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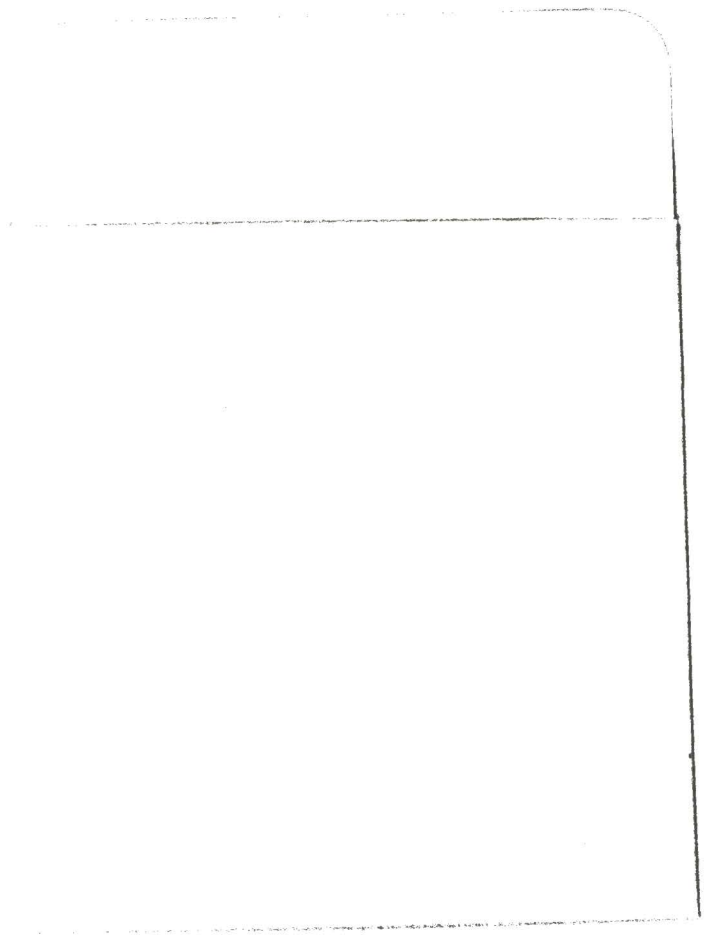

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A variety of plasma and intrinsic cardiac enkephalins were extracted, chromatographed and assayed under control conditions and during two hours of hemorrhagic hypotension. The animals were anesthetized, instrumented and sufficient blood was withdrawn as required to reduce mean arterial pressure and maintain it at 40 mmHg. Central venous blood samples were obtained 15 minutes before and at 30 minute intervals during the experiment. Arterial blood gases remained stable throughout the experiment while pH declined from above 7.4 to near 7.1. Heart rate rose gradually by 100 bpm. Plasma catecholamines were unchanged during two hour time-controls. Plasma norepinephrine and epinephrine increased by 6 and 100 fold respectively, during the first hour of hypotension and remained high through the second hour. All eight enkephalin immunoreactivities monitored were unchanged during the time-controls. Plasma Met-enkephalin (ME) and Peptide-F both gradually increased by 70-100% during the hypotension. Plasma Met-enkephalin-Arg-Phe (MEAP) and Peptide-B concentrations increased 4-5 fold during the same interval. Proenkephalin and other large enkephalin containing peptides though present, were unchanged during hypotension. Myocardial norepinephrine was preferentially concentrated about 3:1 in the atria. Both atrial and ventricular concentrations were reduced by one third or more following two hours of hypotension. Proenkephalin and Peptide-B accounted for 75 % of the intrinsic enkephalins and their ventricular concentrations were 3 to 4 times atrial oncentrations

in the same hearts. Intrinsic cardiac MEAP concentrations were 15-25 times higher than comparable ME concentrations in the same myocardial regions. Hypotension produced a significant increase in Peptide-B and proenkephalin compared to controls. The increase was consistent throughout the heart, thus maintaining the preferential concentration in the ventricles. Myocardial ME, MEAP and Peptide-F were largely unchanged in hypotensives compared to time-controls. The data demonstrate the preferential processing and retention of MEAP rather than ME-immunoreactive enkephalins in heart tissue. The data also indicate the responsiveness of MEAP-ir to changes in the circulatory environment and their subsequent appearance in plasma during hemorrhagic hypotension. Prior data suggests that intrinsic cardiac enkephalins may actively regulate either vagal control of the heart or sympathetic control of vasomotor tone.

HEMORRHAGIC HYPOTENSION ALTERS CIRCULATING
and
MYOCARDIAL ENKEPHALINS and CATHECHOLAMINES

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Thesis

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CHAPTER I

HISTORICAL BACKGROUND: Endogenous opioids are families of peptides with morphine-like activity. Morphine, the prototypical exogenous opiate was isolated form extracts of the opium poppy in 1805. Opiates were considered a miraculus medicine that no one could resist. By 1868 morphine had become so important therapeutically that a leading medical textbook described it as followes:

> Opiates cause a feeling of delicious ease and comfort, with an elevation and expansion of the whole moral and intellectual nature... There is not the same uncontrollable excitement as from alcohol, but an exaltation of our better mental qualities, a warmer glow of benevolence, a higher devotional spirit, and with a stronger self-reliance, and conciousness of power. Nor is this consciousness altogether mistaken. For the intellectual and imaginative faculties are raised to the highest point compatible with individual capacity... Opium seems to make the individual for a time, a better and greater man (7).

The mechanism of action was a mystery at that time but the biological and pharmacological properties of morphine have been widely investigated in the intervening century. Morphine's therapeutic value resides primarily in its potent analgesic and antidiarrheal actions. However morphine's ability to produce euphoria, tolerance and subsequent drug seeking behavior pose significant medical and social liabilities.

In the early nineteen seventies two pentapeptides with opiate-like activity were isolated from porcine brain (10). They were named *methionine enkephalin* (ME) and *leucine enkephalin* (LE). Enkephalin is derived forn the Greek meaning "in the head". The initial four amino acids in both enkephalins are identical and differences between the two pentapeptides reside in the C-terminal amino acid. The first four amino acids in each sequence are also identical in most other endogenous opioids and the N-terminal tyrosine residue is absolutly required for biological activity.

After isolating ME and LE, the obligate opioid sequence was identified in a series of larger peptides with a variety of C-terminal extensions. Precursor peptides which contain multiple opioid sequences were identified, and the constituent opioids were assigned to one of three families as endorphins, dynorphins or enkephalins. Each family of biologically active opioid is thought to derived from one of three distinct precursors, proopiomelanocortin, prodynorphin and proenkephalin (16).

The release of the small biologically active peptides from these precursors involved enzymatic cleavages at pairs of basic amino acids. For example, proenkephalin has seven opioid sequences bounded by arginine and lysine residues. The specific enzymes associated with the release of cryptic enkephalins are tyrosine-like and carboxypeptidase-like activities (17). Most investigators have assumed that the final products are small peptides, but larger intermediates like Peptide-E and Peptide-F are also found in the circulation.

Prior to the discovery of the endogenous peptides, the pharmaceutical chemist had suggested the existance of several different opiate receptors based on structure-activity relationship among alkaloid opiates (14, 15). Martin proposed three primary subtypes which he designed as mu, kappa and delta (12). These receptors appeared to be specific for different peptide families. The dynorphins are considered the natural agonist for kappa receptors (4) and enkephalins favor delta recpetors (13). The mu receptor has significant affinity for morphine, but a specific endogenous agonist for the receptor has not been identified at this time.

Opioid peptides appear to exert most of their activity through their ability to regulate ion transport. The mu and delta receptors have been implicated in the regulation of potassium channels, while kappa receptors are primarily linked to calcium channels (5). Though details of the mechanism are not clear, mu and delta opioid receptors suppress tissue function by increasing potassium influx and hyperpolarizing the

membrane. In contrast, kappa opioids appear to modify neural activity and neurotransmitter release by reducing calcium conductance and calcium dependent exocytosis (3).

One mechanism of action for opioids includes the inhibition of adenyl cyclase (6). This receptor coupling involves standard G-proteins, and two possible mechanisms are suggested. One involves the direct G_i-α mediated inactivation of adenyl cyclase. The second suggest that surplus beta-gamma subunits facilitate the reassociation and inactivation of stimulatory G_s-α subunits.

The distribution of enkephalins has been widely investigated. Proenkephalin has been found in adrenal medulla, spinal cord, pineal gland and sympathetic ganglia. Methionine enkephalin arginine phenylalanine (MEAP), a proenkephalin product of particular interest for us, has been found to be concentrated in globus pallidus, hypothalamus, caudate putamen and amygdala, with very little concentration in cortex and cerebellum. Proenkepahlin derivates have also been identified in the posterior pituitary.

Proenkephalin and its products have been widely associated with other endocrine functions, particularly the secretion of norepinephrine (NE) and epinephrine (Epi). In this regard proenkephalin concentrations in the adrenal medulla are very high and stimulation of the splanchnic nerve results in the co-release of both cathecholamines and opioids (8). Since opiate receptor blockade increases the release of cathecholamines, the

co-release of enkephalins appears to modify the secretion of the cathecholamines through local autocrine or paracrine mechanisms. This appears to be mediated through the inhibition of acetylcholine release.

In 1978, Holaday and Faden, suggested that opioids are involved in the pathogenesis of circulatory shock. They induced circulatory shock by administrating a lethal dose of bacterial endotoxin. They then demonstrated that arterial pressure could be restored to control values by administrating naloxone, a potent opioid antagonist (Figure 1). In 1986 Howells provided evidence that the heart contains more mRNA for preproenkephalin than the brain (9). The location in the heart was not precisly determined, but many scientists have assumed that opioids in the heart were limited to autonomic nerve terminals. Studies in our laboratories, indicate that enkaphalin is distributed throughout the myocardium and immunoflurecent labels suggest that enkephalins in the dog heart are concentrated around intercalated discs (1). Together, these observations suggest that enkephalins in the heart are intrinsic to myocardium and not limited to its autonomic distribution.

In 1959, Kosterlitz and Taylor, demonstrate that morphine reduced bradycardia during stimulation of the vagus nerve in rabbits (11). Experiements done in isolated rabbit hearts provided similar results when DADLE, a delta agonist, was administered in the isolated rabbit atria suggesting the participation of delta opioid receptors (18). These investigators suggested the effect was mediated by a reduction in the rate of the acetylcholine release.

In the process of extracting enkephalins intrinsic to the myocardium, our laboratory has identified a large concentration of the heptapeptide, MEAP. When tested *in vivo*, this peptide was a very potent vagolytic agent (2). Experiments with direct activating cholinergic agents suggested the effect of MEAP was also mediated through the inhibition of the acetylcholine release. At picomolar infusion rates, MEAP eliminated efferent vagal control of heart rate (2), atrial contractility and coronary blood flow. Therefore the receptors associated with this response may be localized on nerve endings within myocardium or within the intracardiac ganglia.

The cardiovascular role of opioids in shock remains unresolved. Previous data demonstrated that enkephalins and endorphins accumulated during hypotension and may have contributed to the circulatory collapse. Exactly which peptides are responsible is unknown. Therefore we conducted a series of experiments to determine if circularing MEAP- immunoreactivity (ir) was increased during circulatory stress and if so in what molecular form.

PRELIMINARY STUDIES: Analysis of enkephalins in blood required an efficent extraction procedure to prevent degradation and processing. We found that more than 80% of added enkephalin was metabolized during the standard separation of red blood cells and plasma. Our extraction strategy consisted of rapid cooling of the whole blood with cold acidified saline. Several acids were evaluated including acidic acid, citric acid and tricarboxylic acid (TCA). The condition of red blood cells followed acidification was evaluated microscopically. The separation of plasma and red cells after acidification with acetic acid was unsatisfactory. TCA produced a clear plasma separation, but the recovery of radiolabled peptide in plasma was less than 20%. Recovery with citric acid was best, almost 75%.

A series of chromatography systems were evaluated for separating the expected peptides by size. Bio-gel P-10 was selected because it separated the three primary peptide fragments efficiently and was compatible with both neutral and acidic extractions. A sample separation of standards is illustrated in Figure 2. Figure 3 diagramatically illustrates the final extracting protocol.

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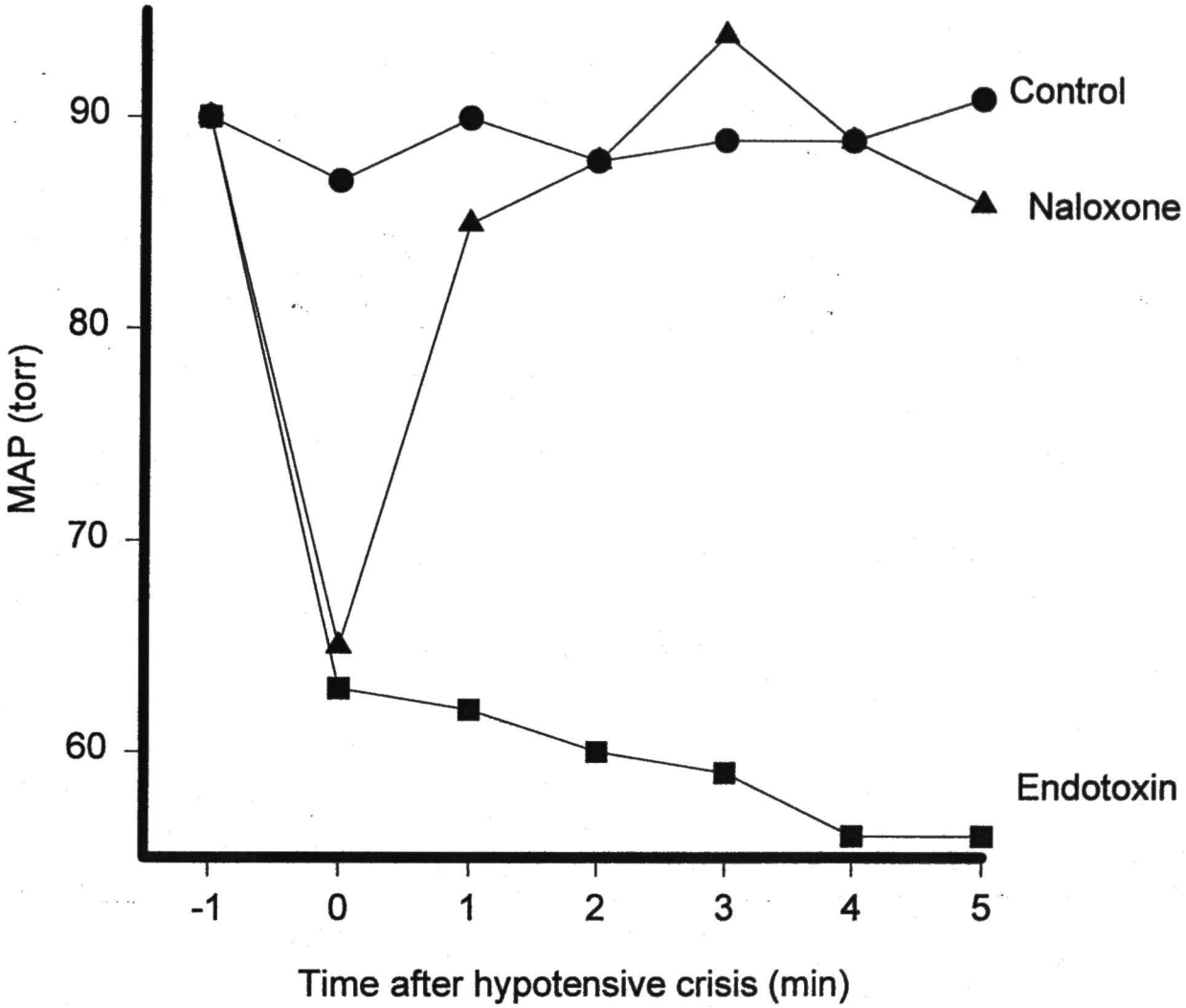

Figure 1. Mean arterial pressure control (circle), during naloxone (triangle) and during administration of endotoxin (squares). Data reproduced from J.W. Holaday and A.I. Faden, 1978.

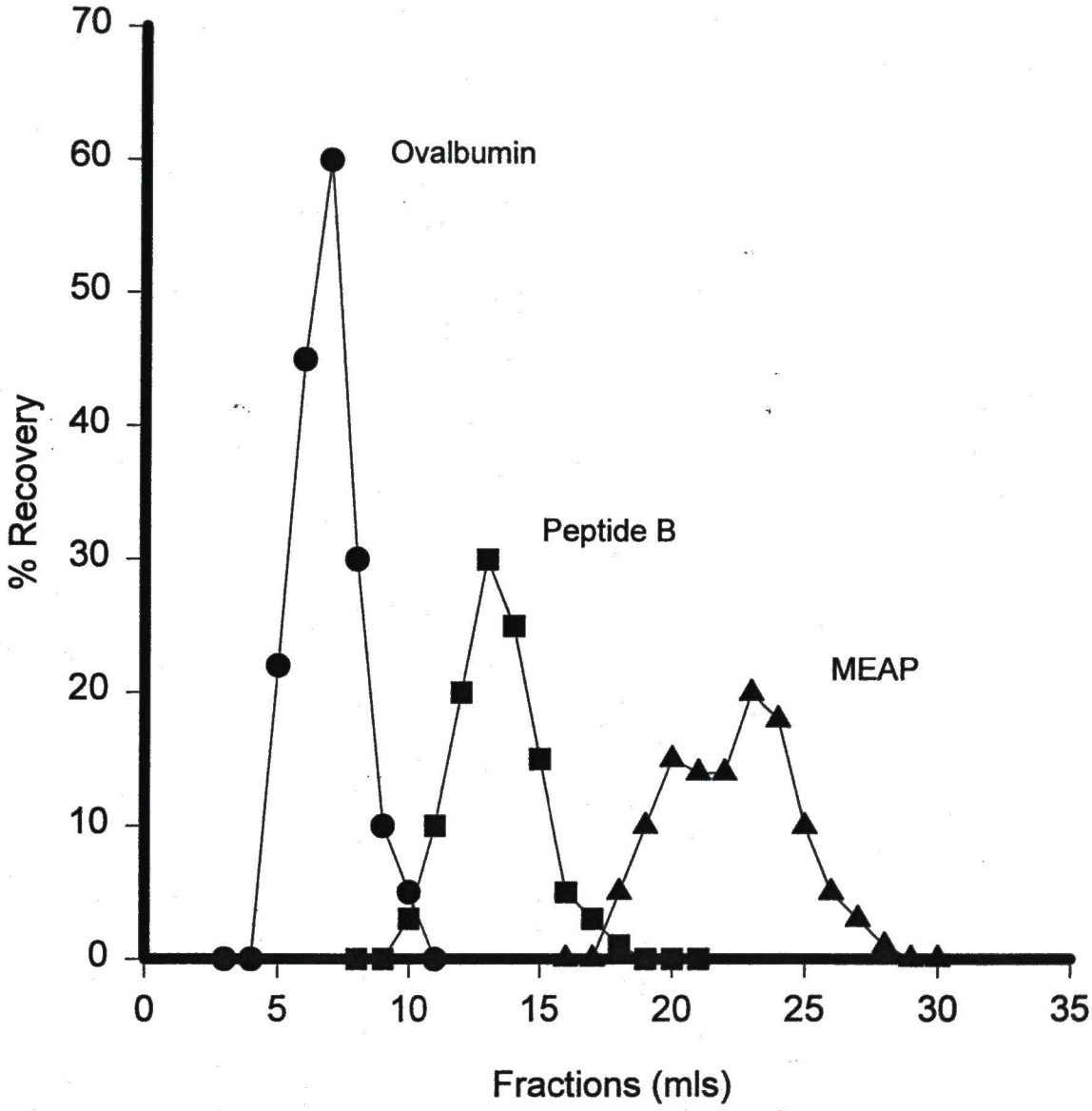

Figure 2. Bio Gel P-10 Chromatography, radiolabeled standards: ovalbumin (circle) Peptide B (squares) and MEAP (triangles).

BLOOD CHROMATOGRAPHY

20 mls blood + 20 mls chilled citric acid

↓

Centrifugation
10 min at 15,000rpm

↓

Separation and Filtration
0.45 μm filters

↓

Porapak columns
1ml etanol & 1ml solution (1:1:1)

↓

Dry vacuum
45min

↓

P-10 columns
fractions freeze or dry under vacuum

↓

RIA

Figure 3. Plasma Peptide Extraction Protocol.

RATIONALE

Endogenous opioids have been implicated in the pathogenesis of circulatory shock for more than fifteen years. This was based upon the hypothesis that endogenous opioids would accumulate during sustained hemorrhagic hypotension and would contribute to the myocardial depression and circulatory collapse which often accompanies the descent into circulatory shock. Much of the support for this proposal came from the beneficial effect of opiate receptor blockade in improving cardiovascular indices during circulatory shock. Some of these salutary effects have proved transient while others appear to be pharmacological effects attributed to naloxone rather than to opiate receptor blockade. However, a spectrum of enkephalin containing peptides are consistently released into the plasma during pathological hypotension. Which peptides are released and where they originate remains undetermined. Our laboratory has determined that the heart contains substantive concentrations of one particular enkephalin immunoreactivity, met-enkephalin-arg-phe, which is a particularly effective vagolytic agent. This observation raised the question does this immunoreactivity circulate during hypotensive stress and if so, in what form. This led us to propose the following hypothesis.

HYPOTHESIS

1. Met-enkephalin-arg-phe circulates in the plasma as the free heptapeptide.
2. Met-enkephalin-arg-phe activity will increase in plasma during hemorrhagic hypotension.

HEMORRHAGIC HYPOTENSION ALTERS CIRCULATING and MYOCARDIAL ENKEPHALINS and CATECHOLAMINES

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ABSTRACT: A variety of plasma and intrinsic cardiac enkephalins were extracted, chromatographed and assayed under control conditions and during two hours of hemorrhagic hypotension. The animals were anesthetized, instrumented and sufficient blood was withdrawn as required to reduce mean arterial pressure and maintain it at 40 mmHg. Central venous blood samples were obtained 15 minutes before and at 30 minute intervals during the experiment. Arterial blood gases remained stable throughout the experiment while pH declined from above 7.4 to near 7.1. Heart rate rose gradually by 100 bpm. Plasma catecholamines were unchanged during two hour time-controls. Plasma norepinephrine and epinephrine increased by 6 and 100 fold respectively, during the first hour of hypotension and remained high through the second hour. All eight enkephalin immunoreactivities monitored were unchanged during the time-controls. Plasma Met-enkephalin (ME) and Peptide-F both gradually increased by 70-100% during the hypotension. Plasma Met-enkephalin-Arg-Phe (MEAP) and Peptide-B concentrations increased 4-5 fold during the same interval. Proenkephalin and other large enkephalin containing peptides though present, were unchanged during hypotension. Myocardial norepinephrine was preferentially concentrated about 3:1 in the atria. Both atrial and ventricular concentrations were reduced by one third or more following two hours of hypotension. Proenkephalin and Peptide-B accounted for 75 % of the intrinsic enkephalins and their ventricular concentrations were 3 to 4 times atrial concentrations in the same hearts. Intrinsic cardiac MEAP concentrations were 15-25 times higher than comparable ME concentrations in the same myocardial regions. Hypotension produced

a significant increase in Peptide-B and proenkephalin compared to controls. The increase was consistent throughout the heart, thus maintaining the preferential concentration in the ventricles. Myocardial ME, MEAP and Peptide-F were largely unchanged in hypotensives compared to time-controls. The data demonstrate the preferential processing and retention of MEAP rather than ME-immunoreactive enkephalins in heart tissue. The data also indicate the responsiveness of MEAP-ir to changes in the circulatory environment and their subsequent appearance in plasma during hemorrhagic hypotension. Prior data suggests that intrinsic cardiac enkephalins may actively regulate either vagal control of the heart or sympathetic control of vasomotor tone.

Key Words: Shock, endogenous opioids, norepinephrine, epinephrine.

INTRODUCTION

A number of laboratories became interested in the role of endogenous opioids in circulatory shock soon after their discovery (27). This was based on the very attractive hypothesis that the accumulation of opioids during circulatory stress would suppress cardiovascular function and contribute to the progressive circulatory collapse which often accompanies shock. Although both enkephalins and endorphins do accumulate during hypotension (5,15,16,18,20,24,30,32,39,41,42), the strongest support for the hypothesis arose from the dramatic recovery of circulatory function in a number of animal models after the administration of the opiate receptor antagonist, naloxone (1,12,13,25,35,40). Although cardiovascular indices usually improved after administering naloxone during shock, comparable improvements in survival were less consistent and therapeutic interest in this approach diminished. Some of the effects of naloxone may in fact be mediated by non-opioid mechanisms (23).

The cardiovascular role of the opioids particularly in shock remains unresolved. When they accumulate, enkephalins may impair peripheral vascular compensations by opposing reflex vasomotor traffic through peripheral sympathetic ganglia (7,10). Circulating enkephalins are elevated during hypotension but chromatography has seldom been employed to identify which enkephalins are present. As a result, which peptides contribute and where they originate is currently unknown. Although the adrenal medulla has been the prime candidate in this regard (11,21), other sources are required since the

peptide patterns in the circulation differ significantly from those observed in the adrenal vein (4, 36,40).

A large abundance of proenkephalin-mRNA in the rat heart suggests that intrinsic cardiac enkephalins may be important. In the process of extracting enkephalins from the dog heart, we observed significant immunoreactivity which cross-reacts with the C-terminal proenkephalin heptapeptide sequence, methionine-enkephalin-arginine-phenylalanine (MEAP-ir). Ventricular MEAP-ir was 30 times greater than methionine-enkephalin (ME) and was 5 times more concentrated in the ventricles than the atria. HPLC analysis indicates that this activity is divided between the free heptapeptide and a number of larger N-terminal extended sequences (2). Suggestions that enkephalins are vagolytic (28,31) led us to infuse MEAP in dogs (9). We found MEAP was more potent than met-enkephalin (ME) and that it reduced vagal bradycardia by more than 70% at physiologically achievable concentrations with an estimated EC_{50} of 1-3 nM.

Since the accumulation of potentially vagolytic and sympatholytic peptides during hypotension could contribute significantly to pressor and chronotropic compensations, we conducted the following study to determine if myocardial and circulating MEAP and any related peptides are increased during sustained hemorrhagic hypotension. In this regard all samples were chromatographically separated prior to assay.

METHODS

General Procedures: Fifteen mongrel dogs weighing between 15-20 kg were maintained with food and water ad libitum for at least one week. The animals were anesthetized with sodium-pentobarbital (32.5 mg/kg), intubated and mechanically ventilated with room air at 300 ml/min/kg. Arterial blood gases and pH were determined (Corning 178 Blood Gas Analyzer) and if required, the PO_2 was adjusted to normal (90-120 mmHg) with supplemental oxygen. The pH and PCO_2 were similarly adjusted to normal (7.35-7.4 and 30-40 mmHg) by administering bicarbonate or modifying the minute volume. Although blood gases were determined at each sampling time during the protocol, no further adjustment were made once the protocols were initiated. Catheters filled with heparinized saline were inserted in the right and left femoral arteries and advanced into the abdominal aorta. One catheter served to withdraw blood for inducing hypotension and to obtain arterial samples during the experiment. A third catheter was inserted into the femoral vein and advanced into the inferior vena cava to administer additional anesthetic as required. A fourth catheter was inserted into the left jugular vein and advanced through the superior vena cava to heart level. This catheter was used to obtain central venous blood samples for peptide and catecholamine analysis. Heparin was administered (1500U/kg) to provide for sustained anticoagulation. The femoral venous catheter and the remaining arterial catheter were attached to Statham PD 23XL transducers to monitor

arterial and central venous pressure. Heart rate was monitored continuously by tachometer tracking pulse pressure tracings. Data were sampled and recorded on-line by computer (Maclab).

Experimental Protocol: A 20 ml prehemorrhage sample was obtained from the jugular catheter. In nine animals arterial blood was withdrawn until an initial 40 mmHg arterial pressure was achieved (10-15 min). A second blood sample was collected at 40 mmHg nd designated as zero time. Arterial blood was withdrawn as required to maintain the arterial pressure at 40 mmHg. Additional venous blood samples for peptide analysis were obtained at +30, +60, +90, and +120 min. Venous samples were collected at -15, 60, & 120 minutes to correlate plasma catecholamines. After two hours the heart was removed, separated into functional compartments and extracted for both enkephalin and catecholamine analysis. Control experiments were performed in 8 dogs without hemorrhage. Blood samples were collected at the same intervals.

Blood Collection: Since we had previously determined that more than 80% of added enkephalin was destroyed during the separation of red cells and plasma, several strategies were evaluated in order to determine the best method for preventing the degradation and further processing of enkephalins immediately after collection. Optimum conditions were obtained when blood samples were collected directly into an equal volume of iced citric acid (20 mg/ml) in saline. This reduces enzyme activity by chelating metal ions and

instantly lowering temperature and pH (34). Blood samples were separated by centrifugation at 15,000 x g for 10 min. The supernatant volume was measured and stored at -90°C.

Tissue Collection: The beating heart was removed and immediately submerged in iced Krebs-Henseleit-bicarbonate (pH 7.45) to reduce enzyme activity and to flush blood from the heart. The heart was dissected on ice and separated into left and right atria, left and right ventricles and the intraventricular septum. The tissue was diced and boiled in 2.5 volumes of 1 N acetic acid with 0.2 N HCl for 30 minutes. After cooling, 0.1% β-mercaptoethanol was added. The tissue was then homogenized (Polytron, Brinkman) and the supernatant separated by centrifugation at 25,000 x g for 30 min. The pellet was rehomogenized with another 2.5 volumes of fresh acid and β-mercaptoethanol. The homogenate was centrifuged again and the two supernatants were combined and stored at -20°C.

Chromatography:

Blood: The supernatant was filtered through 0.45 μm syringe filters (13mm PVDF, Whatman). The peptides from 10 mls of supernatant were extracted and concentrated by adsorption on 0.5 ml Porapak Q columns (Millipore/Waters, Bedford, MA). The columns were washed with 5 mls distilled water to elute unwanted material. The peptides of interest were then eluted with 1 ml of ethanol and 1 ml of a

water:ethanol:acetic acid mixture (1:1:1). The alcohol was evaporated under vacuum in a centrifugal evaporator (Savant) and reconstituted to 2 mls with 0.01 M phosphate buffered saline (PBS), pH 7.0. The peptides in 2 mls of buffered extract were then separated by gel filtration chromatography through Bio-gel P-10 columns (1.5 x 10 cm, 15 ml). The samples were eluted with PBS and fractions were collected corresponding to small (ME & MEAP), intermediate (Peptides B & F), large and very large (proenkephalin) peptides. The columns were calibrated with radioiodinated standards (ME, MEAP, Peptide-B & ovalbumin). The pooled fractions corresponding to the various molecular weight ranges were either aliquoted and frozen or concentrated further by vacuum evaporation depending upon their expected peptide content. All dried samples were reconstituted in 500ul of buffer prior to radioimmunoassay (RIA).

Tissue: The frozen extracts were thawed, neutralized with 10 N NaOH, and filtered through 2 μm syringe filters (Whatman). Peptides in two mls of extract were separated by gel-filtration on P-10 columns and eluted in PBS containing 0.1% gelatin, pH 7.0. Where required, selected fractions were concentrated on Porapak-Q columns, eluted with ethanol:acetic acid:water(1:1:1), dried under vacuum and stored at -20^{0}C.

Radioimmunoassay: Samples were assayed with a C-terminal directed antiserum specific for either ME (14) or MEAP (33). The dried samples were reconstituted with PBS-gel. Iodine-125 labeled radioligands for these two assays were prepared by

chloramine-T and purified by combined gel-filtration/ion exchange chromatography as described previously (8). Synthetic ME and MEAP were obtained from Cambridge Research Biochemicals, Wilmington, DE and Peptide Technologies, Gaithersburg, MD respectively. Bound and free were separated by immunoprecipitation with sheep anti-rabbit gamma-globulin. The antibody for ME cross-reacts 0.1% with MEAP. The MEAP antibody cross-reacts with peptide-B about 300%. Samples were routinely assayed in duplicate.

Catecholamines: Blood samples were collected with 1.5 mg EGTA and 1.0 mg reduced glutathione/ml. The tubes were iced and plasma was separated by centrifugation immediately. Plasma was subsequently further preserved with 40 μl of 50mM sodium metabisulfite ($Na_2S_2O_5$) /ml plasma. Plasma was stored at -90^{o}C for up to one month. Immediately prior to extraction samples were spiked with an internal standard (DHBA) to monitor recovery. Catecholamines in preserved plasma and acid extracts from the myocardium were extracted on acid washed alumina and eluted with 0.1 M $HC1O_4$. The samples were separated by HPLC, compared to authentic standards and quantitated by electrochemical detection (2, 22).

Statistics: All experiments were analyzed using a randomized two factor analysis of variance with multiple levels. Individual time points were then compared with Tukey's protected-T (GB Stat, Dynamic Microsystem Inc., Silver Springs, MD). Differences with probabilities less than 0.05 were considered significant.

RESULTS

Basal cardiovascular parameters for all subjects are listed in Table 1. Initial values for animals assigned to the time-control and hypotensive groups were not statistically different. Figure 1 illustrates the changes in mean arterial blood pressure during the experimental protocol. Figure 2 (a-c) presents the resulting changes in heart rate and plasma pH and the cumulative volume of blood withdrawn to maintain blood pressure at 40 mmHg. There were no significant changes in heart rate in control animals during the protocol. The initial reduction in blood pressure to 40 mmHg required the removal of about 250 ml of blood and produced a comparatively small but significant increase in heart rate. Heart rate continued to rise further throughout the remaining 120 minutes despite the constant baroreceptor input. Compensatory pressor responses necessitated the progressive removal of an additional 500 mls of blood during the two hour protocol. The cumulative volume of blood removed and the heart rate are significantly correlated ($p < 0.001$). No attempts were made to correct blood chemistries during hypotension, but arterial PO_2 and PCO_2 (Table 2) did not vary with time, and hypotensive and time-controls were not significantly different. The plasma pH was significantly reduced during hypotension and the decline was inversely correlated with heart rate.

Plasma catecholamines were determined at zero, 60 and 120 minutes and the results are presented in Figure 3. Catecholamines in the time-controls were unchanged at 60 and 120 minutes. Sympathetic activation during hypotension was confirmed by a six fold increase in plasma norepinephrine at 60 minutes which was maintained through 120 minutes. Evidence for active adrenal participation was provided by a 100 fold increase in plasma epinephrine at 60 and 120 minutes.

Results for plasma opioid determinations in the control animals were not significantly different among sampling times. This was true for all ME and MEAP immunoreactive species including all four molecular weight ranges (data not presented). Peptide determinations in the experimental group prior to hemorrhage were also not different from the time-controls. The majority of the ME-ir elutes chromatographically with authentic ME or with an N-terminal extended intermediate of approximately 3-5kD. Apparent size and crossreaction with the C-terminal ME-antisera suggests this intermediate peptide is the 3.8kD Peptide-F which also has a free C-terminal ME sequence. Both larger molecular weight fractions were consistently near the limits of detection for the assay. The same pattern was evident for MEAP-ir in controls. Values were measurable but low for MEAP and Peptide-B. The values obtained for the larger molecular weight fractions, which should include proenkephalin and any large intermediates, were near the detection limits of the assay.

Figure 4 illustrates plasma ME-ir for the four molecular weight fractions during the hemorrhagic hypotension. Hypotension produced a significant increase in circulating ME and a parallel increase in Peptide-F compared to their pre-hypotensive determination. The progressive increase approached 100% after two hours of hypotension. The results from the two larger fractions were not different from controls suggesting that there was no significant release of large molecular weight species with available C-terminal ME sequences for antibody recognition. Figures 5 & 6 illustrate respectively the direct comparison between Peptide-F, ME and their respective time-controls. In both cases the effect of hypotension was significantly different from control. Labeled (*) time points are different from prehemorrhage determinations.

Figure 7 provides the plasma MEAP-ir summary data for all four size fractions during hypotension. Immunoreactivity in the MEAP and Peptide-B fractions increased approximately four fold during hypotension and appeared to reach a temporary plateau after 60 minutes. By comparison, the larger molecular weight fractions were unchanged at significantly different from time-controls. These data strongly suggest that the larger molecular weight material was selectively retained while an array of intermediate and small peptides were released. Figures 8 & 9 illustrate respectively the direct comparison between Peptide-B and MEAP during hypotension and their time-controls. In both cases

the effect of hypotension was significantly different from control. Once again labeled (*) time points are different from prehemorrhage determinations. Figure 10 illustrates the relative differences in scale between MEAP and ME during hypotension.

Once the experiment was completed, the hearts were collected and analyzed for both catecholamines and opioids. These data are presented in Tables 3, 4 and 5. Atrial norepinephrine (Table 3) was consistently 2 to 3 times higher than comparable measurements in the ventricles in both control and hypotensive groups. Tissue norepinephrine in the hypotensive group declined by approximately 1/4 in all myocardial compartments as a result of hypotension. Contrary to the opioid determinations made in plasma, there was significant ME-ir in all four molecular weight fractions (Table 4). More than 50% of this activity was concentrated in the Peptide F fraction while the remaining activity was divided equally among the other three fractions. In agreement with our prior findings with unchromatographed samples (2) there was no preferential distribution of the ME activity in any specific myocardial compartment. There was no systematic effect of hypotension on myocardial ME-ir.

MEAP-ir determinations (Table 5) were 15-25 times higher than comparable ME determinations in the same fractions. Intermediate sized peptides which eluted with Peptide-B also accounted for the majority of the cardiac MEAP-ir. Approximately two-thirds of the activity eluted with Peptide-B. About two-thirds of the remaining activity

was concentrated with the larger molecular weight fractions. The free heptapeptide represented between 10 and 20% of the immunoreactivity in any myocardial compartment. The large ventricular concentration of MEAP-ir observed earlier in unchromatographed samples was most apparent here in the peptide-B fractions where ventricular content was 3-4 times greater than that in the atria. This difference was less apparent but still significant in the MEAP and proenkephalin fractions. There was a consistent increase in MEAP-ir following hypotension in all heart fractions. This increase was most dramatic for peptide-B in the atria and proenkephalin in the ventricles.

DISCUSSION

The experiments presented above were conducted to answer several fundamental questions regarding the nature and quantity of enkephalins in the heart and circulation. The study first sought to determine if the high intrinsic myocardial MEAP-ir reported earlier (2) was accompanied by a similar peptide presence in the plasma. The data presented clearly indicate that the free heptapeptide circulates in the blood stream at or above the concentrations routinely found for ME. The study next attempted to determine the relative size of the MEAP-ir in plasma. The data suggest that after chromatography, the free heptapeptide and Peptide-B account for most of the circulating MEAP-ir. The activity was divided approximately equally between the two. Very little circulating MEAP-ir was associated with the larger molecular weight fractions suggesting that little intact proenkephalin was released. ME-ir followed much the same pattern with the majority of the circulating activity divided equally between the free pentapeptide and a larger intermediate which elutes in the same fractions as peptide-B. The size and free C-terminal ME sequence suggest the intermediate may be the canine equivalent of the 3.8 kd, Peptide-F. Larger molecular weight forms were again absent by comparison suggesting that there was little secretion of unprocessed precursor.

Other investigators reported that proenkephalin accounted for as much as two-thirds of the circulating ME when the fractions were digested (41). Assuming a six fold

yield following the enzymatic release of all cryptic ME, the large peptides in the present study would account for only about 15-20% of the total ME-ir. Similar to the findings of the present study however, Watson et al (41) found approximately equal concentrations of small and intermediate sized peptides in plasma during hemorrhage. A second group also in partial agreement, found little proenkephalin-sized material in the circulation (30). They, however, also found little intermediate sized material. We believe the instantaneous dilution, acidification, and reduction in temperature achieved by drawing the blood directly into syringes preloaded with equal volumes of iced preservative produces a substantial improvement in peptide recovery in the current study. Some of the differences noted between this and prior studies may be attributable to a much better recovery for small and intermediate sized peptides thus reducing proportionately the relative contribution by unprocessed precursor.

The study also sought to determine if circulating MEAP-ir was dynamically responsive to changes in the cardiovascular environment. The sharp four fold increase in both plasma MEAP and Peptide-B during hemorrhage confirms their exquisite sensitivity to the hypovolemic challenge. The parallel but smaller increases in plasma ME and Peptide-F under the same circumstances were similar in magnitude to that reported by a number of other investigators (15,16,17,18,19). The much larger MEAP response was somewhat paradoxical considering the composition of proenkephalin. Since

proenkephalin contains four ME sequences for each MEAP, one must propose that either the post-translational processing of proenkephalin is non-uniform or MEAP is significantly more resistant to degradation.

The failure to detect an increase in either ME-ir or MEAP-ir in the large molecular weight fractions suggests that they are not actively secreted with the smaller fragments. Their background presence in the circulation may then be a consequence of some non-secretory process, perhaps as a result of cell lysis.

The 6-100 fold increase in circulating catecholamines during the hemorrhage is indicative of the intensity of the neuroendocrine response. The 2:1 ratio of epinephrine to norepinephrine indicates significant peripheral sympathetic and adrenal medullary contributions. The clear preferential atrial/ventricular distribution of myocardial norepinephrine was consistent with our prior findings (3) and the established distribution of sympathetic nerve terminals within the myocardium. The decline in norepinephrine throughout the heart despite soaring plasma concentrations suggests a continuous myocardial norepinephrine overflow into the circulation during hypotensive challenge.

Each of the ME- and MEAP-immunoreactivities identified in plasma were also identified in heart. The large molecular weight fractions were however, better represented in tissue than in plasma. Approximately one-half of the tissue ME-ir was

eluted in the Peptide-F fraction and remaining 50% was evenly divided among ME, the very large, and large molecular weight fractions. As reported previously, tissue ME was evenly distributed throughout the muscle suggesting a myocardial rather than neural origin. Cardiac ME-ir was unaltered after 2 hours of hypotension. Myocardial MEAP-ir was 15-25 times higher than comparable ME measurements and was concentrated approximately three to one in the ventricles. These results were in agreement with our previous observations for unchromatographed samples (2). The proenkephalin and Peptide-B accounted for more than 75% of the tissue enkephalin and for most, if not all, of the atrial/ventricular difference. The large concentrations of cardiac Peptide-B suggest it serves as a storage form, perhaps protected from rapid degradation. In addition, its presence in the circulation suggests it may have a function of its own. The prior identification of the proenkephalin-mRNA in isolated cardiomyocytes clearly indicates the myocytes are capable of making proenkephalin (29,37,38). The presence of the precursor in the heart and the large tissue to plasma ratios for cardiac enkephalins suggest that the heart may secrete enkephalin and may contribute significantly to the circulating peptide concentrations.

Three of the four myocardial MEAP-ir fractions were increased by hemorrhage. Only MEAP failed to reach statistical significance, perhaps as a result of its increased secretion. Coincident increases in tissue proenkephalin and plasma MEAP suggest that hemorrhage induced an increase in proenkephalin expression.

Several important questions remain unanswered. What is the critical stimulus for enkephalin release? While hemorrhagic hypotension is a highly effect stimulus, it is also very complex. In addition to the decrease in afterload, hemorrhage is accompanied by intense sympathetic activation, extremely high catecholamines, reduced pH, increased heart rate, reduced atrial stretch and presumably a host of other neurohumoral djustments including the release of renin, angiotensin, aldosterone, cortisol, and vasopressin. Second, where does circulating enkephalin originate? Prior studies have established that the adrenal glands cannot explain the pattern of circulating enkephalin observed in shock or stress (4,36,41). We estimate that the dog adrenal contains approximately 10 nmoles of MEAP-ir and the heart contains another 3-5 nmoles. Given that coronary blood flow is approximately 10 times that in the adrenal, we suggest that the heart could easily make significant contributions to circulating enkephalin, if myocardial enkephalin is secreted.

Enkephalins are potent regulators of vasomotor traffic in the periphery (7). If the heart releases enkephalins, they may serve to regulate afterload and therefore allow the heart to influence its own workload. MEAP is also a very potent inhibitor of efferent vagal control of the heart (9). This effect probably involves the local inhibition of acetylcholine release and may represent either an endocrine or paracrine effect. Enkephalin mediated inhibition of vagal control could explain the progressive tachycardia observed during hypotension when circulating catecholamines and blood pressure remained relatively constant.

In summary, a series of different sized enkephalins have been identified in both the heart and plasma. ME, MEAP Peptide-B, and Peptide-F represent the majority of the enkephalins in the blood and only these four are significantly increased in plasma during hemorrhagic hypotension. Proenkephalin and Peptide-B account for most of the intrinsic cardiac enkephalin and both are increased after hemorrhage. The dynamic responses in circulating and intrinsic cardiac enkephalins during alterations in the cardiovascular environment suggest the heart may be a functional source of enkephalin which may then serve to regulate autonomic responses.

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TABLE 1

BASAL CARDIOVASCULAR PARAMETERS (means ± SE)		
	CONTROL	HYPOTENSION
NUMBER OF SUBJECTS	8	9
HEART RATE (bpm)	104 ± 11.27	107 ± 9.61
MEAN ARTERIAL PRESSURE (mmHg)	95 ± 7.29	101 ± 3.03
SYSTOLIC PRESSURE (mmHg)	120 ± 7.64	128 ± 4.29
DIASTOLIC PRESSURE (mmHg)	84 ± 6.86	88 ± 3.06
CENTRAL VENOUS PRESSURE (mmHg)	3.3 ± 1.11	3.8 ± 2.56

TABLE 2

BLOOD GAS MEASUREMENTS			
TIME	PROCEDURE	PCO_2	PO_2
-15 Minutes	Control	33.13 ± 3.05	116 ± 8.26
	Hypotension	30.1 ± 1.35	118 ± 3.57
0 Minutes	Control	35.15 ± 3.13	105 ± 6.74
	Hypotension	29.6 ± 1.24	102 ± 6.80
30 Minutes	Control	34.53 ± 1.90	111 ± 3.73
	Hypotension	32.3 ± 1.72	108.6 ± 8.05
60 Minutes	Control	33.58 ± 1.86	111 ± 7.56
	Hypotension	31.81 ± 1.76	106.9 ± 6.76
90 Minutes	Control	31.53 ± 1.85	116 ± 5.71
	Hypotension	30.16 ± 1.59	110 ± 6.94
120 Minutes	Control	30.86 ± 1.75	114 ± 6.13
	Hypotension	32.12 ± 1.89	110 ± 9.51

Values are means ± SE.

N=8 Control; n=9 Hypotensive

TABLE 3

Heart Tissue Norepinephrine (nmols/gm)					
	Right Atrium	Left Atrium	Right Ventricle	Left Ventricle	Septum
Control	15.2 ± 1.6*	13.5 ± 1.0*	6.7 ± 0.6	5.8 ± 0.7	7.0 ± 0.9
Hypotension	11.9 ±1.4*,**	10.1 ±1.2*,**	4.7 ± 0.5**	3.9 ± 0.6**	3.4 ± 0.4**

Means ± SE, n = 8 control; n=9 hypotensive.

* Significantly different from ventricles.

** Significantly different from control.

TABLE 4

TISSUE ME-ir (pmol/gram)					
	Procedure	**Very Large**	**Large**	**Peptide F**	**ME**
Right Atrium	control	0.35 ± 0.08	0.22 ± 0.04	0.98 ± 0.16	0.17 ± 0.04
	hypotension	0.20 ± 0.03	0.24 ± 0.05	0.84 ± 0.12	0.23 ± 0.03
Left Atrium	control	0.22 ± 0.03	0.13 ± 0.04	0.80 ± 0.13	0.23 ± 0.04
	hypotension	0.28 ± 0.07	0.15 ± 0.03	0.90 ± 0.16	0.12 ± 0.03
Left Ventricle	control	0.15 ± 0.03	0.20 ± 0.03	0.88 ± 0.12	0.30 ± 0.06
	hypotension	0.21 ± 0.04	0.18 ± 0.04	0.94 ± 0.16	0.18 ± 0.02
Right Ventricle	control	0.28 ± 0.07	0.15 ± 0.03	0.76 ± 0.09	0.23 ± 0.07
	hypotension	0.31 ± 0.04	0.14 ± 0.04	0.82 ± 0.13	0.21 ± 0.03
Septum	control	0.18 ± 0.04	0.17 ± 0.04	0.74 ± 0.10	0.14 ± 0.04
	hypotension	0.23 ± 0.04	0.21 ± 0.05	0.66 ± 0.07	0.19 ± 0.04

Met-enkephalin-immunoreactivity (Me-ir) was not significantly different between treatments or among tissue types.

Values are means ± SE.

TABLE 5

TISSUE MEAP-ir (pmol/gram)					
	Procedure	ProEnk	Large	Peptide B	MEAP
Right Atrium	control	2.56 ± .81	1.75 ± .47	5.67.38	3.19 ± .38
	hypotension	2.67 ± .39	2.91 ± .48	9.20 ± 1.73	2.27 ± .30
Left Atrium	control	2.49 ± .50	2.15 ± .48	5.97 ± 1.02	1.89 ± .23
	hypotension	3.35 ± .69	3.82 ± .99	9.05 ± 1.32	1.93 ± .36
Left Ventricle	control	7.30 ± 1.14	4.37 ± .99	25.15 ± 2.01	6.35 ± .88
	hypotension	10.14 ± 1.45	4.04 ± .67	28.29 ± 1.81	4.37 ± .74
Right Ventricle	control	7.53 ± .76	2.15 ± .39	20.99 ± 1.92	3.56 ± .75
	hypotension	12.32 ± 1.85	3.49 ± .94	26.78 ± 2.38	2.70 ± .32
Septum	control	6.19 ± .60	1.55 ± .37	21.30 ± 1.59	4.14 ± .90
	hypotension	9.35 ± 1.39	4.06 ± .69	28.58 ± 1.74	4.25 ± .76

There was a significant effect of hypotension ($P < 0.05$) on met-enkephalin-arg-phe-immunoreactivity (MEAP-ir) across tissue types for all molecular weight fractions except MEAP ($p < 0.054$). The ventricular Proenkephalin (Pro-Enk) and Peptide B concentrations were significantly greater than those in the atria ($p < 0.05$).

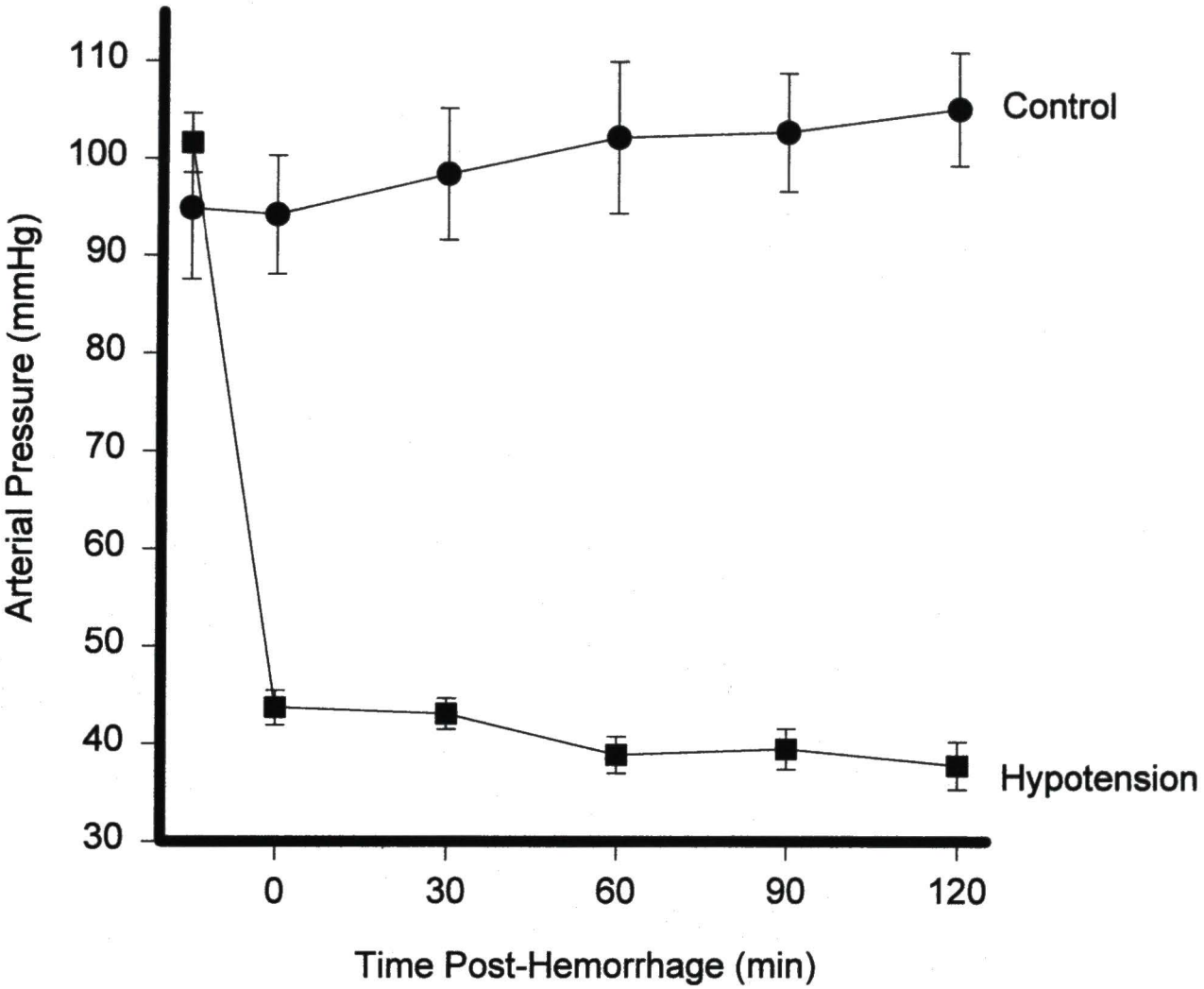

Figure 1. This figure illustrates changes in mean arterial pressure between control and hypotension during 2 hr experimental protocol.

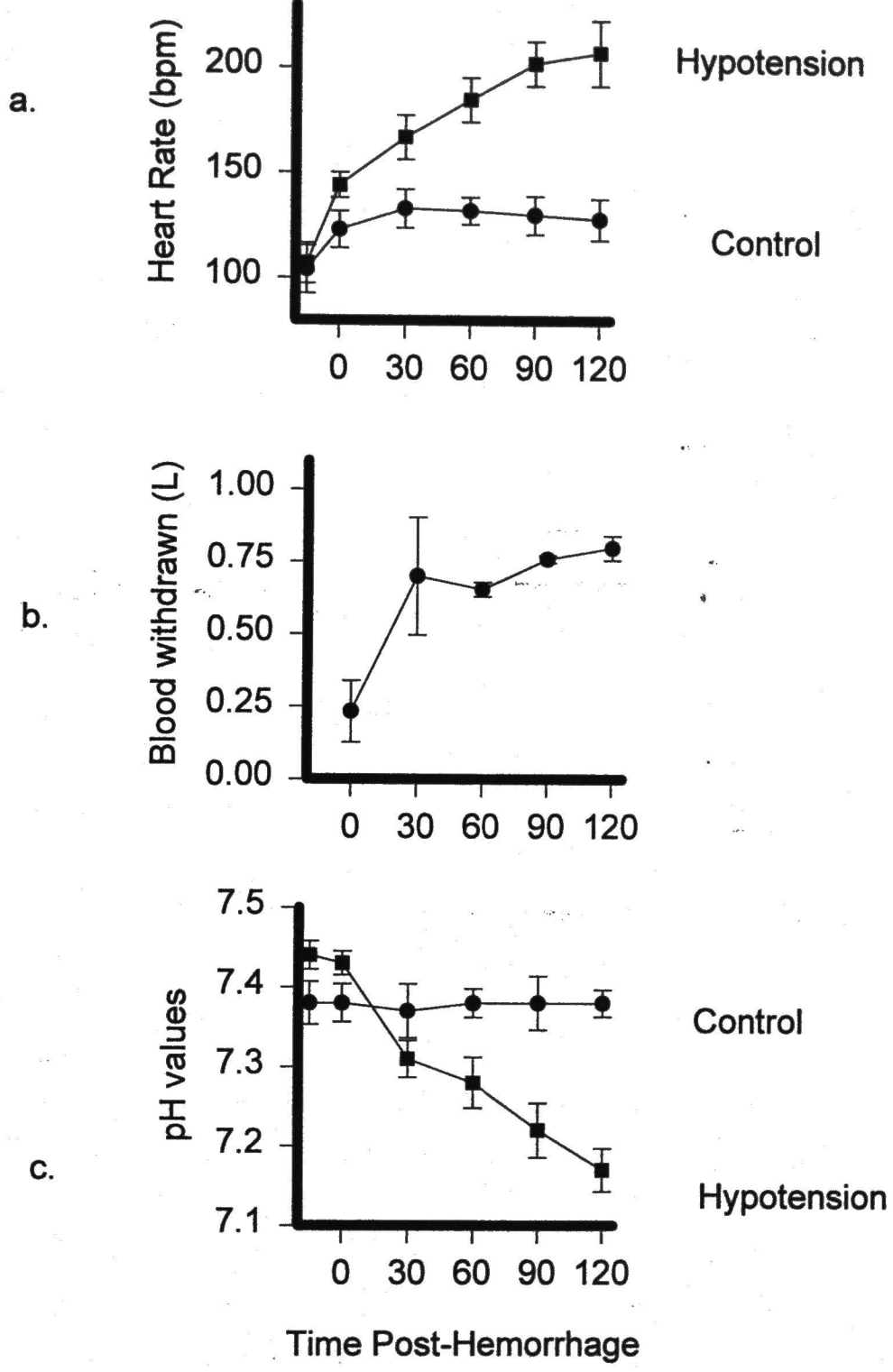

Figure 2a. Heart rate, control vs hypotensive animals. Values are means and SE, n=8 for control and n=9 for hypotension. Control vs hypotension, P< 0.05. Figure (2b) provides representative data for the cummulative blood withdrawn to maintain arterial pressure at 40 mmHg (n=3). Figure (2c) arterial pH, control vs hypotension. Values are means and SE, n=8 & 9 respectively. Control vs hypotension, P< 0.05.

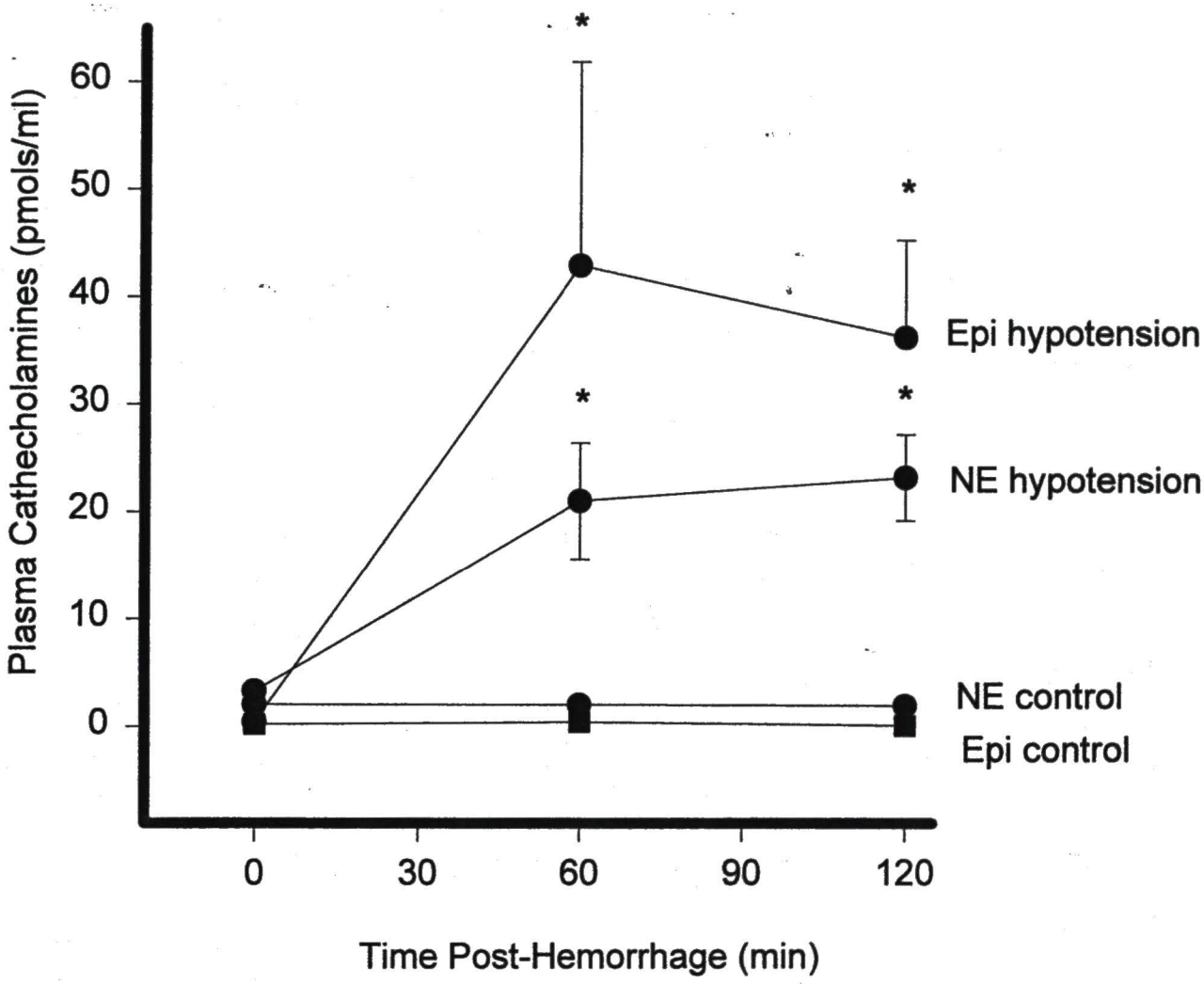

Figure 3. Plasma cathecholamines, control vs hypotensive animals. Values are means and SE, n=8 & 9 respectively. Epi and NE are significantly different from control, $P < 0.05$.

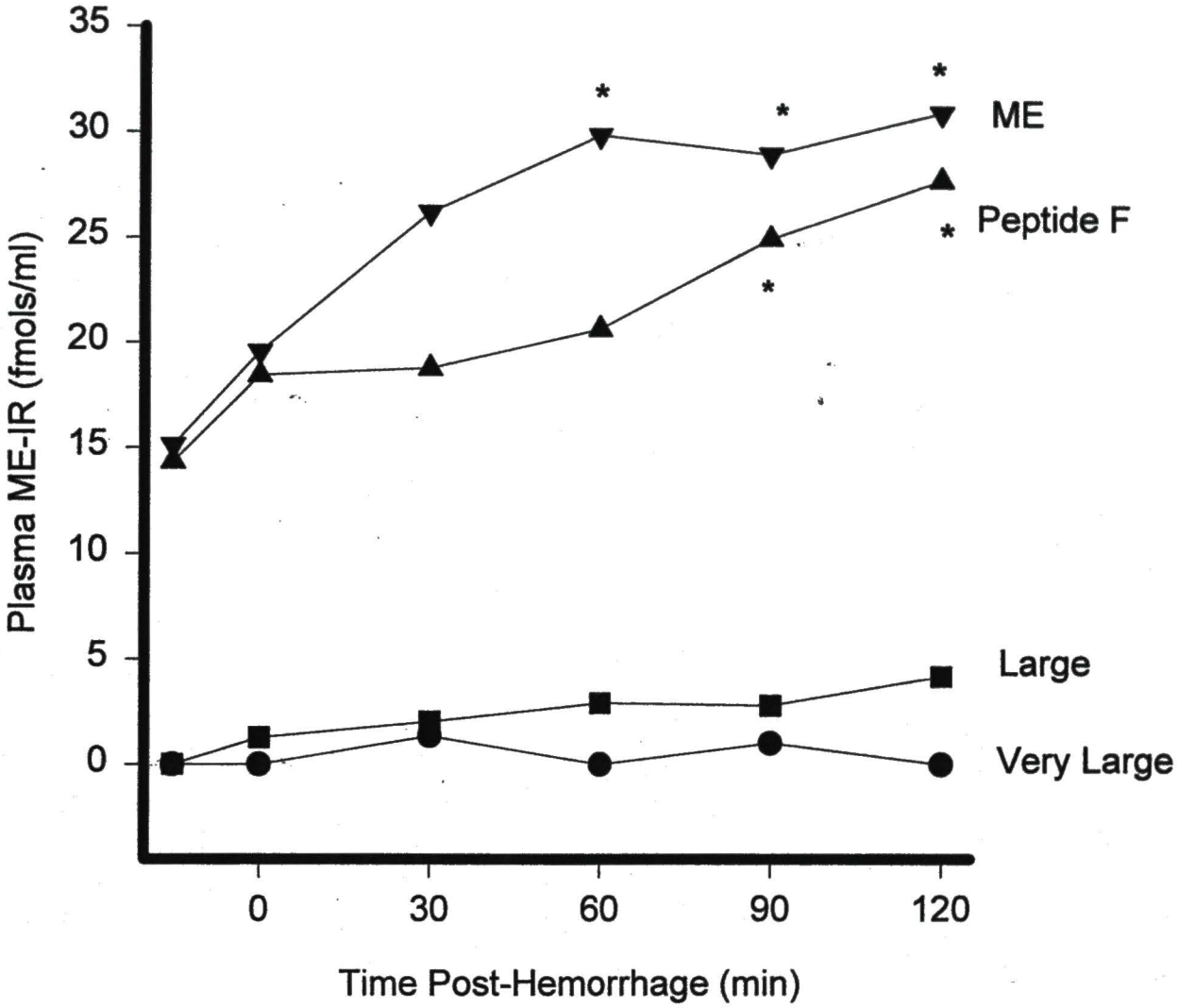

Figure 4. Plasma met-enkephalin immunoradiactivity during hypotension. Values are means ± SE, n=9. Error bars have been omitted for clarity.
* Significantly different from hemorrhage control, $P < 0.05$.

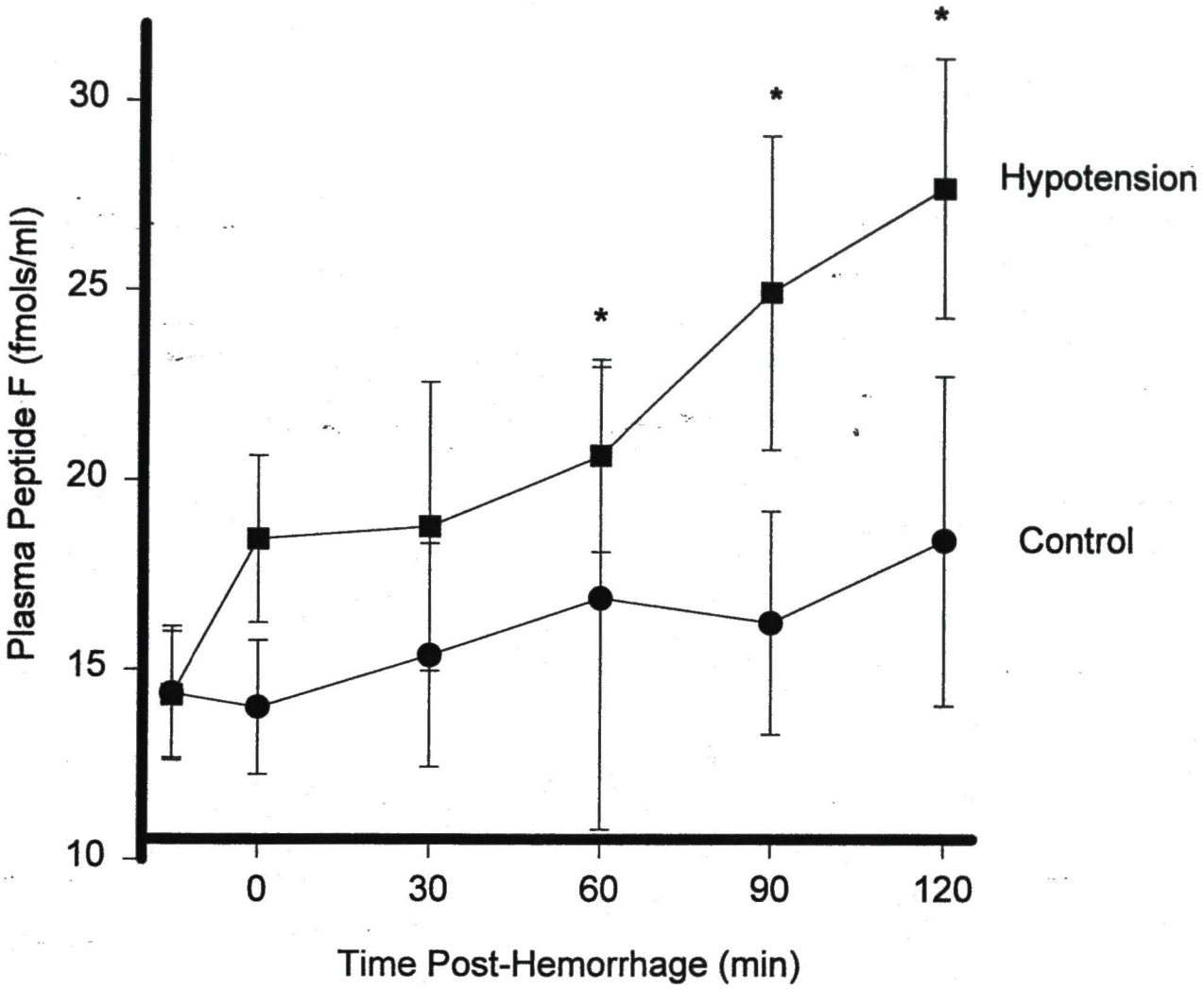

Figure 5. Plasma Peptide-F, control vs hypotensive animals. Values are means ± SE, n=9 for hypotension and n=8 for control. *P < 0.05 compared to prehemorrhage. Control vs hypotension, P<0.05.

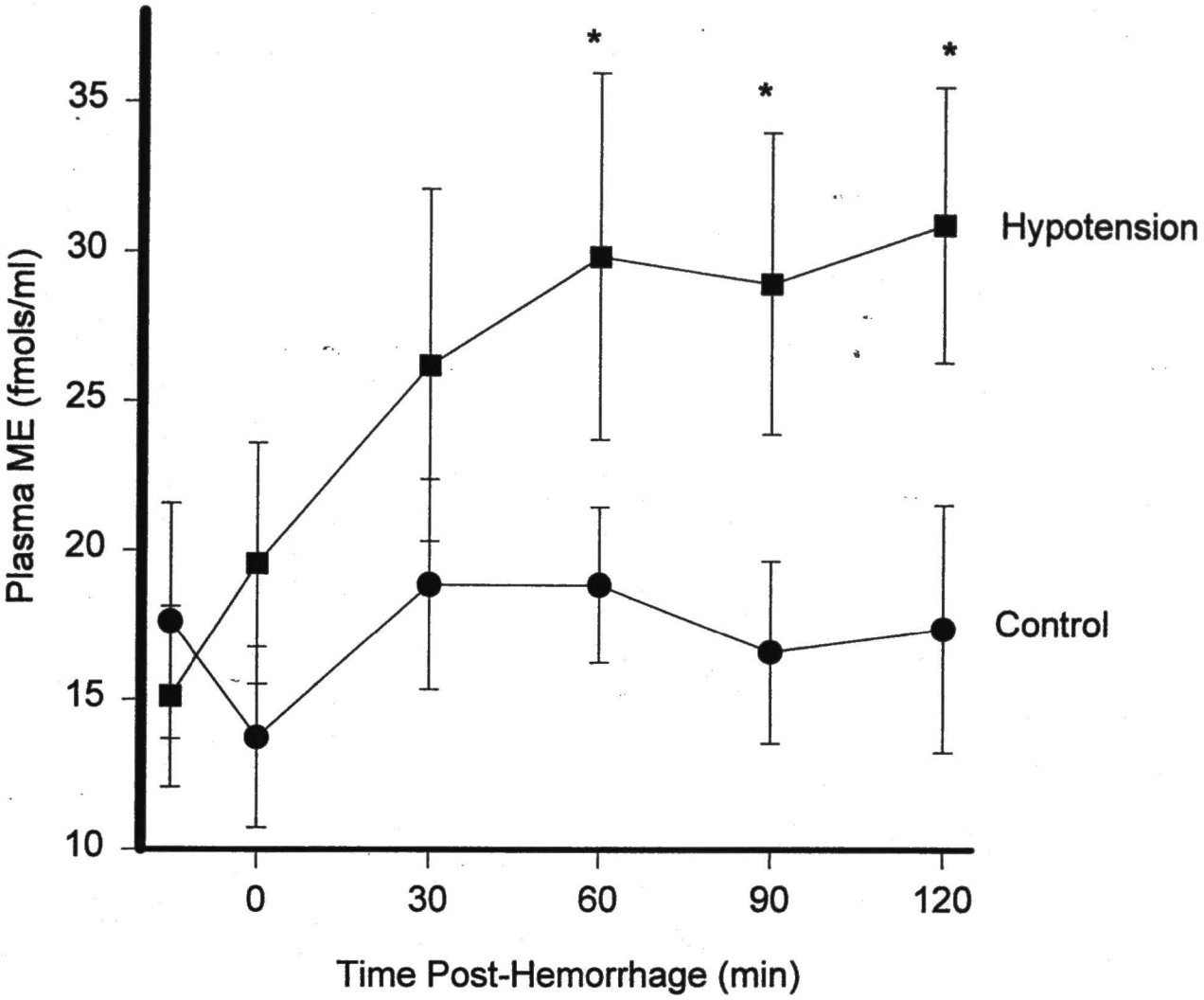

Figure 6. Plasma met-enkephalin, control vs hypotensive animals. Values are means ± SE, n=8 for control and n=9 for hypotension, *P<0.05 compared to prehemorrhage determination. Hypotension vs control, P<0.01.

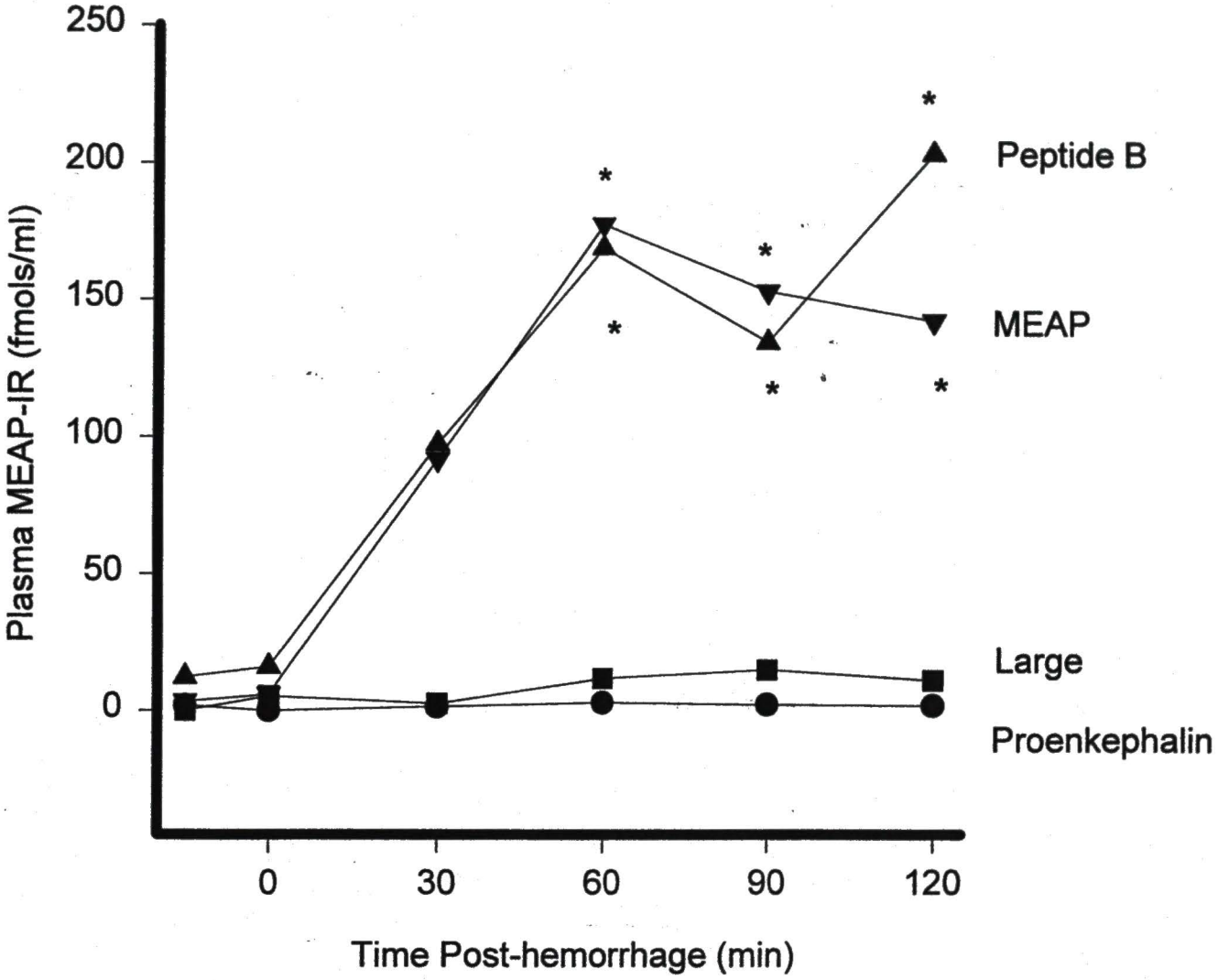

Figure 7. Plasma met-enkephalin-arg-phe and related peptides during hypotension. Values are means, n=9. Error bars were omitted for clarity.
* Significantly different from prehemorrhage determination, $P<0.05$.

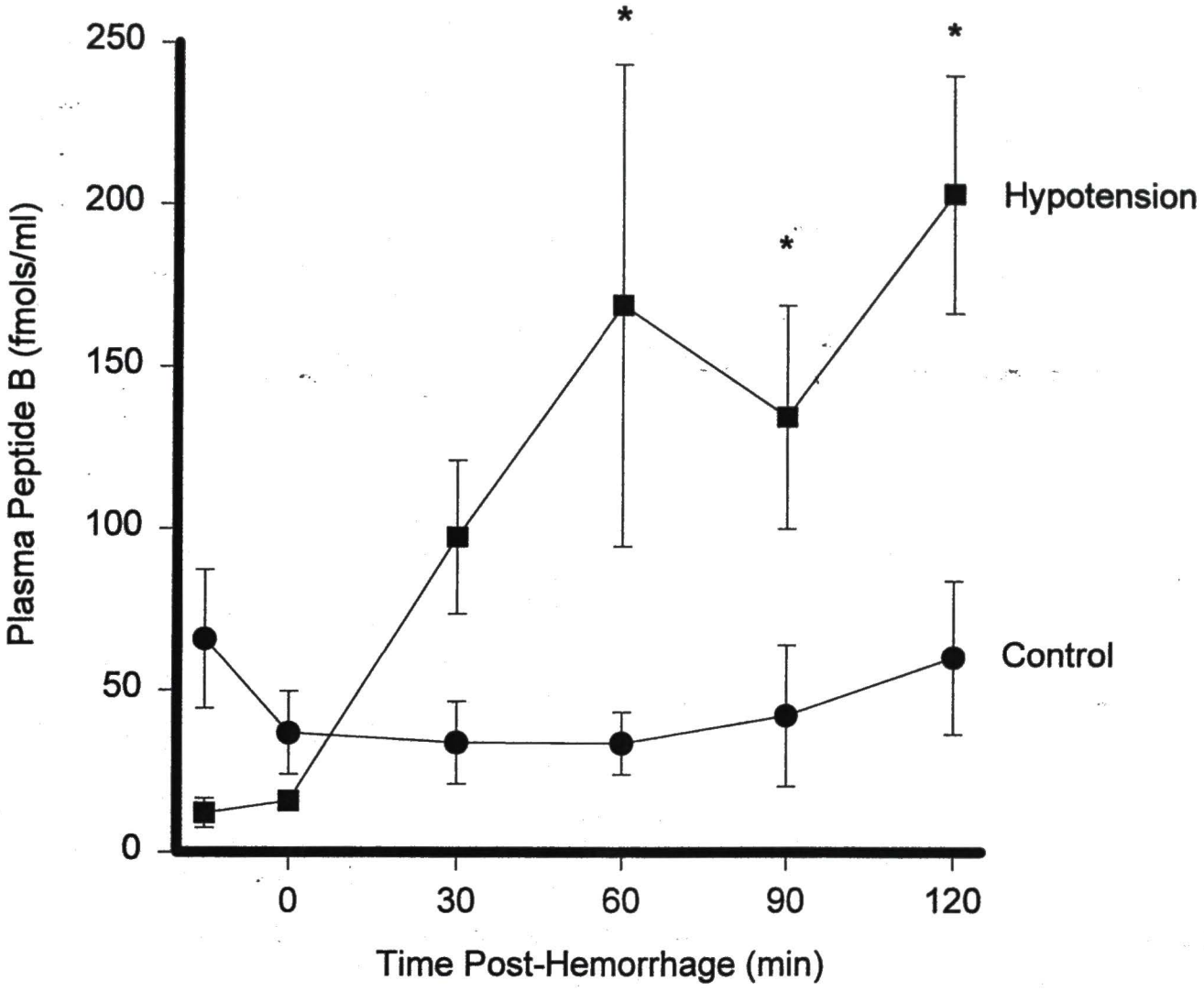

Figure 8. Plasma Peptide-B, control vs hypotensive animals. Values are means ± SE, n=8, *P< 0.05 compared to prehemorrhage determination. Control vs hypotension, P<0.01.

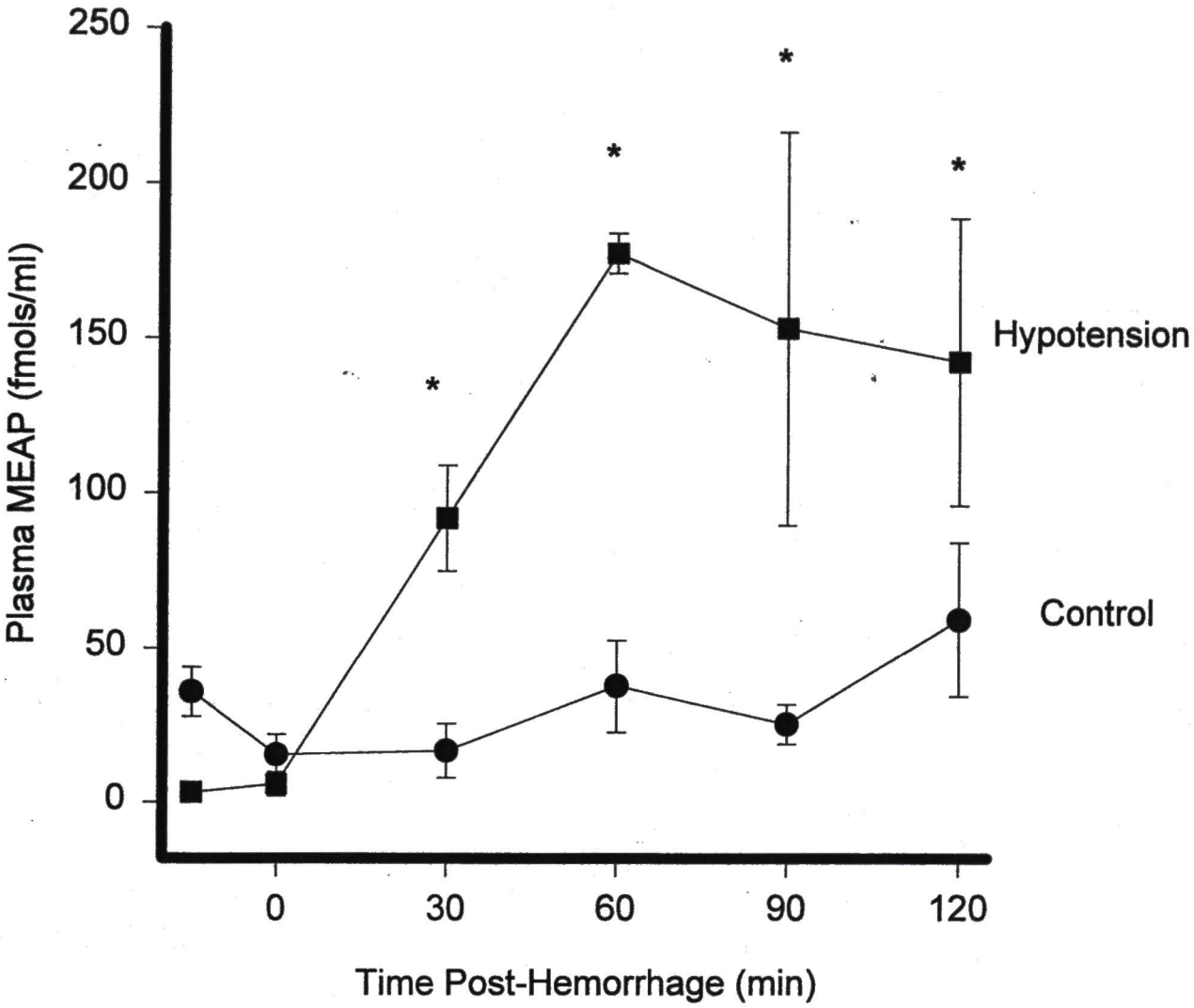

Figure 9. Plasma MEAP, control vs hypotensive animals. Values are means ± SE, *P< 0.05 compared to prehemorrhage determination. Hypotension vs Control, P< 0.01.

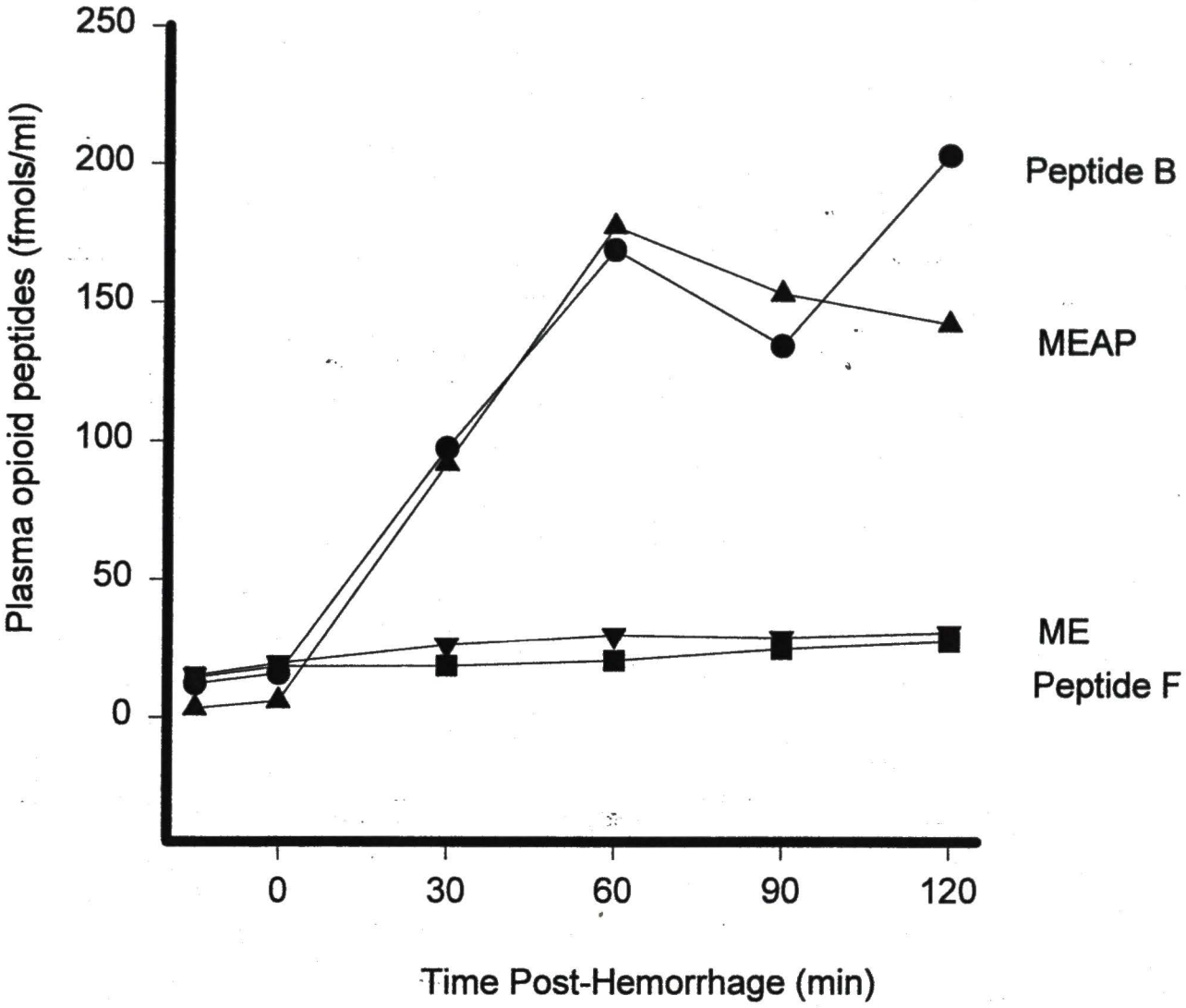

Figure 10. Comparison of relative change in ME-ir and MEAP-ir during hypotension. N=9 for ME-ir and n=8 for MEAP-ir. Error bars have been omitted for clarity.

CONCLUSIONS

1. The majority of met-enkephalin-arg-phe immunoreactivity circulates in the plasma as the free heptapeptide and the N-terminally extended intermediate, Peptide-B. Limited quantities of larger MEAP containing peptides are present as well.

2. Plasma MEAP, ME, Peptide-B and Peptide-F all increase significantly during hemorrhagic hypotension. Relative and absolute increases in MEAP and Peptide-B are far greater than those for ME and Peptide-F. This sharp rise in circulating opioids suggests that enkephalin actively participates in the circulatory adjustment to hemorrhagic hypotension.

3. Intrinsic myocardial enkephalins are predominantly comprised of Peptide-B and Proenkephalin.

4. The increases in plasma enkephalins during hemorrhagic hypotension are accompanied by significant increases in several large and intermediate sized precursors including Proenkephalin and Peptide B. This suggests that myocardial enkephalins may contribute to the rise in plasma enkephalin during hypotension.

5. The close correlation between circulating enkephalin and increasing heart rate during hypotension suggests this response may result from the vagolytic effect of these peptides.