{3}H-MERF across the canine heart vascular bed. Propranolol, however, does increase the intact ^{3}H-MERF in the plasma, but additional β adrenergic blockade agents need to be investigated to determine the mechanism by which this takes place.

MET-ENKEPHALIN-ARG-PHE (MERF)

AND METABOLISM OF MERF ACROSS

THE CANINE HEART VASCULAR BED

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MET – ENKEPHALIN – ARG – PHE (MERF) AND METABOLISM OF MERF ACROSS THE CANINE HEART VASCULAR BED

THESIS

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LIST OF ABBREVIATIONS

ANF	:	Atrial natriuretic factor
CHF	:	Congestive heart failure
CPM	:	Counts per minute
CVP	:	Central venous pressure
dP/dt	:	Change in pressure over change in time
HR	:	Heart rate
IV	:	Intravenous
LVP	:	Left ventricular pressure
MAP	:	Mean arterial pressure
MERF	:	Methionine-enkephalin-arginine-phenylalanine
ME	:	Methionine enkephalin
NE	:	Norepinephrine
NS	:	Normal saline
NPY	:	Neuropeptide Y
RIA	:	Radioimmunoassay
T-G-G-F-M-R-F	:	Tyrosine-glycine-glycine-phenylalanine-methionine-arginine-phenylalanine
V-A	:	Venous – arterial difference

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CHAPTER 1

INTRODUCTION

Methionine-enkephalin-arginine-phenylalanine (MERF) is an endogenous opioid heptapeptide found in almost all tissues in the body (duodenum, ileum, lung, heart, brain, adrenal gland, pancreas, etc.) (18). Endogenous opiates modulate the effects of classical neurotransmitters, with their effects determined by the state of autonomic balance at the time of a stimulus (3). The role of enkephalins in tissue other than nerves is not completely understood or known. MERF appears to be concentrated in the myocardium and is well positioned to function as a local paracrine regulator (2).

Proenkephalin is produced in canine cardiomyocytes. The proenkephalin is then cleaved by a prohormone convertase to peptide B and other intermediate products (19). Peptide B is further cleaved into MERF. MERF is the carboxyl terminus of peptide B and proenkephalin (19). The amino acid sequence of MERF is T-G-G-F-M-R-F. MERF immunoreactivity is five times more concentrated in the ventricles than atria in contrast to met enkephalin, which is evenly distributed throughout the canine myocardium (2). MERF concentration is four to five times greater than met enkephalin in the atria and thirty times greater in the ventricles as shown in Table I (2). This leads us to believe that MERF has a more significant role in the canine myocardium than met enkephalin.

Assay	R Atrium	L Atrium	R Ventricle	L Ventricle	Septum
MERF RIA	24.9 ± 2.9	23.2 ± 2.6	128 ± 15.6*	112 ± 11.5*	116 ± 12.7*
ME RIA	3.6 ± 0.3	4.2 ± 0.6	4.8 ± 0.6	4.4 ± 0.5	3.5 ± 0.4

Table I
Opioid content in canine heart tissue. Values are in fmol/mg protein. Values are mean ± SEM. n=30, * $p < 0.001$ (2).

Enkephalins have also been located in nerve endings in sympathetic ganglia, the vagus nerve, and the splanchnic nerve (14). Previous data suggests that enkephalins are co-stored in vesicles with catecholamines and act as co-transmitters (14). Data has suggested that cardiovascular responses to enkephalins are dependent upon the dominating branch of the autonomic nervous system (5). It is important to note that the catecholamines, epinephrine and norepinephrine, also have been shown to be capable of inhibiting the degradation of enkephalins and could serve to increase the concentration of biologically active enkephalins (5). Endogenous opioids also modulate catecholamine release. Opioids limit norepinephrine release during sympathetic nerve stimulation (12). This is part of an intrinsic feedback mechanism that regulates norepinephrine release and cardiac excitability (12). Enkephalins also modulate the atrial responsiveness to norepinephrine as well as the calcium kinetics within cardiac myocytes (20). Neuromodulatory opioids are also capable of influencing cardiac excitability by restraining vagal control of heart rate and contractility (3). Enkephalins are contained in the heart. There is evidence of enkephalin action to modulate autonomic function in the

heart (3,12,20). There is also evidence that these opioids work in conjunction with atrial natriuretic factor (ANF) in the control of blood pressure (11).

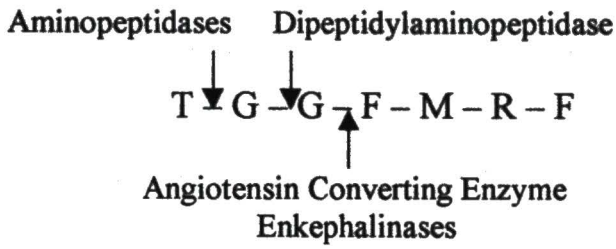


Figure I
Cleavage sites of enkephalin degradation by enzymes (16).

Aminopeptidases, enkephalinases, and angiotensin converting enzyme are responsible for the degradation of MERF (Figure I). The major breakdown product of MERF is dependent upon the tissue in which the degradation takes place. In plasma, these enzymes cleave the peptide bond of tyrosine in position 1 (Figure I)(2,8). Free tyrosine is also the main degradation product in brain tissue with lower levels of T-G-G found in the brain (Figure I)(8). The primary mechanism for breakdown of met-enkephalin is the T-G bond in both plasma and the brain (Figure I)(8). The importance of this bond for the action of the degrading enzymes, aminopeptidases, has been shown by the long lasting activity of enkephalin analogs substituting D-alanine for glycine at position 2 [T-A*-G-F-M] (8). Currently, the processing or degradation of enkephalins across the heart vascular bed is unknown and is one of the aims of the current investigation.

Spillover is a phenomenon in which a substance, for example catecholamines, enters the plasma. This occurs when the rate of release of the substance in question exceeds the rate of uptake or degradation, thus spillover into the plasma is observed and can be measured. Two major types of spillover are known. First, there is regional spillover. This is the spillover from one particular organ or region, such as the heart. Second, there is total body spillover, which is the cumulation of all of the regional body spillover values. Esler et al. has described spillover of other constituents released with catecholamines, such as the peptide, neuropeptide Y (NPY) (9). Although no definitive importance of the value of the overflow of NPY is known, it has been suggested that NPY spillover may have special significance when measured as a cardiovascular effector (9). Several factors are important in determining spillover. These include the rate of release and the activity of the competing disposition mechanisms (9). These mechanisms include uptake, degradation, and diffusional flow into the circulation (9). This spillover into circulation is influenced by regional blood flow and the exchange conductivity of the capillary and postcapillary venular bed (9). In order to evaluate spillover, the substance in question must be at steady state. In this experiment, the spillover values for MERF were calculated based on the assumption that cardiac extraction of MERF is negligible. The kinetics of MERF were at steady state at the times used to evaluate MERF spillover.

Opioids possess clinically significant roles. Some autonomic imbalances and associated pathologies are associated with disturbances of endogenous opioid

neuromodulators (3). Elevated endogenous opioid levels could contribute to cardiovascular disease by reducing vagal activity and shifting autonomic balance towards greater sympathetic influence (3). This is the case in both congestive heart failure (CHF) and aging. Vagal dysfunction and elevated opioid levels have been reported in CHF (3). Plasma ANF, endogenous opioid, and NE levels are significantly higher in acute CHF patients (11). Opioid peptides do not modulate ANF release in healthy humans, but it is involved in the control of ANF release in acute CHF (11). In cases of severe acute CHF, opioids inhibit NE release from sympathetic terminals (11). The administration of the opioid antagonist, naloxone, reverses this inhibitory effect and results in NE hypersecretion, thus stimulating ANF release (11). Plasma values of ANF, NE, and enkephalin for healthy human subjects and those in severe acute CHF are shown in Table II. Aging reduces reflex bradycardia during pharmacologically induced increases in blood pressure (3). This is accompanied by a parallel increase in cardiac concentrations of enkephalin and the mRNA for its precursor, proenkephalin (6). The opioid, morphine, is the most commonly used analgesic in patients suffering from chest pain and acute myocardial infarctions. Al'absi et al. reported a correlation between men at risk for hypertension with a positive hypertensive parental history and attenuated pain sensitivity (1). Al'absi et al. also reported that the stimulation of the baroreflex by elevated blood pressure can in turn stimulate the release of endogenous opiates in the medulla, leading to attenuation of pain perception (1).

Subject	ANF	ANF + N	NE	NE + N	Enkephalin
Healthy	7.5 ± 0.5	7.8 ± 0.7	151.3 ± 3.6	153.5 ± 3.7	15.0 ± 1.4
Severe Acute CHF	53.8 ± 1.0*	68.0 ± 1.4*	563.8 ± 13.4*	776.6 ± 18.7*	41.0 ± 3.2*

Table II.

Basal plasma levels of atrial natriuretic factor (ANF, pg/ml), norepinephrine (NE, pg/ml), and enkephalin (fmol/ml), and effects of naloxone (N) administration in healthy subjects and congestive heart failure (CHF) patients. * $p < 0.01$ (11).

Both enkephalins and catecholamines are important regulators of cardiovascular function. Much research has been performed on catecholamines and their role in this regulation. Catecholamine spillover has been shown to be an indicator of sympathetic function. Relative to the amount of research performed on catecholamines, the role of MERF is much less known and has been investigated in far less detail.

These experiments are designed to provide information necessary to determine MERF spillover across the cardiac vascular bed. In order to evaluate the ability of catecholamines to change extraction, if any exists, or degradation independent of beta-receptors, the effect of beta-adrenergic blockade was first evaluated. A beta-receptor blockade was necessary to prevent changes in cardiac function, such as HR, MAP, and coronary blood flow, confounding the results. The beta-adrenergic blocker DL-propranolol was chosen to induce a total beta-receptor blockade. The effects of cardiac beta-receptors on ^{3}H-MERF spillover were evaluated with isoproterenol in combination with propranolol.

Purpose of the Study

The purpose of this study was to determine whether the canine heart shows a spillover phenomenon for MERF. If it does show MERF spillover, does the heart take up, or extract enkephalins from the plasma as it does catecholamines? If so, can MERF spillover also be an indicator of cardiac function or an indicator of a pathologic condition?

Specific Aim and Hypothesis

The specific aim of this study was to investigate the metabolism of MERF across the canine heart vascular bed with the hypothesis being that ^{3}H-MERF is either degraded in the plasma or taken up by the heart, but is not re-released by the heart. The venous-arterial difference of ^{3}H-MERF was analyzed across this vascular bed, as well as the percent intact ^{3}H-MERF. This data will be used to support or disprove the hypothesis. I further hypothesize that the exogenous catecholamine, isoproterenol, inhibits or reduces the rate of MERF degradation. This inhibition is done via a mechanism independent of beta-adrenergic receptors, possibly through structural interactions with MERF or the degrading enzymes. The purpose of using isoproterenol is two fold. First, only mixed adrenergic agonists, epinephrine and norepinephrine, have been previously investigated. Isoproterenol is a pure beta receptor agonist and the effect of its structure, independent of the beta receptor properties, was studied by inducing a complete beta-receptor blockade.

CHAPTER 2

METHODS AND PROCEDURES

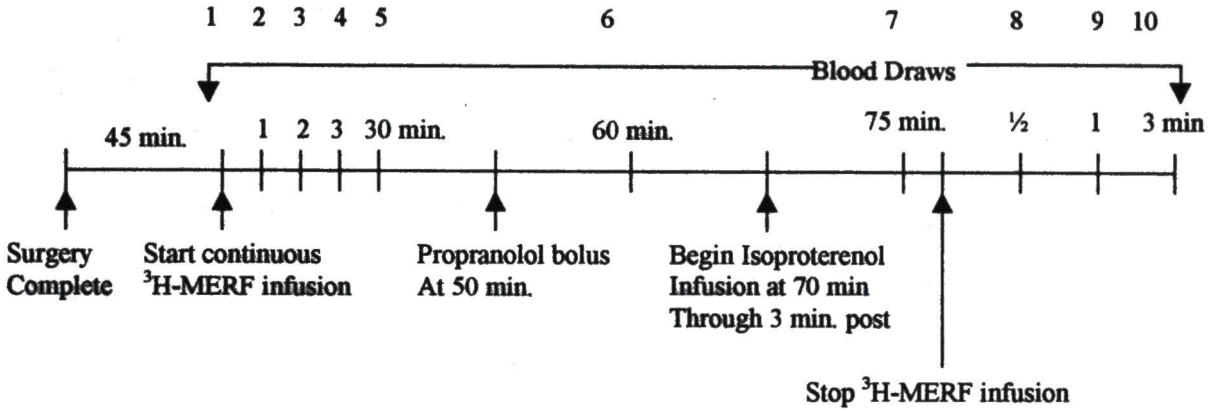


Figure II.
Experimental Protocol

The experimental protocol (figure II) was designed to address the hypotheses by continuously infusing ^{3}H-MERF into a canine subject. The infusion of isoproterenol in the presence of beta adrenergic blockade by propranolol was done in order to investigate the effect of cardiac beta receptors on MERF processing and the structural effect of isoproterenol on the degradation of ^{3}H-MERF. Plasma MERF kinetics were evaluated by calculating the venous-arterial difference to determine whether net degradation, extraction, or re-release of ^{3}H-MERF was taking place. The isoproterenol and propranolol treatments were initiated after the plasma concentrations of ^{3}H-MERF reached steady state kinetics.

SURGICAL PROCEDURE

Mongrel dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg) IV. The dogs were endotracheally intubated and mechanically ventilated with supplemental O_2 as required by monitoring the blood gases. Inguinal incisions were made and the femoral artery and vein were catheterized for administration of fluids, anesthetics, and continuous monitoring of arterial pressure and central venous pressure. A cervical incision was made to expose the left jugular vein, which was cannulated with a catheter. This catheter was advanced into the coronary sinus by feel after opening the chest and exposing the heart. This catheter was used to obtain blood from the heart's venous drainage. The dog was given succinylcholine (1 mg/kg) IV to induce a temporary neuromuscular blockade during surgical opening of the chest. The chest was opened through the left fourth intercostal space. Ribs were cut at the sternal interface as needed, and the heart was exposed through an incision in the pericardium. A Millar pressure transducer was placed in the left ventricle through the left atrium to obtain dP/dt, left ventricular pressure (LVP), and heart rate (HR). The left anterior descending (LAD) coronary artery was isolated and an external Transonic flow probe was placed around the vessel to monitor changes in coronary blood flow. Another catheter was placed in the left atrium for administration of [^{3}H]-MERF. After completion of the surgical preparation, a rest period of 45 min was given prior to beginning the MERF infusion in order to let catecholamine levels and other parameters to return to baseline values.

EXPERIMENTAL PROTOCOLS

[^{3}H]-MERF was administered continuously with a syringe infusion pump into the left atrial catheter at 50 nCi/kg/min (0.4 ml/min), or 0.9 pmol/min. An isoproterenol infusion (3 μg/min; 0.5 ml/min) with beta-receptor blockade (propranolol, 0.2 mg/kg intravenous bolus) was also administered in some of the dogs. Beta-receptor blockade was confirmed with a 5 μg intravenous bolus of isoproterenol prior to beginning isoproterenol infusion. Other subjects received a normal saline (NS) infusion with beta-receptor blockade. The effect of isoproterenol/β-blockade on heart rate, change in MAP, change in coronary flow, percent intact [^{3}H]-MERF, and V-A difference was compared to the subjects receiving beta-receptor blockade/NS and those receiving NS/NS. Maintenance doses of pentobarbital were administered to maintain complete anesthesia as needed throughout the experiment. This was monitored by corneal reflexes, jaw tension, heart rate, and blood pressure. Ventilatory changes were also made as needed to return blood gas values towards normal values by increasing or decreasing ventilatory rate, tidal volume, or O_2 flow rate. Blood gas analysis continued until successive readings showed no change in the normal values of pH, pO_2, and pCO_2. Blood samples were taken at the times indicated in figure 2 throughout the experiment to determine any differences in arterial and venous concentrations of the [^{3}H]-MERF across the heart. The dog was euthanized with a bolus injection of KCl at the termination of the experiment.

SAMPLE COLLECTION

Arterial and venous blood samples were collected prior to, and at 1, 2, 3, 30, 60, and 75 minutes after [^{3}H]-MERF infusion, and ½, 1, and 3 minutes after the [^{3}H]-MERF infusion was stopped. Arterial and venous samples, 5 ml each, were collected simultaneously into 5 ml of chilled citric acid in saline (20 mg/ml). This prevents degradation and further processing of endogenous opioid peptides by instantly lowering the temperature and pH and by chelating metal ions required for enzyme activity. The blood samples were centrifuged at 15,000 X g for 10 minutes at 4° C. The supernatant was collected, filtered through a Whatman 0.45 μ filter, aliquoted, and stored at -90° C until processed.

SAMPLE PROCESSING

Poropak Q was used to separate intact MERF from degraded MERF. Poropak Q is a gas chromatography packing that binds phenylalanine under neutral conditions. The phenylalanines were eluted from the column by acidified organic solvent. The plasma samples were thawed at room temperature until the sample was completely in the liquid phase. The sample was centrifuged again at 3200 X g for 15 minutes at 4° C. The supernatant was measured, and each sample was loaded onto a separate column packed with 0.5 ml of Poropak Q. This volume was collected into a new collection tube (load sample). Each column was then washed with 4 ml of double distilled water, and that volume was collected into a new collection tube (wash sample). Next, each column was washed with 3 ml of 1:1:1 ethanol, glacial acetic acid, and double distilled water to elute

the [^{3}H]-MERF. This volume was also collected into a new collection tube (intact sample). An aliquot of each arterial and venous sample from all three passes through the column (1 ml from each sample) were placed into a scintillation vial filled with scintillation fluid (10 ml for the load sample and 5 ml for the water and intact samples). These vials were then placed into a beta counter (Beckman LS7000) to obtain counts per minute. These values were used to determine the V-A difference of the infused [^{3}H]-MERF across the heart and the percent of intact peptide.

SAMPLE ANALYSIS

HPLC: The blood samples were further analyzed to verify what is being measured, for example, intact ^{3}H-MERF, ^{3}H-Tyr, ^{3}H-Tyr-Gly, or ^{3}H-Tyr-Gly-Gly. An aliquot of each sample was dried under vacuum on a speed vac concentrator (Savant). The aliquots were reconstituted with 0.1% trifluoroacetic acid (TFA) and injected on C18 300Å reverse phase HPLC column. The column was eluted with 0.1% TFA/H_2O with a gradient of 0.1% TFA/acetonitrile (ACN) 0-60% over 30 minutes after 10 minute isocratic elution at 1 ml/min. This was done automatically using an ISCO gradient programmer. Detection of radioactive fragments was done by an online Packard radiomatic scintillation detector.

RIA: Dried aliquots of the venous and arterial blood samples were reconstituted with phosphate buffered saline (PBS), pH 7.0 and assayed with antisera specific for MERF. The RIA was used to determine the endogenous MERF concentration in fmol/ml of blood as well as the specific activity of the radiolabled MERF. The specific activity was

calculated as nCi/pmol of the ^{3}H-MERF, and was determined using the preparation of ^{3}H-MERF from each experiment.

ANALYSIS OF DATA

Data were analyzed with a two-way analysis of variance (ANOVA). Dependent variables were accepted as significant with an α level of 0.05. If $p < 0.05$, a one-way analysis of variance (ANOVA) and Tukey's post test was performed to determine between which groups the significant difference exists at an individual time point. The three groups were compared during steady state (60 and 75 min) and at ½ min after stopping the ^{3}H-MERF infusion. Data are presented as the mean ± standard error of the mean. The first 30 min of collection were analyzed to determine the plasma half-life.

CHAPTER 3

RESULTS

Cardiovascular parameters: The mean ± SEM for baseline MAP, HR, coronary flow, dP/dt, LVP, and CVP for all three groups are summarized in Table III. There were no significant differences in any of the baseline cardiovascular parameters between the experimental groups ($p > 0.05$).

Table III. *Cardiovascular data*

	n	MAP mmHg	HR bpm	Coronary Flow ml/min	dP/dt mmHg/sec	LVP MmHg	CVP mmHg
Control	10	113.2 ± 4.7	146.6 ± 5.8	27.39 ± 1.50	2024 ± 137	129.7 ± 5.3	2.5 ± 0.7
Prop	6	107.5 ± 8.9	151.5 ± 10.7	33.61 ± 5.18	1806 ± 182	118.5 ± 8.9	2.1 ± 0.6
Prop + Iso	9	107.2 ± 4.7	147.2 ± 5.6	36.74 ± 3.41	1786 ± 202	125.4 ± 9.3	0.9 ± 0.9

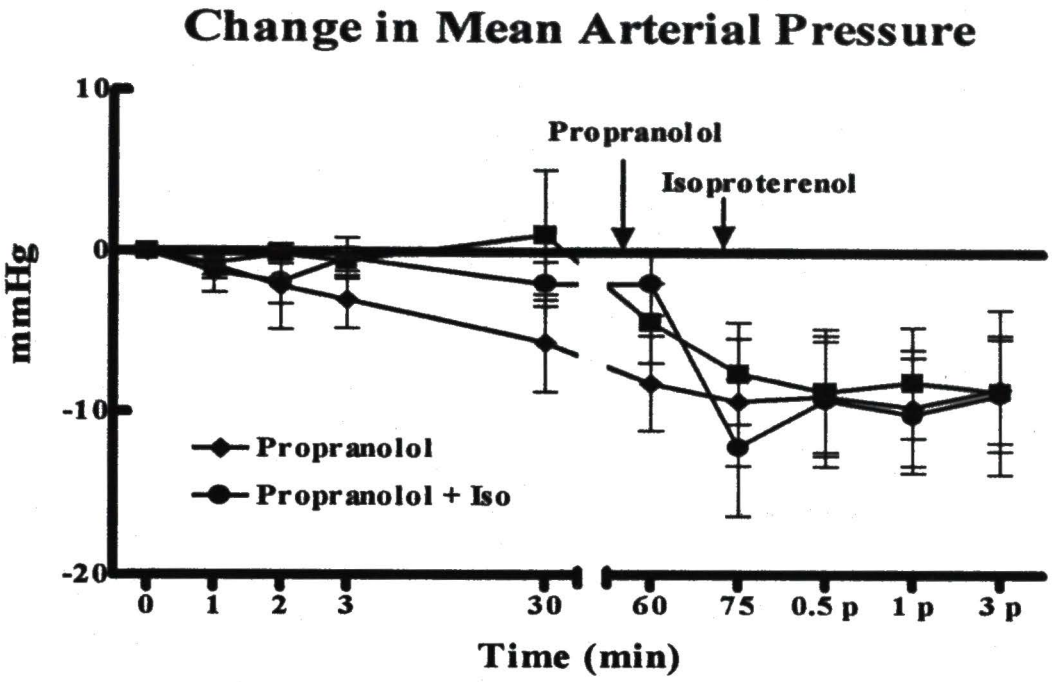
MAP – Mean Arterial Pressure; HR – Heart Rate; dP/dt – Change in Pressure over Change in Time; LVP – Left Ventricular Pressure; CVP – Central Venous Pressure

The data in figure III depicts the change in MAP and change in heart rate as the mean ± SEM. Over time, there is a slight decrease in MAP shown by all three groups. However, there is no significant difference in the change in MAP among the groups for any time point ($p > 0.05$). There is no change in heart rate over time in the control group

($p > 0.05$). Propranolol and propranolol + isoproterenol both significantly lower heart rate compared to control at time points 60 and 75 min ($p < 0.05$). Propranolol also significantly lowers heart rate compared to control at 0.5, 1, and 3 min after stopping the MERF infusion ($p < 0.05$). Isoproterenol has no significant effect on heart rate in the presence of propranolol ($p > 0.05$).

The data in figure IV depicts the changes in dP/dt and left ventricular pressure (LVP) from baseline values as the mean ± SEM. Overall, there is a slight decrease in dP/dt over the course of the experiment. With the exception of the time point 3 min after stopping the infusion, there is no difference in the change among the three groups ($p > 0.05$). There is a decrease in dP/dt for the propranolol + iso group compared to control at time point 3 min after stopping infusion ($p < 0.05$). There is also a decrease in LVP over time for all three groups. There is a decrease in LVP in the propranolol + iso group vs. propranolol at the 75 min time point ($p < 0.05$). There is no significant difference among the three groups for the remaining time points ($p > 0.05$).

The data in figure V depicts the change in coronary flow as a percent from the baseline value as the mean ± SEM. There is a slight decrease in coronary flow over time, as seen with the changes measured in MAP. There is no significant difference in the percent change in coronary blood flow among any of the groups at any time point ($p > 0.05$).



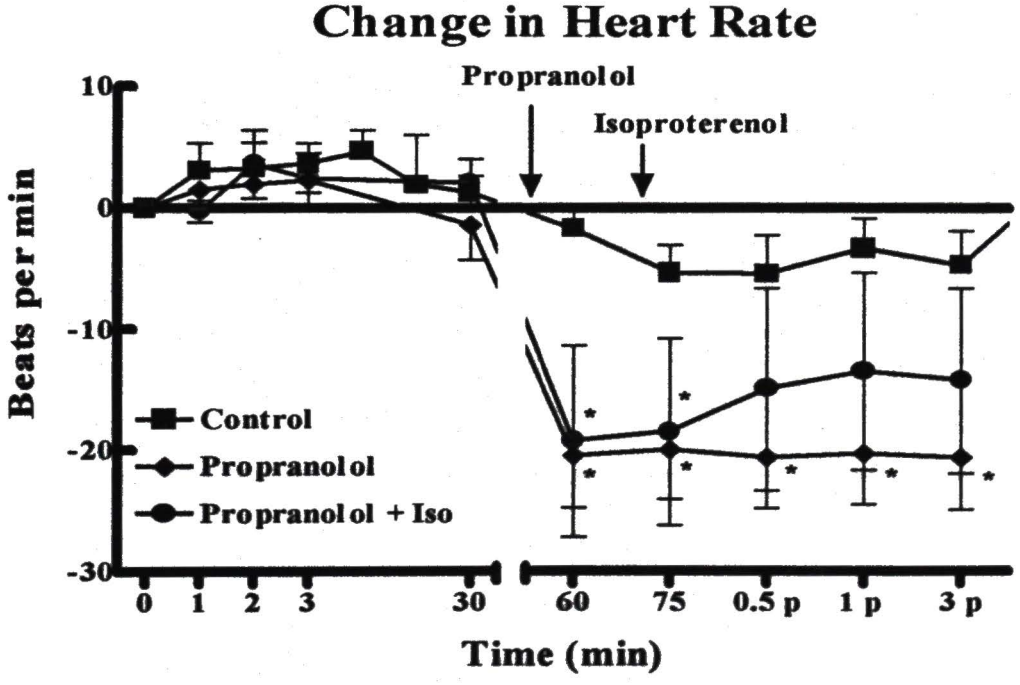
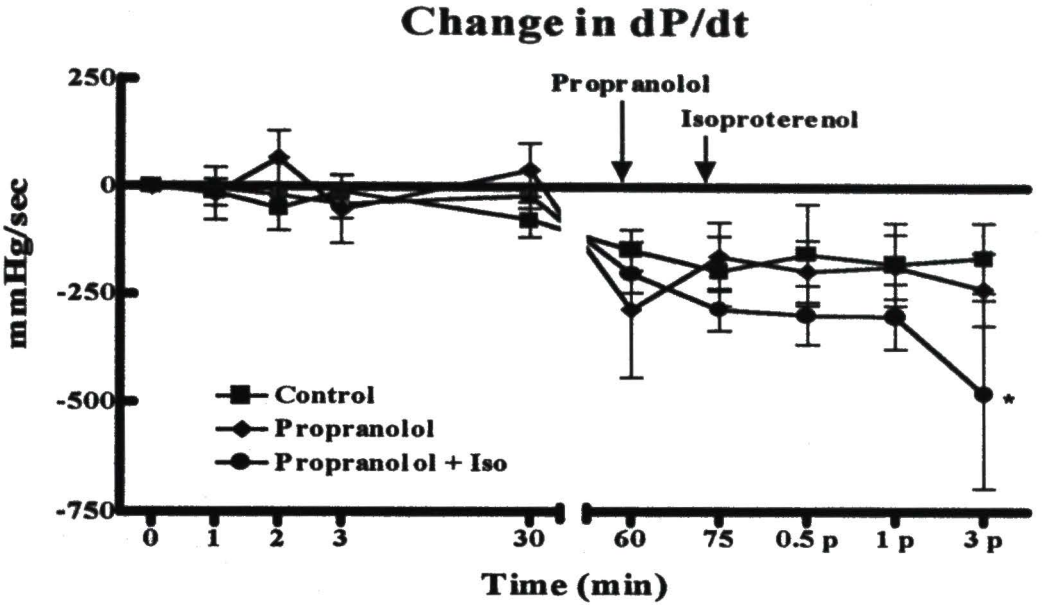


Figure III

Average ± SEM change in mean arterial pressure (MAP) and heart rate (HR) for the three groups. *p < 0.05 vs. control.



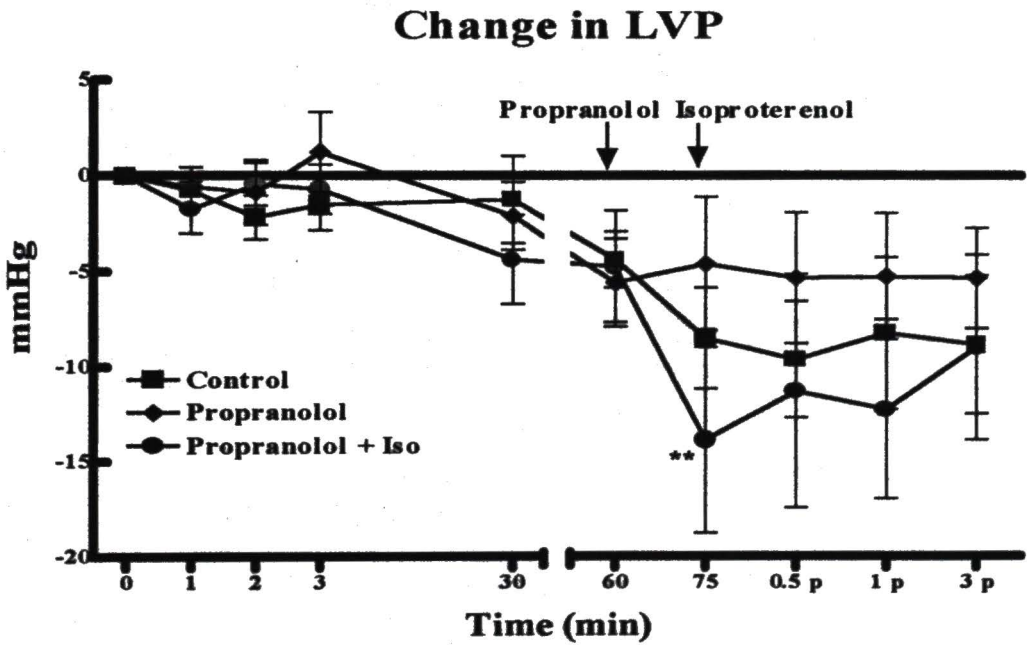
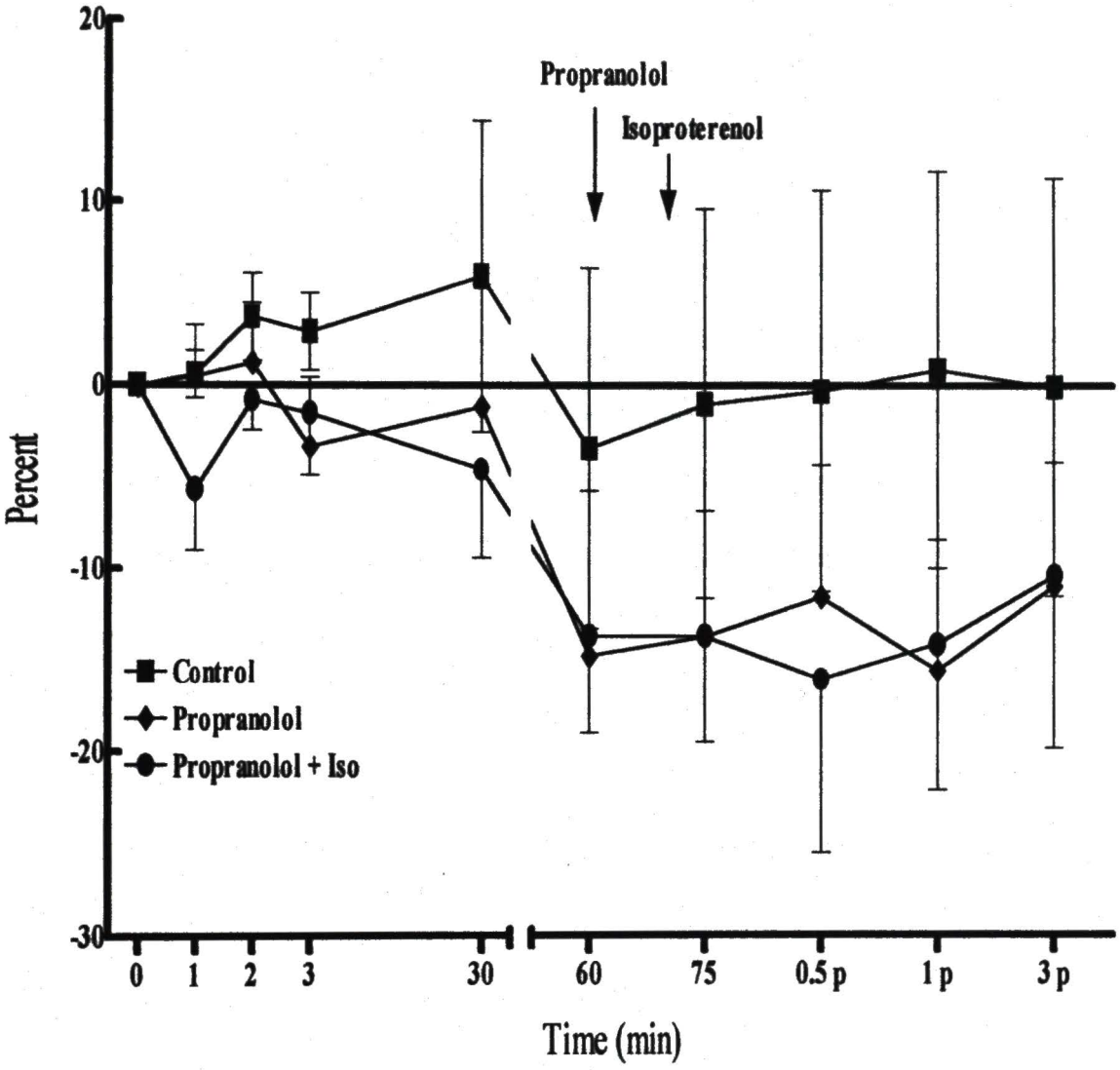


Figure IV

Average ± SEM for the change from baseline in left ventricular pressure (LVP) and dP/dt for all three groups. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. propranolol.



Baseline CF	Avg ± SEM	n
Control	27.39 ± 1.50	6
Propranolol	33.61 ± 5.18	6
Propranolol + Iso	36.74 ± 3.41	7

Figure IV

Average ± SEM percent change from baseline of coronary blood flow for the three groups.

The data in figure VI depicts the venous – arterial (V-A) difference across the heart in counts per minute (CPM) as the mean ± SEM. All groups were treated alike until time 60 min (n = 16), since no treatments had yet been performed. At time 60 min the control n = 8 and propranolol n = 12. For the remainder of the experiment, propranolol n = 4 and propranolol + iso n = 8. The V-A difference is negative during infusion of only ^{3}H-MERF. This indicated either a net uptake or degradation of the ^{3}H-MERF. There is no significant difference in the V-A difference between control and propranolol + iso at any time point ($p > 0.05$). At time point 75 min, there is a significant increase in the V-A difference compared to control ($p < 0.05$). There is no difference between propranolol, propranolol + iso, or control for the remaining time points ($p > 0.05$).

The data in figure VII depicts the percent intact arterial and venous (coronary sinus) ^{3}H-MERF as the mean ± SEM. As with the V-A data, all groups were considered control through the 30 min time point since no treatments had yet been performed. There is a rapid degradation or uptake of the infused ^{3}H-MERF within the first three minutes of infusion. Steady state kinetics are achieved, and this occurs between 30 and 60 minutes after beginning infusion. The half-life of ^{3}H-MERF has been measured to be between 2 and 3 minutes. There is no significant difference in the percent intact ^{3}H-MERF between control and isoproterenol in the presence of propranolol ($p > 0.05$). There is an increase in the arterial and venous percent intact ^{3}H-MERF with propranolol vs. both groups at time point 60 min ($p < 0.05$), but there is no difference between propranolol and the other groups for the remaining time points ($p > 0.05$).

The data in figure VIII depicts the RIA analysis of endogenous MERF in fmol/ml of blood ± SEM. There is a significantly greater amount of endogenous MERF in the propranolol + iso group compared to the other groups at the termination of the experiment ($p < 0.05$). However, there is no difference in the amount of endogenous MERF among the three groups before the MERF infusion was started ($p > 0.05$). Over the course of the experiment, there is a slight decrease in the amount of endogenous MERF in the plasma. Control n = 7, propranolol n = 5, and propranolol + iso n = 8.

The data in figure IX depicts the V-A difference of endogenous MERF in fmol/ml of blood ± SEM for all three groups before starting the MERF infusion and at the termination of the experiment. There is no significant difference in the V-A difference of the endogenous MERF among the three groups ($p > 0.05$).

The data in figure X depicts the spillover of ^{3}H-MERF as the mean ± SEM. At the 60 min time point, the spillover in the propranolol + iso and propranolol groups is significantly lower compared to control ($p < 0.05$). The spillover of ^{3}H-MERF is significantly lower in the propranolol + iso group compared to propranolol at the 75 min time point ($p < 0.05$). No significant difference exists among the three groups at the 0.5 min post time point ($p > 0.05$).

Figure XI shows two HPLC tracings of MERF. Panel A is a tracing of a 20 μl sample of 5 μCi ^{3}H-MERF / 2 ml 0.1% TFA/H_2O. The large single peek seen with a retention time of approximately 45 min. is intact ^{3}H-MERF. Panel B is a tracing of 1 ml of the elution from an arterial sample at steady state. The largest peak, which has a retention time of approximately 45 min., is the same peak seen in tracing A. These tracings confirm that the CPMs obtained for the intact MERF is indeed intact MERF.

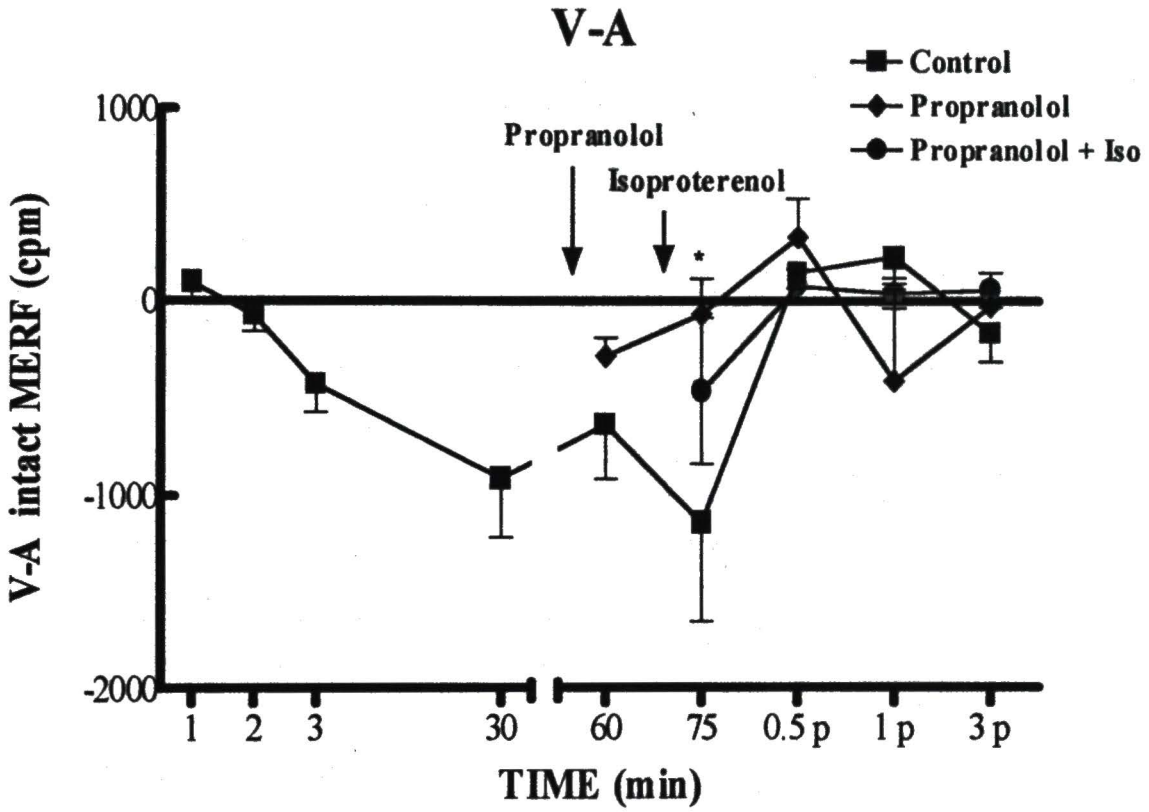


Figure VI

Average ± SEM of venous - arterial difference for all three groups.
*** $p < 0.05$ vs. control.**

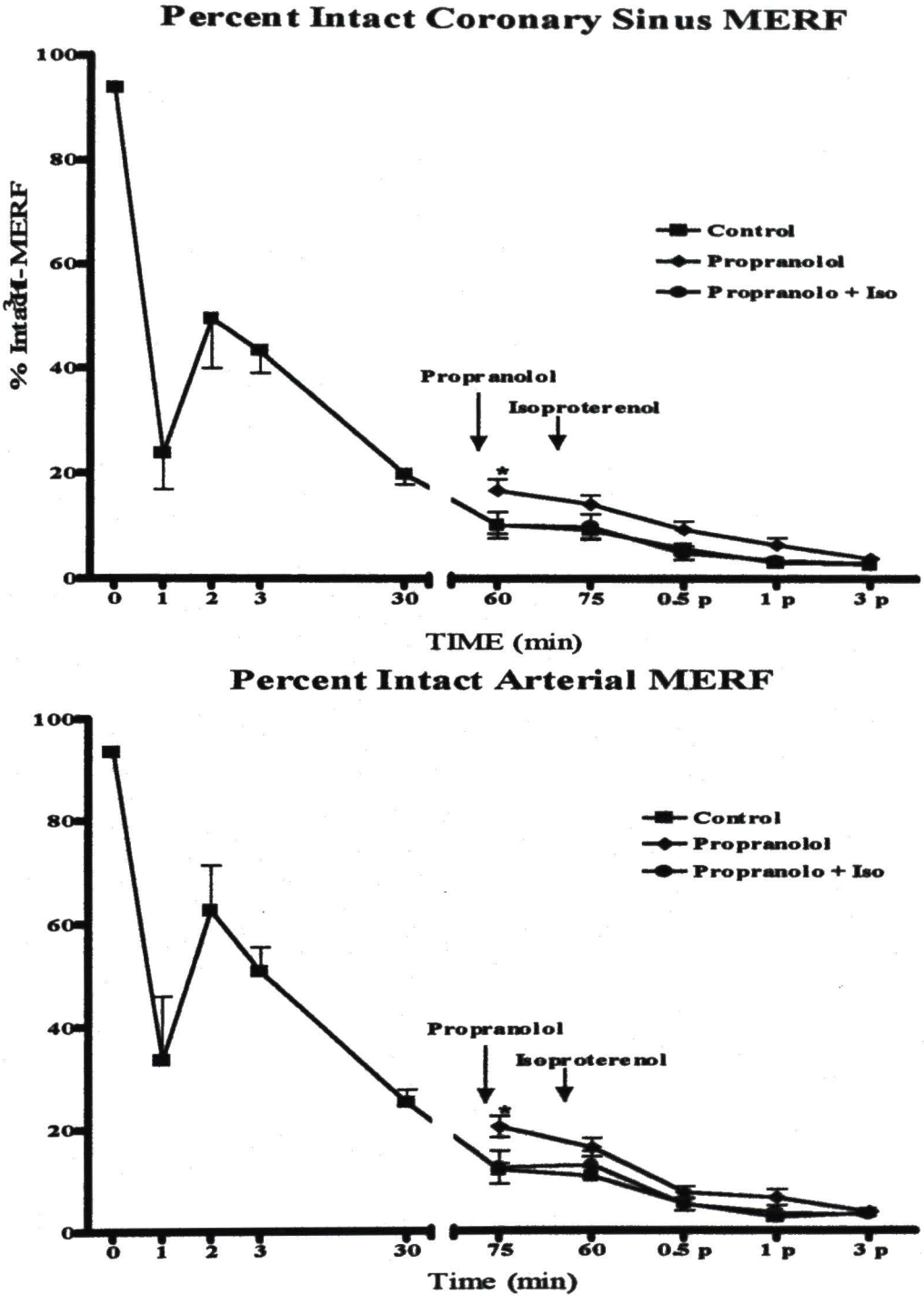


Figure VII

Average ± SEM of the percent intact MERF of all three groups. * $p < 0.05$ vs. control and propranolol + Iso.

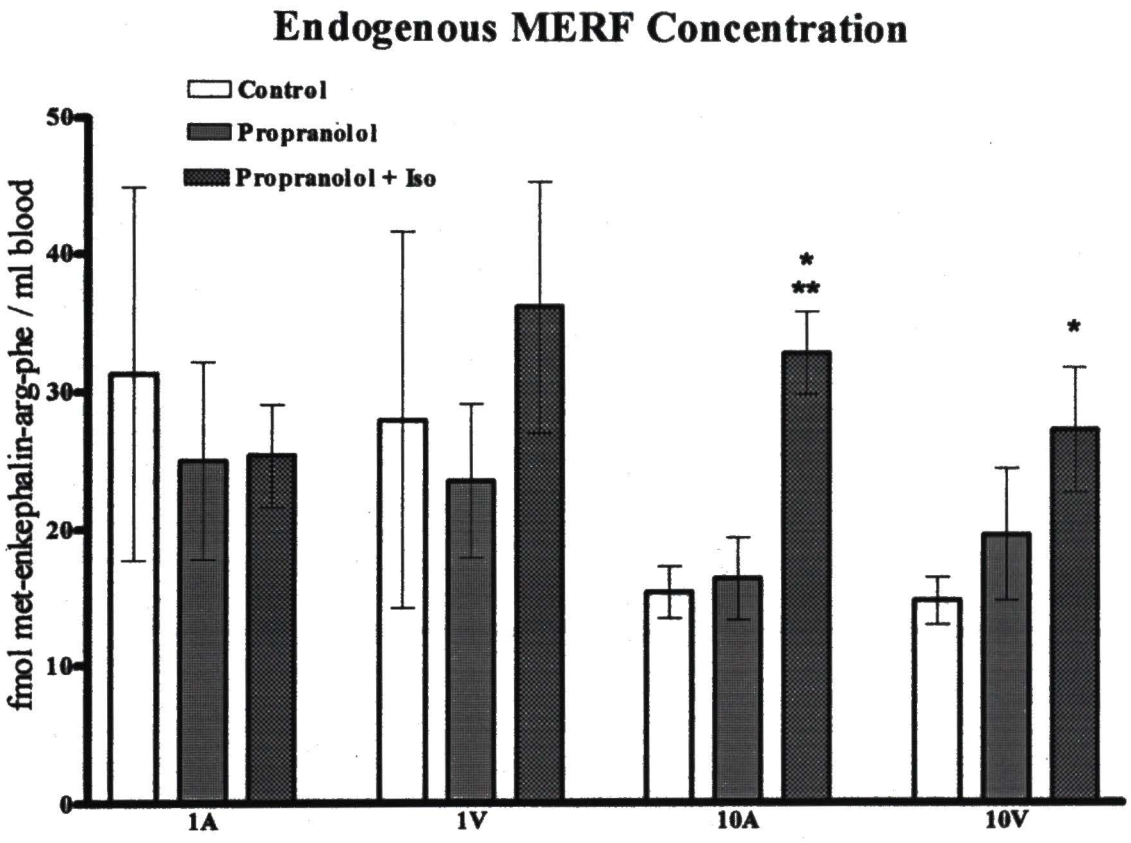


Figure VIII

Average ± SEM of endogenous MERF for all three groups before (1A and 1V) and following (10A and 10V) MERF infusion and treament with propranolol or propranolol + iso. * $p < 0.05$ vs. control. ** $p < 0.05$ vs propranolol.

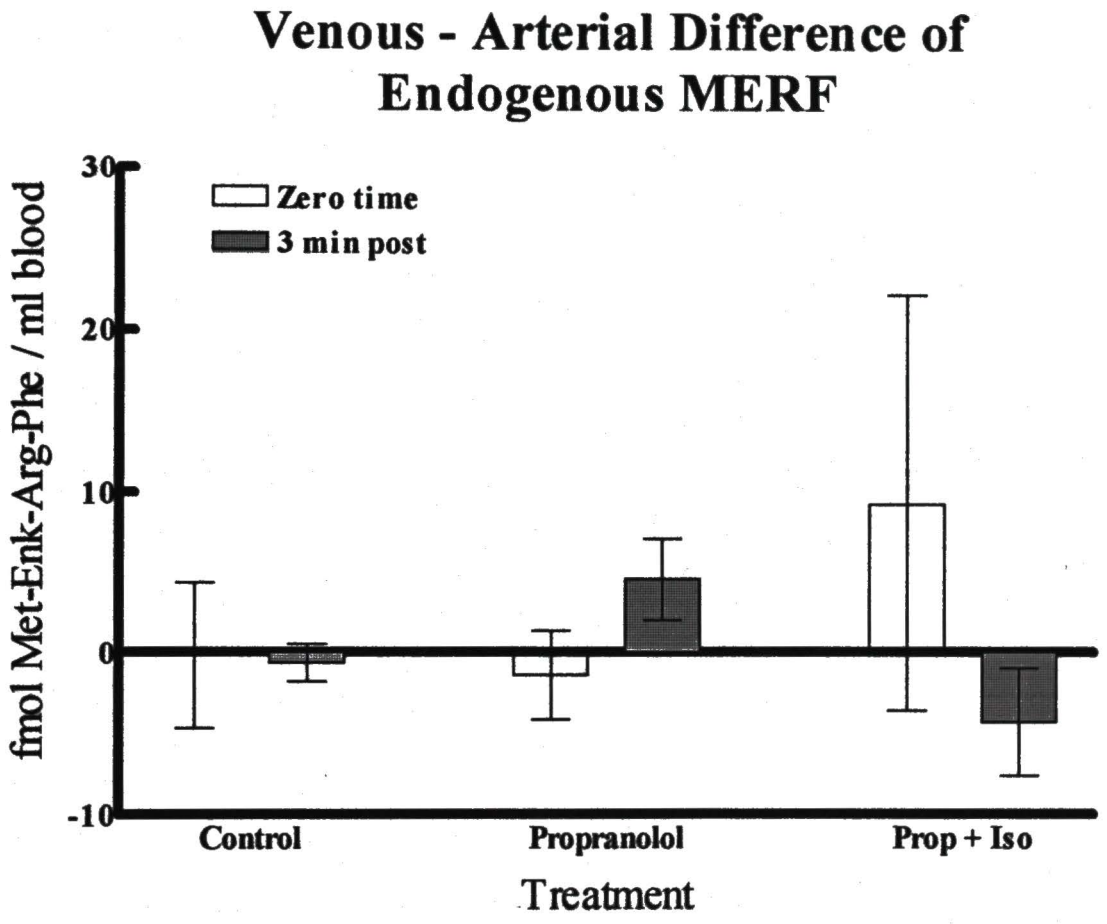


Figure IX

Average ± SEM of the venous - arterial difference of endogenous MERF for all three groups measured by RIA. No significant difference among the three groups.

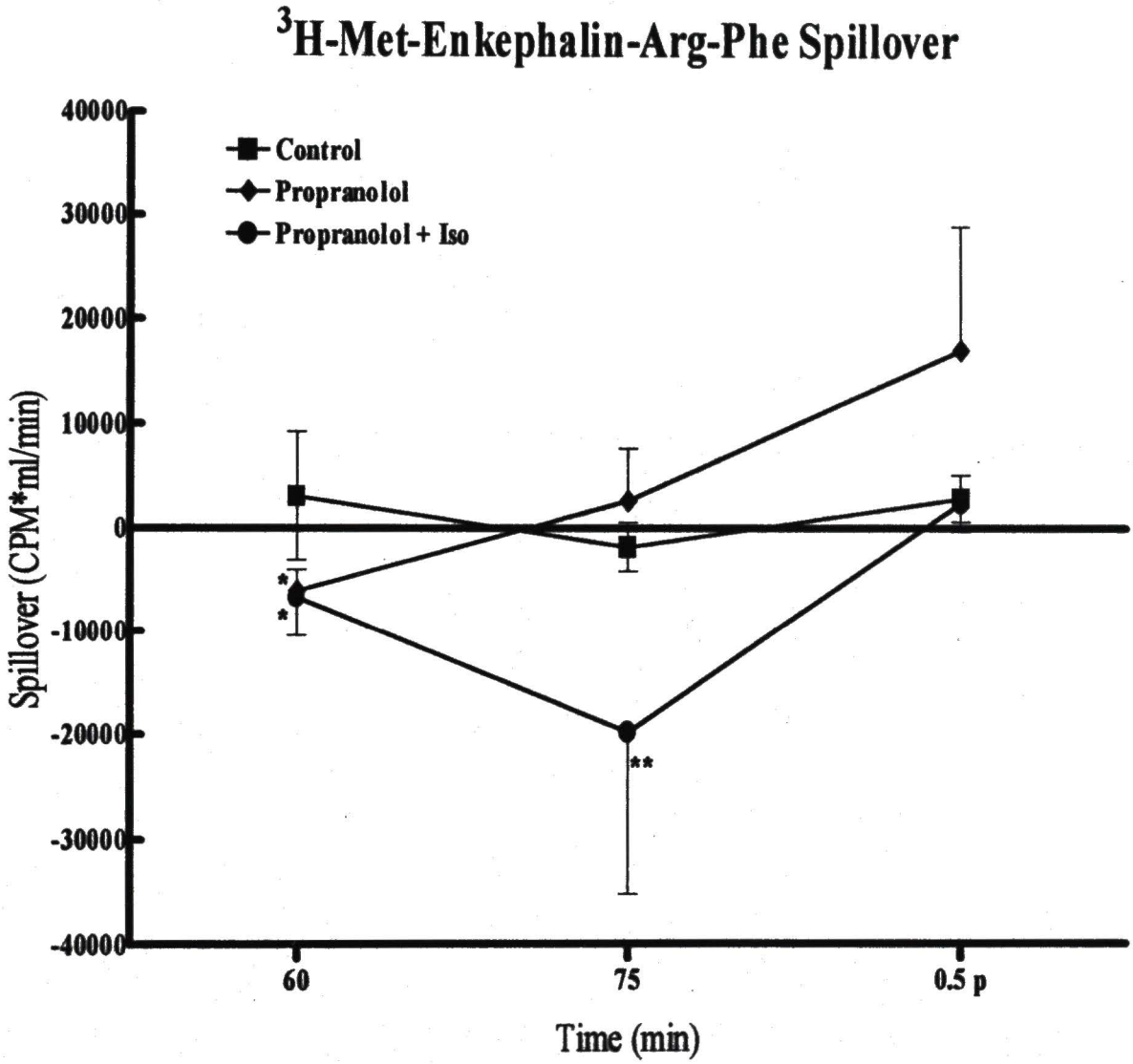


Figure X

Average ± SEM of MERF spillover for all three groups. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. propranolol.

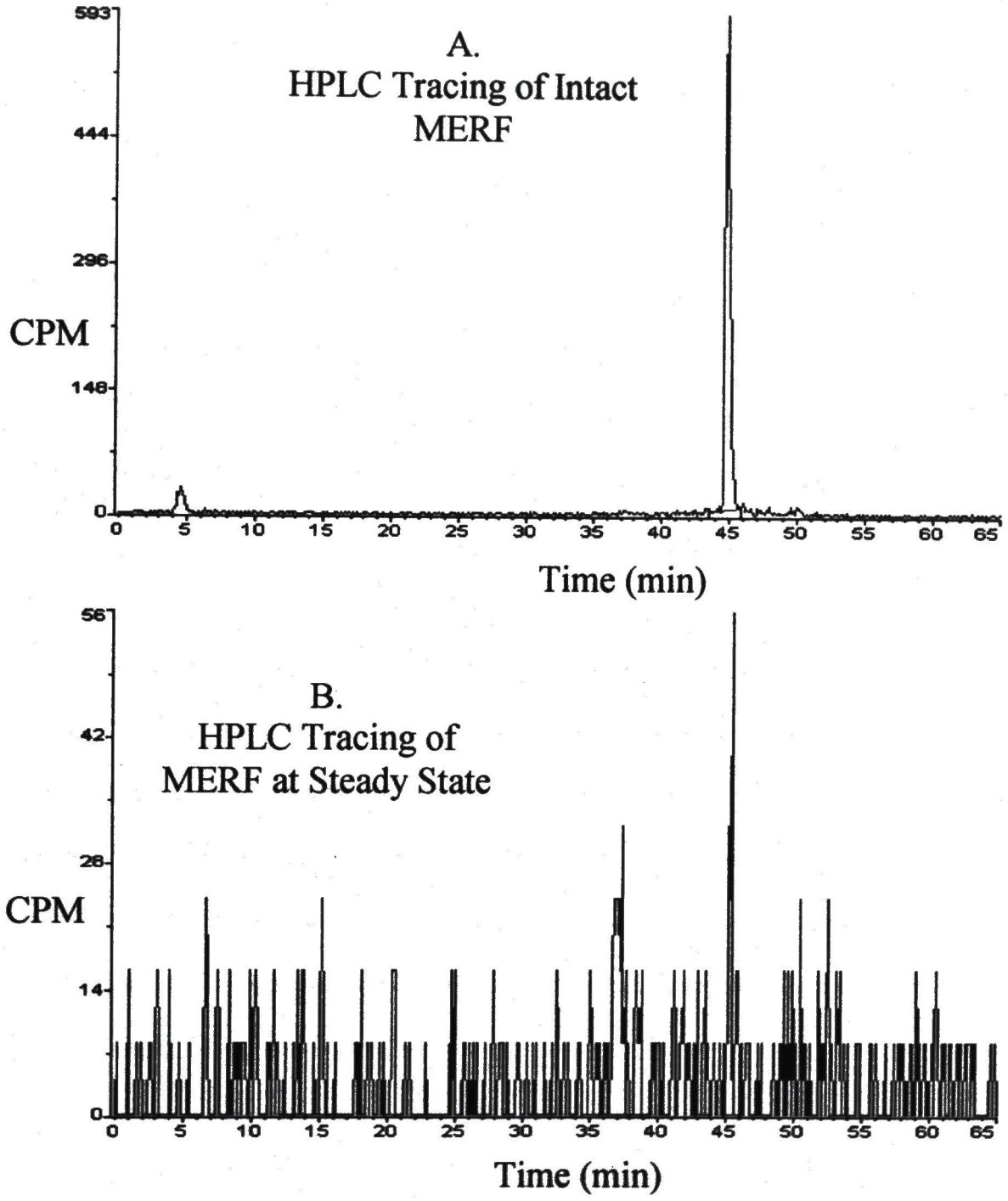


Figure XI

A. HPLC tracing of intact MERF preparation showing retention time of approximately 45 min. B. HPLC tracing of intact MERF elution from plasma at steady state, showing intact MERF was actually measured.

CHAPTER 4

DISCUSSION

Opioids bind to δ, κ, and μ opioid receptors in the heart and vasculature (17). The receptors for MERF are located in intracardiac parasympathetic ganglia or on vagal nerve terminals innervating the SA node, and are most likely of the δ subtype (4). When MERF binds to these opioid receptors, release of acetylcholine is inhibited, thus decreasing the vagal induced bradycardic effect. The decrease of this bradycardic effect may prevent abrupt changes in heart rate and potential episodes of asystole (3). This effect of MERF holds true when the parasympathetic control of the heart is dominant over sympathetic control. There is evidence that vagolytic enkephalins are released during sympathetic stimulation and may function to provide a smooth transition to an increased heart rate (3). MERF, however, does not alter resting coronary blood flow, myocardial O_2 consumption, or atrial contractile force directly (3). MERF, therefore, functions as a classic neuromodulator by opposing the direct actions of neural inputs with little direct effect of its own under resting conditions (3).

Little is known about the actual biological significance of MERF in the body. However, an increasingly greater amount of information is being discovered about MERF and its biological roles and functions. Since MERF is found in cardiomyocytes (2) and is even produced by cardiomyocytes (13), MERF appeared to have a possibly important

biological function in the heart. This study set out to establish the metabolic fate of MERF across the canine heart vascular bed. The MERF could experience multiple fates. These include degradation in the plasma, degradation in the heart or other tissue, uptake by the heart or uptake by other tissue. If uptake is present, the fate of MERF after uptake could be degradation or re-release. We know that there are degratory enzymes, enkephalinases, aminopeptidases, and angiotensin converting enzyme (ACE), located in the plasma and tissues. We have shown the plasma half-life of infused MERF to be approximately 3 minutes. We can assume that this short half-life can be attributed to rapid degradation of infused MERF. In future studies, this hypothesis can be tested by infusing known inhibitors such as kelatorphan, acetorphan, RB38A, or an ACE inhibitor such as captopril. No known uptake inhibitor exists for enkephalins. Therefore, we cannot block any possible uptake mechanism and see if that increases the concentration of MERF in the blood. In future studies, we will have to examine the heart tissue to determine if uptake exists. The heart will have to be removed from the dog and frozen. Sections of the heart will be stained to determine presence of MERF using immunocytochemistry technique. Additionally, autoradiographic techniques will have to be used to determine whether any MERF present is radiolabled, thus indicating uptake.

Previous research has been done showing that epinephrine and norepinephrine decrease the rate of degradation of enkephalins. In this study we sought to determine the effect of an exogenous β-agonist, isoproterenol, on the rate of MERF degradation. Isoproterenol did not decrease that rate of degradation, but we did unexpectedly find that the β antagonist, propranolol, increased the amount of MERF in the blood. We need to

infuse another β-blocker with the MERF to determine whether this increase of MERF is caused by a receptor mediated process, or if it is a structural property of propranolol. If this "protection" is receptor mediated we would expect to see a similar increase in plasma MERF with other beta blockers, such as atenolol or metaprolol. If it is a property of propranolol, then we would find no effect from other β-blockers. Another study to perform would be to use stereospecific forms of propranolol if we determine this to be a propranolol effect. The type used in this study was a racemic mixture of D- and L-propranolol. We would also use L-propranolol, the active enantiomer, and D-propranolol, the inactive isomer, to see if the effect is stereospecific.

Another question that we proposed was regarding the possibility of MERF spillover. Spillover is a phenomenon commonly used with catecholamines to evaluate sympathetic nerve function. Some factors influencing catecholamine (norepinephrine) spillover include regional blood flow, rate of norepinephrine (NE) release, and activity of the competing mechanisms of uptake and metabolism of NE (9). The spillover value at steady state can be summarized by the following formula:

$$\text{Organ Spillover} = [(V-A) + A_{\text{extraction}}] \times \text{Organ Plasma Flow} \qquad (9)$$

Since we have been able to achieve a steady state during the ^{3}H-MERF infusion and we can measure blood flow, it is possible to be able to measure MERF spillover in the canine heart. Figure 10 shows the spillover of MERF at the 60, 75, and 0.5 post min time points,

accounting only for the V-A difference and coronary blood flow. In order to make any calculations of spillover, it is necessary for the system to be at steady state. This condition was achieved during the experiment, allowing the following assumptions to be made. At steady state, the possible uptake and degradation of MERF is balanced with its possible release and degradation. Therefore, a negative V-A difference indicates a net uptake or degradation of MERF, whereas a positive V-A difference indicates a net release or a decrease in the degradation of MERF. Is propranolol affecting this balance? We have all variables of the equation except for the possible mechanism for uptake, if any exist. The V-A difference seen in MERF in the control group and the propranolol + iso group is negative during the infusion, indicating net uptake or degradation of the MERF. However, this difference is positive in the propranolol group at the 0.5 min post infusion time point and several dogs had a positive V-A difference at the 75 min time point, indicating release of MERF from the heart tissue or an inhibition of degradation. This raises the question, what does this indicate, if anything, about the handling of plasma MERF in the absence of β-adrenergic signaling. Does β-adrenergic signaling increase uptake of MERF into tissues, increase degradation, or decrease its release? If this is the case, we would expect isoproterenol to reverse the effects of the propranolol. This scenario was supported by my data. It is also possible that the propranolol alters the volume of distribution of the MERF.

Although the importance of MERF is not completely understood, it does appear to have cardiovascular effects (3,4,5,11,17,20). Since the ventricles produce a relatively

large amount of MERF, we can assume that it has an important role in cardiovascular function or modulation. When we are able to determine all of the metabolic fates of MERF, we can look further at the spillover of MERF into the circulation and determine if this has any significance, as does catecholamine spillover. As this is still a new area of research, much work still remains to be done and many questions to be answered before we have a complete understanding about the function and biological significance of MERF.

CHAPTER 5

LIMITATIONS

Administration of DL-propranolol (0.2 – 0.4 mg/kg IV) produced a complete β-adrenergic blockade. A significant decrease in HR (-20 ± 4 bpm and a decrease in MAP (-8 ± 3 mmHg) was seen in the dogs that were administered propranolol. With the accompanying decrease in cardiac output, an increase in sympathetic nerve activity might be expected to try to increase the cardiac output. If this was the case, an increase in plasma norepinephrine (NE) and possibly even epinephrine (epi) levels may lead to the increase in percent intact MERF seen in the dogs administered only propranolol. Keeten et al. have shown that the plasma NE concentration in conscious rats is reduced from 172 ± 6 pg/ml control to 151 ± 8 pg/ml in rats administered propranolol (1mg/kg) (12). They have also shown that propranolol reduced NE spillover by reducing NE clearance from the plasma (Table 3) (12). The plasma epinephrine concentration was unchanged (12). Richardt et al. has shown that propranolol dose dependently suppressed NE uptake (15). Neither atenolol nor timolol, β-adrenergic blockers, had a significant effect on NE uptake (15). Propranolol's effect on plasma NE levels is independent of its β-adrenergic blocking properties and is rather due to an interaction of propranolol with its uptake mechanism (15). Dean et al. showed that in humans, there was not a significant increase in plasma NE after β-adrenergic blockade alone (7). Esler et al. also demonstrated in

humans that the plasma NE concentration rose marginally with propranolol administration, from 1.65 ± 0.65 nmol/l to 1.85 ± 0.75 nmol/l (10). Increases in plasma NE concentrations are seen during the administration of propranolol. However, these increases are not significant. Although it is possible that even these slight increases could cause the increase seen in the V-A difference and percent intact MERF of the dogs administered propranolol only, this is not the most likely source of the increase. Conflicting data on propranolol's effect on plasma NE levels suggests that the change in NE concentration was not responsible for my observed change in the percent intact MERF seen with the administration of propranolol only.

Another limitation to this study is that it is not known whether or not extraction of peptides exists. If an uptake mechanism does exist, no blockers of this mechanism are known. Blockade of the uptake of peptides would be able to show that the extraction of MERF does or does not play a role in the metabolism of MERF across the canine heart vascular bed.

Since MERF is a small peptide consisting of only seven amino acid residues, it is possible that the MERF can diffuse across the coronary capillary membrane. We are not able to determine whether or not this is occurring, nor can we measure the amount of MERF diffusing out of the vasculature into the interstitium.

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