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THE ROLE OF NOREPINEPHRINE IN THE DEVELOPMENT OF HEPATOCELLULAR DYSFUNCTION FOLLOWING TRAUMA-HEMORRHAGE AND CRYSTALLOID RESUSCITATION

By

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ABSTRACT

THE ROLE OF NOREPINEPHRINE IN THE
DEVELOPMENT OF HEPATOCELLULAR DYSFUNCTION
FOLLOWING TRAUMA-HEMORRHAGE
AND CRYSTALLOID RESUSCITATION

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Liver dysfunction is a relatively common occurrence following trauma-hemorrhage. Liver dysfunction may contribute to multiple organ failure, leading to death after injury. The sympathetic adrenal system is rapidly activated in response to trauma-hemorrhage. This study explores an association between the catecholamine response and hepatocellular dysfunction observed following hemorrhage. A fixed pressure model of trauma-hemorrhage and resuscitation in the rat was used. Briefly, rats were subjected to a midline laparotomy to induce trauma and were bled to and maintained at a mean arterial pressure of 40 mmHg until 40% of the maximal bleedout volume was returned in the form of Ringer's lactate. The hypotension time was approximately 90 minutes. The animals were then resuscitated with Ringer's lactate over 60 minutes using a volume equal to 4 times the volume of the maximal shed blood. The alterations in hepatic β-adrenergic receptor binding characteristics and plasma catecholamine levels were studied during different stages of this model. The results show that 1.5 hours following trauma-hemorrhage and resuscitation the β-

adrenergic receptor maximal binding capacity (B_{max}) of whole liver plasma membrane preparations and isolated hepatocytes was depressed while the B_{max} of Kupffer cells was increased. The dissociation constant (K_d) of β-adrenergic receptors was unaltered in isolated hepatocytes, Kupffer cells, and whole liver plasma membrane preparations following hemorrhagic shock and crystalloid resuscitation. The plasma levels of epinephrine were elevated ~100-fold during hemorrhage. Following resuscitation, the epinephrine levels began to decrease and they were normalized between 4 and 8 hours following resuscitation. Plasma levels of norepinephrine were elevated ~10-fold during hemorrhage and remained elevated up to at least 24 hours following resuscitation. Since the norepinephrine levels remained elevated, while those of epinephrine were normalized, the effects of elevated plasma levels of norepinephrine (similar to those observed following hemorrhage) on several cardiovascular and hepatic parameters were then examined. To determine the effects of chronically elevated plasma norepinephrine in normal rats, Mini-osmotic pumps (consistently releasing norepinephrine) were implanted into the peritoneal cavity for a period of 24 hours. Chronic elevation of plasma norepinephrine caused depressions in stroke volume and cardiac output, which resulted in a depressed mean arterial blood pressure despite an increase in total peripheral resistance. This chronic elevation in plasma norepinephrine, similar to that observed following

hemorrhage, caused significant depressions in active hepatocellular function and hepatic microvascular blood flow. Elevated plasma levels of GOT and GPT were observed, indicating an altered hepatocyte integrity. Chronic infusion of norepinephrine reduced hepatocyte β-adrenergic receptor B_{max}. In addition, plasma levels of IL-6, an inflammatory cytokine, were shown to be significantly elevated due to the chronic norepinephrine infusion. These findings suggest that the elevation of norepinephrine may play a role in the development of hepatocellular dysfunction and altered hepatocyte integrity following trauma-hemorrhage and crystalloid resuscitation.

To Isaac Asimov and Pink Floyd;

for teaching me to look beyond my nose.

To my wife, Stacie;

for helping me to see my hand in front of my face.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

18. Differences in mean arterial blood pressure at 24 hours following infusion of norepinephrine
19. Differences in total peripheral resistance at 24 hours following infusion of norepinephrine
20. Differences in microvascular blood flow at 24 hours following infusion of norepinephrine
21. Differences in systemic hematocrit at 24 hours following infusion of norepinephrine
22. Body weight loss at 24 hours following infusion of norepinephrine
23. Differences in active hepatocellular function at 24 hours following infusion of norepinephrine
24. Hepatocellular damage, as measured by plasma levels of GOT/GPT at 24 hours following infusion of norepinephrine
25. Differences in plasma glucose levels at 24 hours following infusion of norepinephrine
26. Differences in the maximal binding capacity of β-adrenergic receptors on hepatocytes at 24 hours following infusion of norepinephrine
27. Differences in the dissociation constant of β-adrenergic receptors on hepatocytes at 24 hours following infusion of norepinephrine
28. Differences in plasma levels of tumor necrosis factor at 24 hours following infusion of norepinephrine
29. Differences in plasma levels of interleukin-6 at 24 hours following infusion of norepinephrine

INTRODUCTION

LITERATURE REVIEW

CATECHOLAMINES

The term "catecholamine" refers to phenylethylamines which have hydroxyl groups at the 3 and 4 positions of the aromatic ring (147). The hydroxyl groups form the "catechol" portion of the catecholamine, which is required for the maximum potency of the molecule as an adrenergic agonist (147). The ethylamine portion of catecholamines is associated with the affinity of the catecholamine to a specific adrenergic receptor, while the aromatic ring structure is involved with the activity of the catecholamine (147). In other words, the side chains associated with the ethylamine portion of the molecule affect the affinity of that molecule to a specific adrenergic receptor, while the side chains associated with the aromatic ring determine if the compound acts as an agonist. Norepinephrine and epinephrine are catecholamines that are produced and secreted in the body (11). Norepinephrine is released by most, but not all, postganglionic nerve fibers of the sympathetic nervous system (11). Epinephrine, otherwise known as adrenaline, is released into the circulation by the adrenal medulla, which sits superior to the kidney (11). Norepinephrine is also released from the adrenal medulla, albeit to a lessor degree than epinephrine (42). The

adrenal medulla cells are pseudopostganglionic neurons which are innervated by preganglionic sympathetic nerve fibers (11).

ADRENERGIC RECEPTORS

Historical perspective and pharmacology

The receptors for catecholamines have been termed "adrenergic" receptors. The existence of subtypes of adrenergic receptor subtypes was first proposed in 1948, when Raymond Ahlquist observed that adrenergic agonists exhibited two distinct responses based on the order of potency of the compounds in causing a response in different organs (1). Since then, multiple subtypes have been discovered, namely, α_1, α_2, β_1, β_2, and β_3, depending upon their relative affinity for each of the adrenergic ligands (146,147).

These subtypes of adrenergic receptors are all globular, plasma membrane spanning proteins, that have the same general structure (119). The adrenergic receptors consist of one amino acid chain with seven transmembrane α-helices (119). The N-terminal end extends off the extracellular surface and has many N-linked glycosylations (119). The C-terminal end extends into the cytoplasm and has many serine and threonine sites, which can be phosphorylated by β-adrenergic receptor kinase causing receptor desensitization (54). The seventh transmembrane

helix is involved with agonist binding and parts of the third intracellular loop and C-terminal end appear to be involved with G protein interactions (54).

Agonist binding to β_1, β_2, and β_3 adrenergic receptors activates G_s proteins, which stimulate adenylate cyclase to produce the second messenger cAMP (11). α_2 adrenergic receptors activate G_i proteins, which block the activation of adenylate cyclase (11). α_1 receptors activate an unknown G protein which activates phospholipase C and causes an increase in the intracellular levels of Ca^{2+} (11). The α and β adrenergic receptors are found in many tissues throughout the body and typically both subtypes will be found within each of these tissues (42).

Although epinephrine and norepinephrine act as agonists for each of the adrenergic receptor subtypes, there are differences in the relative affinity for each of these catecholamines specific to each of the adrenergic receptor subtypes (147). Due to this receptor crossover, the particular physiological effect of either epinephrine or norepinephrine is a function of the relative amounts of each catecholamine found in the circulation and the adrenergic receptor distribution and sensitivity. In general, epinephrine is known to cause inotropic and chronotropic increases in cardiac output, vasodilation resulting in decreased total peripheral resistance, increased systolic pressure, and decreased diastolic pressure (95).

Norepinephrine has moderate inotropic and chronotropic effects on the heart (95). However, cardiac output is either unchanged or slightly depressed, which is due to the pronounced vasoconstriction that results in increased total peripheral resistance and increased arterial blood pressure (95). Both epinephrine and norepinephrine cause an increase in hepatic glucose output by stimulating glycogenolysis and gluconeogenesis, and by inhibiting glycogenesis (42).

Radioligand binding studies

In 1974, three groups independently developed methods that allowed for the study of β-adrenergic receptors using radioligands with high specific activities (6,75,76). The use of several thermodynamic principles, including the law of mass action, allows for the derivation of equations that describe the ligand-receptor interactions (26,53). The binding data that is observed during a membrane filtration assay can then be interpreted by these equations and characteristics of the ligand-receptor interactions can be determined for the specific tissue. A Scatchard plot can then be used to linearize this data and determine the maximal binding capacity and affinity of the receptor population (14,110).

Activation of adenylate cyclase

When agonist is bound to β-adrenergic receptors there is a resultant rise in intracellular levels of cAMP. The mechanism of β-adrenergic receptor stimulation causing increased cAMP involves a stimulatory G protein (G_s) and adenylate cyclase (52). Adenylate cyclase is an enzyme which lowers the activation energy needed to convert ATP into cAMP (121). G_s is a membrane associated heterotrimeric G protein consisting of an α, β, and γ subunit (93). When agonist bound β-adrenergic receptors associate with G_s, a GDP molecule is released from the α subunit, causing dissociation from the β and γ subunits (120). Once the α subunit dissociates into the cytoplasm, it is quickly bound to a GTP molecule, which activates the α subunit (16). This activated α subunit associates with adenylate cyclase, thus activating it to convert ATP to cAMP (87). The cAMP acts as an intracellular messenger (second messenger), activating or inactivating specific protein kinases (122). The α subunit has an intrinsic GTPase activity, which is relatively slow (52). When the GTP that is associated with the α subunit is cleaved to form GDP the α subunit dissociates from the adenylate cyclase, thus inactivating it (52). The α subunit (with GDP bound) then associates once again with a β and γ subunit (52).

Receptor desensitization

A general but not universal property of many biological systems is that prolonged exposure to an agonist produces attenuation of the biological response to that agonist, which is referred to as desensitization. β-adrenergic receptors have been shown to become desensitized to catecholamines in a number of tissues (147). This desensitization can occur by a homologous or heterologous mechanism. Heterologous desensitization refers to a decrease in adenylate cyclase activity so that other hormones which act via adenylate cyclase are also said to be desensitized (51). Homologous or receptor specific desensitization refers to the modification of agonist bound receptor so that there is a functional uncoupling of receptor from its second messenger system (51). This involves the changing of the receptor structure by phosphorylation or the sequestration and degradation of receptors (23). β-adrenergic receptor kinase and cAMP-dependent protein kinase are responsible for the phosphorylation of the carboxyl end of the β-adrenergic receptors (43). These phosphorylations are followed by the binding of β-arrestin, which leads to sequestration and degradation of the β-adrenergic receptor (99). Prior to stimulation, β-adrenergic receptor kinase is associated with the peripheral side of cytoplasmic microsomal membranes (44). Following β-adrenergic receptor stimulation the β-

adrenergic receptor kinase dissociates from the microsomal membranes, diffuses through the cytoplasm and associates with the $\beta\gamma$ subunits of G_s (66). The G protein heterodimer $\beta\gamma$ anchors the β-adrenergic receptor kinase to the membrane and promotes agonist- dependent phosphorylation of receptors (100).

THE LIVER

Cells

The liver is comprised mainly of hepatocytes (2), while sinusoidal endothelial cells, Kupffer cells, Ito cells, and Pit cells make up the other cells of the liver (2).

Hepatocytes, primarily responsible for hepatic metabolism, appear homogeneous under the light microscope. However, there is a great deal of heterogeneity of hepatocytes, as determined by structure, function, and location (45,61). Hepatocytes perform a diverse number of metabolic functions, including: formation of bile, carbohydrate storage and release, formation of urea, production of plasma proteins, metabolism of fats, inactivation of polypeptide hormones, reduction and conjugation of steroid hormones, and detoxification of many drugs and toxins (2). Sympathetic nerve fibers, which release norepinephrine, make contact with virtually every hepatocyte and have a profound influence over their metabolic activity (109). Stimulation of these nerve fibers

leads to an increase in hepatic glucose output (109).

Sinusoidal endothelial cells line the sinusoids formed by the hepatocytes (68). These cells have numerous fenestrae, which are grouped together in sieve plates (68). The size of these fenestrae determines the amount of exchange between blood and the perisinusoidal space (70). The size of these openings is controlled by both hormones and neurotransmitters, via contractions in cytoskeletal components (70). The sinusoidal endothelial cells have numerous functions in various biological reactions, including inflammation, immune responses, regulation of hepatic microvascular blood flow, and lipid metabolism (107).

Kupffer cells account for approximately 1/3 of the sinusoidal cell volume, constitute 80 to 90% of the fixed tissue macrophages of the reticuloendothelial system, are found on both sides of the endothelial cells, and are highly active in the phagocytosis of unnecessary, damaged, altered, and foreign material (70,85). The Kupffer cells produce and secrete a number of mediators with potent biological effects, including proteases and cytokines, which influence hepatocytes as well as other sinusoidal cells (70).

Ito cells are located in the perisinusoidal space, with cytoplasmic extensions wrapped around the sinusoidal endothelial lining (149). Due to their contractile properties, they may play an important role in the

regulation of sinusoidal blood flow by acting as sinusoidal sphincters (98). The Ito cells not only function to maintain the normal extracellular matrix (5), but they also play a major role in liver fibrosis, which is stimulated by proteins released from damaged hepatocytes (17).

Pit cells are similar to large granular lymphocytes and are located on the sinusoidal endothelial lining (17,149). These Pit cells exhibit natural killer cell activity (17,149).

Circulation

The liver normally receives approximately 25% of the cardiac output, with a dual blood supply; ~75% is poorly oxygenated blood from the hepatic portal vein and ~25% is oxygenated arterial blood from the hepatic artery (90). Blood from both sources mixes in the sinusoids and through arterial-portal anastomoses (89). The sinusoids are also connected to one another via numerous gates in a net-like fashion (89). Because all of these connections (arterial-portal, arterial-sinusoid, portal-sinusoid, sinusoid-sinusoid) are independently gated by contractile components, the sinusoids can receive varying mixtures of portal and arterial blood in addition to local variations within the liver itself (89). In fact, there is evidence that there are variations in the fraction of arterial/portal blood flow in different sections of hepatic lobes, which reiterates

that there exists a heterogeneity of hepatocytes (27). Blood from the sinusoids enters the inferior vena cava via a common hepatic vein (90).

In the liver, neither the portal system nor the arterial system autoregulate; i.e., as flow and pressure increase, resistance either remains constant or decreases (47). Sympathetic nerve stimulation constricts the pre-sinusoidal resistance vessels in the portal venules, hepatic arterioles, sinusoids, and central venules, which is mediated by α-adrenergic receptors (108). β-adrenergic agonists tend to be dilatory, particularly in the hepatic arterioles (108).

Hepatic microvascular injury occurs following hepatic ischemia/reperfusion partly due to the inflammatory response, which causes the adhesion of leukocytes to the endothelial lining of the venules and sinusoids (88). Kupffer cells release a variety of vasoactive and toxic mediators, such as prostaglandins, leukotrienes, interleukin-1, tumor necrosis factor, and nitric oxide (33). It is suspected that many of these substances are involved with the regulation of hepatic microvasculature and contribute to the microvascular dysfunction during low flow conditions (33). However, there is little data regarding the precise mechanisms by which these mediators affect the hepatic microcirculation. Following low flow conditions, Kupffer cells and sinusoidal endothelial cells may become

swollen, possibly impairing flow (91). This leads to leukocyte adhesion, further impeding microvascular blood flow (91).

Hepatic dysfunction following trauma-hemorrhage

Liver dysfunction following trauma-hemorrhage is a relatively common occurrence and its presence is associated with a adverse outcome when this dysfunction manifests as liver failure (129). Hepatic dysfunction following trauma-hemorrhage can be categorized into that which occurs immediately (primary hepatic dysfunction) following trauma-hemorrhage and that which occurs later (secondary hepatic dysfunction) usually as a result of a systemic inflammatory response (129).

Immediately following the onset of shock as well as after resuscitation, there is a reduction in liver perfusion (132). This leads to a decrease in lactate and amino acid clearance as well as total hepatocellular protein synthesis (129). Also, there is a depression in the energy charge of the liver with an accumulation of inorganic phosphate and lower adenine nucleotides, which leads to the leakage of inorganic phosphate from the hepatocytes (129). The presence of intrahepatocellular enzymes glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT) in the circulation is characteristic of hepatocellular damage following hemorrhage (139). The degree of primary

hepatic dysfunction produced is related to the degree and duration of the microcirculatory compromise, in addition to the type and adequacy of resuscitation (129).

Later following the completion of resuscitation, the secondary hepatic failure is due to physiological responses to endogenous mediators such as cytokines increased during hemorrhage (129). These endogenous mediators cause a systemic inflammatory response (129). During this stage the amino acid clearance of the liver is increased as a result of increased acute phase protein production, while the production of non-acute phase proteins, such as albumin and transferrin, is decreased (19). Both gluconeogenesis and glucose release are increased, although there is a reduction in the production and release of ketone bodies (148). The redox state of hepatocytes, as reflected by the lactate/pyruvate and ketone body ratios are normal (18). The increased synthesis and release of acute phase proteins is characteristic of the acute inflammatory response, which can be induced by the cytokines TNF and IL-6 (73). These cytokines can be released by the closely associated Kupffer cells (94).

Determination of active hepatocellular function

Jaundice, hypoglycemia, and bleeding due to a depression in the circulating levels of various clotting factors produced by the liver are all indications of severe

hepatic failure (22,48). Elevated levels of liver enzymes (GOT and GPT) in the plasma are indications of alterations in the integrity of the hepatocellular plasma membrane (hepatocyte damage) and not hepatocellular dysfunction (22,138). A more sensitive indicator of hepatocellular function, removal of injected indocyanine green (ICG) from the plasma, is useful in detecting abnormalities in hepatic function. Indocyanine green is a dye that has several properties which make it useful in the assessment of hepatocellular function. It is nontoxic at low doses and bound to albumin in the circulating plasma (24,74,97). ICG is cleared exclusively by the liver via an energy-dependent receptor mediated membrane transport process (46,74). Michaelis-Menton kinetics can be applied to the initial uptake of ICG into the liver and a theoretical maximal velocity of clearance (V_{max}) can be determined (74). This maximal velocity of clearance would be attained when all of the receptor sites are occupied, and would be irrespective of hepatic blood flow (74). An *in vivo* hemoreflectometer can be used for the determination of indocyanine green concentration, performed without the removal of blood samples (50). Several studies have employed the indocyanine green clearance technique in order to examine the alterations in active hepatocellular function (46,84,97,123). Furthermore, this technique has been utilized successfully for numerous studies measuring active

hepatocellular function under various low flow conditions (49,50,130,131,133,135,136,142).

CARDIOVASCULAR RESPONSE TO TRAUMA-HEMORRHAGE

During hemorrhage there is a biphasic cardiovascular response pattern (112). In the early stages of hemorrhage (loss of up to 15% of blood volume) there is an increase in heart rate and vascular resistance which maintains the mean arterial blood pressure near pre-hemorrhage values (i.e., compensation). However, as the degree of blood loss increases, both the heart rate and total peripheral resistance decrease causing a depression in mean arterial blood pressure (i.e., decompensation). Throughout the entire course of hemorrhage, during both phases, the cardiac output decreases. The explanation of the cardiovascular response to hemorrhage involves the interaction of three cardiovascular reflexes: the arterial baroreceptor reflex, the activation of cardiac C-fiber afferents, and the arterial chemoreflex.

The arterial baroreceptor reflex (ABR) responds to both the degree and rate of stretch in the arterial walls of the aortic arch and the carotid sinuses (28). Thus, the ABR can respond to changes in both the pulse pressure and the mean arterial pressure. During hemorrhage there is a decrease in both the pulse pressure and mean arterial pressure causing a depression in the baroreceptor afferent activity. This

decrease in activity in the afferent limb of the ABR following hemorrhage causes an increase of activity in the sympathetic efferent limb of the ABR and a decrease of activity in the vagal efferent limb of the ABR (67). A combination of decreased vagal and increased sympathetic drive to the heart increases both the rate and force of cardiac myocyte contractility. The increase in sympathetic activity constricts the arterioles throughout the body by means of norepinephrine interacting with α-adrenergic receptors, causing an increase in the total peripheral resistance. Also, in response to the decreased afferent activity from the arterial baroreceptors, there is increased sympathetic stimulation of the adrenal gland to produce and secrete epinephrine into the circulation. The ABR is able to minimize the depression in mean arterial blood pressure during blood losses of up to about 15% of total blood volume (78). However, as blood loss exceeds this amount, MAP begins to fall. This is not due to the failure of the ABR, but due to the activation of a second cardiovascular reflex; the activation of cardiac vagal C-fiber afferents.

The cardiac vagal C-fibers are afferent fibers stemming from a group of receptors in the left ventricular myocardium (10). These receptors can be activated, mechanically, by deformations in the cardiac wall due to vigorous contractions around incompletely filled chambers. Activation of these receptors causes bradycardia due to

increased vagal efferent activity to the heart and reductions in skeletal muscle and renal vascular resistance due to a withdrawal of sympathetic vasoconstrictor tone (31). This "depressor" reflex may protect the heart by reducing cardiac work at a time when coronary blood flow is compromised.

The third reflex of importance in the cardiovascular response to hemorrhage is the arterial chemoreceptor reflex (ACR). The arterial chemoreceptors are found in the carotid and aortic bodies and they respond to falling oxygen tension by increasing afferent activity. Also, increases in carbon dioxide tension, and falls in blood pH increase the sensitivity of these chemoreceptors to hypoxia. In addition to causing increased respiratory activity, stimulation of the chemoreceptors causes vagally mediated bradycardia and increased sympathetic vasoconstrictor tone (30). This response is modified by the increased respiratory activity, which inhibits both the vagal activity to the heart and the sympathetic vasoconstrictor activity. Therefore, the increased respiratory activity due to chemoreceptor afferent activity may attenuate the bradycardia seen following severe hemorrhage and prevent the MAP from further decreasing.

In contrast to hemorrhage, tissue trauma alone causes an increase in MAP accompanied by tachycardia (55). This pressor response is largely due to increased sympathetic vasoconstrictor activity; as this response is unaffected by

complete cardiac autonomic blockade, but is abolished by phentolamine (α-adrenergic antagonist) (106). There is a reduction in sensitivity and an increased setpoint of the baroreceptor reflex following tissue trauma. The afferent pathway of the cardiovascular response to trauma seems to be in somatic fibers running from the damaged tissues (106). However, the precise mechanism of this reflex remains unknown.

The bradycardia that occurs following hemorrhage is attenuated by the presence of tissue trauma (77). This attenuation seems to protect against the hypotensive effects of severe hemorrhage. However, this protection may be more apparent than real, as trauma in conjunction with hemorrhage causes greater decreases in cardiac index and systemic oxygen delivery than hemorrhage alone (105). Also, animals that are subjected to hemorrhage in conjunction with electrical stimulation of the sciatic nerve (to simulate tissue trauma) have a lower survival rate compared to animals that are subjected to hemorrhage alone (96). It is possible that the improved maintenance of blood pressure during hemorrhage, in the presence of tissue trauma, is attained by intense peripheral vasoconstriction. This response may lead to ischemia of the visceral organs, thus, compounding the severity of visceral organ damage. The cardiovascular response to the combination of both trauma and hemorrhage has obvious clinical significance, as

hemorrhage is frequently associated with tissue trauma.

IL-6 and TNF

Interleukin-6 (IL-6) is an immunologically active protein which is secreted by many different cell types, particularly Kupffer cells following hemorrhage and resuscitation (94). The primary role of IL-6 in the cytokine network is the stimulation of B-cell proliferation and antibody secretion (150). IL-6 enhances the production and release of hepatic acute phase proteins and prostaglandins (126). There appears to be a correlation between IL-6 and tumor necrosis factor (TNF). Peak IL-6 levels usually follow those of TNF (128) and IL-6 release has been shown *in vivo* to be stimulated by TNF (113).

TNF is a cytokine which causes a catabolic state that leads to extensive weight loss (cachexia; hence the common name of TNF as cachectin) (69), in addition to exerting autocrine, paracrine, and endocrine control of the inflammatory response by inducing numerous cell types to secrete various cytokines (114). TNF enhances the phagocytic activity of macrophages and neutrophils, and acts as a cytotoxic peptide (71). Plasma levels of TNF are elevated during hemorrhage and up to 4 hours following resuscitation (9). Also, both TNF and IL-6 have been shown to be elevated at 24 hours following hemorrhagic shock (64).

TNF has been proposed to be one of the key pathological

mediators producing the shock-like state associated with the pathophysiology following hemorrhage (20). Infusion of TNF (1.0 mg/kg) has been shown to produce decreased systemic vascular resistance and hepatocellular dysfunction (111). Administration of 1.8 mg/kg TNF produced significant hypotension, metabolic acidosis, and tissue necrosis (124). It has also been shown that infusion of a low dose of recombinant murine TNF-α (one which does not cause a depression in cardiac output, blood pressure, or tissue microvascular perfusion) produces hepatocellular dysfunction (145).

AIMS

In spite of aggressive resuscitation carried out at the scene of the accident and in the emergency room, a large number of trauma victims subsequently die of sepsis and multiple organ failure (MOF). Half of the individuals who survive the initial traumatic insult die within a few days or weeks due to sepsis and MOF (20). In the United States, trauma is the leading cause of death in the first three decades of life, and it causes the loss of more productive years of life than AIDS, heart disease, and cancer combined (125).

Because of the liver's central role in metabolism and the reticuloendothelial system, it may be a key organ in the manifestation of MOF following trauma-hemorrhage (20). Studies have shown that following trauma-hemorrhage and crystalloid resuscitation, there is a decrease in hepatic microvascular blood flow and a decrease in active hepatocellular function (130,139,141,142). It is essential that we understand the pathophysiological alterations which occur in the liver following trauma-hemorrhage, so that specific therapeutic approaches can be developed in order to improve the survivability of trauma victims.

One of the earliest responses to trauma-hemorrhage is sympathetic adrenal activation, causing an increase in circulating catecholamine levels (83,101,151). However, there are discrepancies in the catecholamine levels

observed, depending upon the model used. Teleologically the purpose of this "stress response" is to direct blood from the visceral organs to the skeletal muscles, dilate the pupils, and increase the plasma glucose levels; all of which ready the animal for accurate physical movement within its environment, hence the name "fight or flight response". However, during trauma-hemorrhage the internal environment in which the sympathoadrenal activation is occurring is different from the normal state. Namely, there is a diminished volume of blood that can be used to perfuse the various organs. Since the sympathoadrenal activation that occurs during hemorrhage is acting on a smaller blood volume, it may actually be detrimental to the survivability of the animal by magnifying the amount of hypoxia in the visceral organs. **Therefore it is an objective of this study to determine the alterations in plasma levels of epinephrine and norepinephrine during trauma-hemorrhage and following crystalloid resuscitation.**

β-adrenergic receptors, which are stimulated by catecholamines, are well documented as performing a key role in the control of hepatic glucose, ketoacid, free fatty acid, and amino acid metabolism (25,37,58,60). β-adrenergic receptor binding characteristics have been shown to be altered in various tissues in a number of adverse circulatory conditions (40,62,79,92,103,127). However, it remains unknown whether there are alterations in the hepatic

β-adrenergic receptors following trauma-hemorrhage and crystalloid resuscitation. Kupffer cells are tissue macrophages that reside in close association with the hepatocytes. The Kupffer cells are strategically located to first encounter the pathogens that translocate from the intestine and travel through the hepatic portal vein. It remains unknown whether there are any alterations in Kupffer cell β-adrenergic receptor binding characteristics following trauma-hemorrhage. **Therefore, it is an objective of this study to determine whether the binding characteristics of hepatocyte and Kupffer cell β-adrenergic receptors (i.e.; maximum binding capacity and affinity) are altered during trauma-hemorrhage and following crystalloid resuscitation.**

The results obtained from the first aim indicate that the plasma levels of norepinephrine remain elevated following resuscitated hemorrhagic shock, while epinephrine levels are normalized after resuscitation. Because norepinephrine is secreted mainly from nerve terminals, plasma levels actually illustrate a diluted amount of the noradrenergic activity. Norepinephrine has profound microvascular effects, via α-adrenergic receptors, causing constriction of arterioles perfusing the visceral organs, thereby decreasing the blood flow to these organs. Norepinephrine affects the rate and force of contractility in cardiac myocytes. The noradrenergic stimulation, seen following hemorrhage and resuscitation, may have deleterious

effects on the visceral organs. In particular, it may play a role in the development of hepatic dysfunction following trauma and hemorrhage. **Therefore, it is an objective of this study to determine if chronic elevation of plasma norepinephrine, similar to that which is observed following hemorrhage and resuscitation, causes alterations in various cardiac parameters, such as cardiac output, heart rate, and mean arterial pressure. I will determine the effects of chronic administration of norepinephrine on various hepatic parameters, including: hepatocellular damage (as measured by plasma GOT and GPT levels), plasma glucose levels, microvascular blood flow, active hepatocellular function, and β-adrenergic receptor binding characteristics.**

The increase in circulating levels of TNF observed during hemorrhage is in the absence of detectable endotoxin in the general circulation (7). Norepinephrine may be a factor which stimulates the release of TNF during hemorrhage. Studies have shown that rats chronically infused with norepinephrine showed cachexia and increased temperature, while those infused with epinephrine did not exhibit these alterations (104). Both the cachexia and the fever may be attributable to the effects of TNF; as TNF causes cachexia and stimulates macrophages to release Interleukin-1, which is a cytokine that stimulates the hypothalamus to induce fever. The proliferative response of whole blood lymphocytes, in shocked animals, shows a

diminished response that can be obliterated by using adrenergic blockers (29). **Therefore, it is an objective of this proposal to determine whether chronic infusion of norepinephrine causes an increase in the plasma levels of TNF and/or IL-6.**

The liver plays a key role in the development of MOF and is sensitive to adrenergic stimulation. I am therefore determining the effect of chronically elevated levels of norepinephrine on hepatic function. In doing so, I hope to better understand the physiological derangements that occur in the liver following resuscitated hemorrhagic shock. This knowledge can then lead to the further development of treatments which will help prevent MOF following trauma and hemorrhage, thus, reducing the leading cause of death in the first three decades of life.

MATERIALS AND METHODS

HEMORRHAGE MODEL

A nonheparinized, fixed pressure, model of trauma-hemorrhage and crystalloid resuscitation in the rat was used, as previously employed in our lab (132,134,141). Male Sprague-Dawley rats (275-325g) were fasted overnight (~16 hr) prior to the experiment, but allowed water ad libitum. The animals were anesthetized with ether, following which, a 5 cm midline laparotomy was performed to induce tissue trauma prior to hemorrhage. The abdominal incision was closed in two layers and care was taken to suture the wounds securely to make it as painless as possible. Both the left femoral artery and the right femoral vein were cannulated with PE-50 tubing. The incisions needed for these cannulations were bathed with 1% lidocaine to provide analgesia during the study period. The arterial catheter was used for bleeding and the venous catheter for crystalloid resuscitation. The right carotid artery was cannulated with PE-50 tubing and was used for measurement of mean arterial pressure (MAP) and later for blood sampling. This tubing was tunneled subcutaneously to the dorsal side of the neck, where a swivel was attached to allow the animals free movement without the chance of tangling the tubing. The animals exhibited no movements in response to the surgery, indicating that they were not in pain or distress. Following recovery from anesthesia, the animals

were bled to a MAP of 40 mmHg within 10 minutes. The rats were maintained at this pressure until 40% of the maximal bleedout (MB) volume was returned in the form of Ringers lactate (RL). This period of hypotension lasted approximately 90 minutes. Immediately following hemorrhage the rats were resuscitated with four times (4X) the volume of MB with RL over a period of 60 minutes at a constant infusion rate. Sham-operated rats underwent the same surgical procedure, but were neither bled nor resuscitated. Samples were taken from sham animals at 3 hours following surgery, which corresponds to 1.5 hours following hemorrhage and resuscitation. Blood samples acquired at 4, 8, and 24 hours following resuscitation were taken from the animals carotid artery, as the femoral catheters were tied off. The carotid line was kept clear of clots by infusing RL through the line at a rate of 0.5ml/hour. The maintenance fluid intake for a normal rat is ~10ml/100g body weight/day, i.e., a 300g rat imbibes 1.25ml/hour. Therefore, one can give 0.5ml/hour of fluid, in addition to giving water *ad libitum*, without exceeding the normal maintenance fluid intake. When a 0.5ml blood sample was taken, 1ml of RL was returned in order to replace the fluid loss. None of the rats died during the course of the experiment until they were euthanized by an overdose of ether. Rats were excluded from the experiments (before data was collected) only if technical mishaps occurred during the procedures. The

experiments were performed in adherence to the National Institute of Health Guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of Michigan State University.

CATECHOLAMINE ASSAY

Arterial blood samples (0.5ml) were collected in tubes containing EGTA and reduced glutathione to prevent clotting and catecholamine degradation. The plasma was separated and frozen for subsequent measurement of catecholamines. Catecholamines were separated and measured using a commercially available kit (CAT-A-KIT, Amersham) which utilized thin layer chromatography to separate the various catecholamines and a tritiated radioenzymatic assay to measure the quantity of each.

Catechol-O-methyltransferase was used to catalyze the transferral of a [^{3}H]-methyl group from [^{3}H]-S-adenosyl-L-methionine to a hydroxyl group on the various catecholamines. The products of these methylations of epinephrine, norepinephrine, and dopamine were [^{3}H]-metanephrine, [^{3}H]-normetanephrine, and [^{3}H]-methoxytyramine, respectively. These tritiated products were extracted from the inorganic phase by quickfreezing in a dry-ice bath and then separated using thin layer chromatography. A liquid scintillation counter was used to measure the amount of radioactivity in the samples of isolated catecholamines.

[^{3}H]-methoxytyramine was measured immediately following separation, while [^{3}H]-metanephrine and [^{3}H]-normetanephrine were converted to [^{3}H]-vanillin prior to measurement of radioactivity. The radioactivity of the samples was then compared to that of a catecholamine standard provided in the kit and used to determine the amount of catecholamines present.

HEPATIC PLASMA MEMBRANE ISOLATION

A modification of the method of Blitzer and Donovan (13) was used to isolate hepatic plasma membrane. The whole liver was removed, cleaned of excess fatty and vascular tissue, and washed in ice-cold normal saline. The liver was diced in 30ml of ice-cold Buffer A (0.25M Sucrose, 10mM Tris-HCl, 0.5mM EGTA, pH 7.4). Homogenization was performed under ice-cold conditions using 15 up-down strokes of a loose fitting teflon homogenizer at 3500 rpm. The homogenate was centrifuged at 7,710 x g for 10 minutes, after which the supernatant was harvested and mixed with Percoll® to form a 45% Percoll solution. This solution was then placed at the bottom of a continuous Percoll gradient (30%,25%,18%,10%, and 0%) in a 38ml polycarbonate tube. The gradient preparation was spun at 62,000 x g for 60 minutes at 4°C, using a SW27 rotor at 21,000 rpm in a Beckman L5-65 ultracentrifuge. At the end of centrifugation, a 5ml aliquot was taken from the interface between the 0% and 10%

Percoll gradients and placed in another 48ml polycarbonate tube, to which approximately 25ml of Buffer B (10mM $NaHCO_3$, 5mM Histidine, pH 7.4) was added. This solution was mixed thoroughly and then spun at 62,000 xg for 90 minutes at 4°C. Following centrifugation, the supernatant was discarded and the remaining pellet was mixed with 500μl of Buffer B. An aliquot of this sample was used to determine protein concentration according to the method of Lowry *et al.* (82) using bovine serum albumin as a standard. The plasma membrane suspension was stored at -70°C until it was utilized for the marker enzyme assay or the receptor binding assay. In order to maintain a low non-specific binding, this storage time was never greater than 3 weeks. This method effectively purified plasma membrane from the whole liver homogenate without purifying endoplasmic reticulum. This was determined by measuring the activities of specific enzymes which are indicative of the plasma membrane (5'-nucleotidase) or the endoplasmic reticulum (glucose-6-phosphatase). The methods used to determine the activities of these enzymes were similar to those used by Aronson and Touster (4).

β-ADRENERGIC RECEPTOR BINDING ASSAY

The binding characteristics of the β-AR's on the liver plasma membranes were determined using a membrane filtration assay, similar to that used by Liu and Ghosh (79). The plasma membrane suspension was diluted to a final protein concentration of 5mg/ml (final amount: 200μl). A solution of 50μl Buffer R (200mM Tris-HCl, 50mM $MgCl_2$, pH 7.4), 20μl of varying concentrations (final concentrations:0.0625nM to 2.5nM) of the radioactively labeled β-AR specific antagonist [^{125}I]-(-)Iodopindolol (New England Nuclear; specific activity, 2200 Ci/mmol), and distilled water to a total volume of 160μl was prepared. Alprenolol (10μM) was added to those tubes that were used to determine non-specific binding. To this mixture, 40μl of the 5mg/ml plasma membrane suspension was added and immediately incubated at 37°C for 20 minutes in a shaking water bath. The effects of different incubation times on specific binding activity of ^{125}I-Iodopindolol to whole liver plasma membrane preparations, were obtained from sham-operated animals, using a concentration of 1.5 nM. Incubation was arrested by the addition of 4ml ice-cold Buffer R to the sample, which was then filtered through a glass microfiber filter (GF/B, Whatman). The incubation tube was washed twice with 4ml ice-cold Buffer R and filtered through the same GF/B filter. Radioactivity of the filter was determined using a LKB 1282 Compugamma-Universal gamma counter. The maximum binding

capacity (B_{max}, i.e., the maximum receptor number) and dissociation constant (K_d, 1/affinity) were determined by Scatchard analysis (147). Figure 1. illustrates a representative plot of the data obtained.

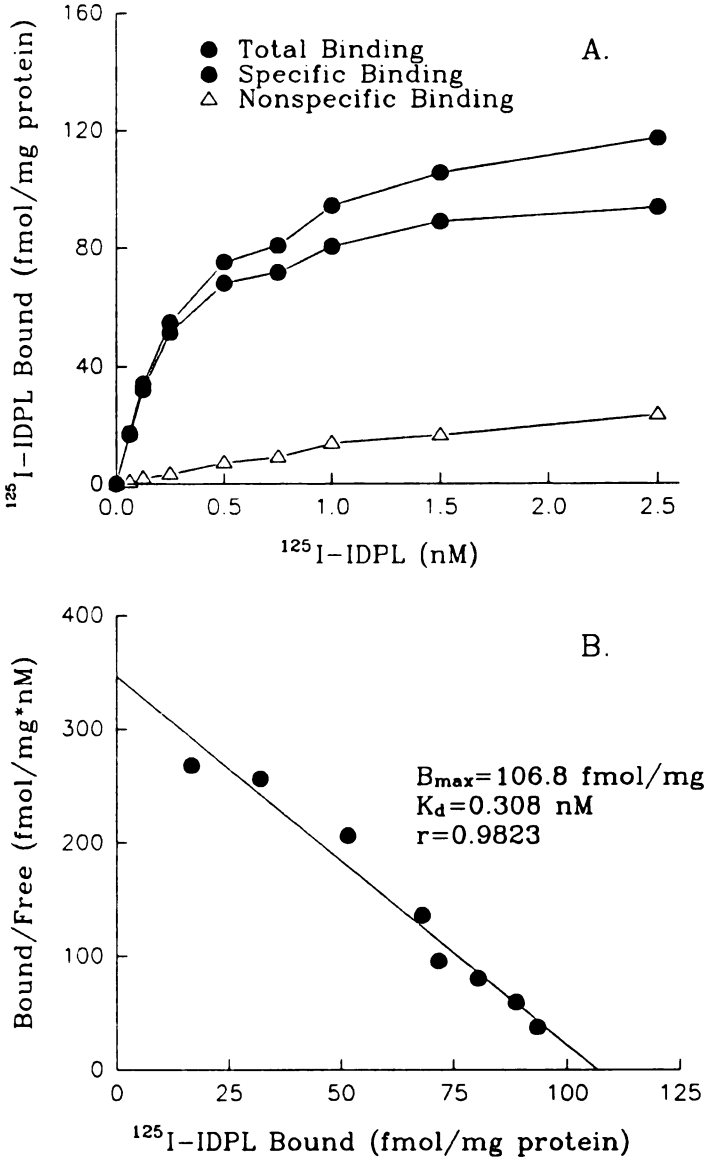

Figure 1. **A)** Saturation curve of specific ^{125}I-IDPL binding capacity to whole liver plasma membrane preparations obtained from a sham-operated animal, and **B)** Scatchard plot of the above specific binding data. The specific binding capacity is equal to the difference between the total binding capacity and the nonspecific binding capacity. Scatchard analysis was employed to determine the dissociation constant (K_d) and the maximum binding capacity (B_{max}) of the β-adrenergic receptors. In this representative experiment, the B_{max} was 106.8 fmol/mg protein, as indicated by the x-intercept. The K_d was 0.308nM, as determined by -1/slope.

KUPFFER CELL AND HEPATOCYTE ISOLATION

Kupffer cells were isolated using a method similar to that of Keogh *et al.* (63). The portal vein was cannulated and the inferior vena cava was severed. The liver was perfused *in situ* with 200 ml heparinized Hanks balanced salt solution (HBSS) (Ca^{++}/Mg^{++} free, 37°C) at a rate of 28 ml/min followed by 200 ml HBSS containing 0.05% collagenase (Worthington Type IV, 162 U/mg, low tryptic activity in order to limit damage to membrane receptors) (Ca^{++}/Mg^{++} free, 37°C). The liver was removed, rinsed with 25 ml HBSS (Ca^{++}/Mg^{++} free, 37°C), minced in 25 ml HBSS containing 0.05% collagenase, and incubated at 37°C for 20 min. The cell suspension was then passed through a sterile 150 mesh stainless steel screen into cold HBSS containing 10% heat inactivated fetal bovine serum. This cell suspension was centrifuged (50xg, 2 min, 4°C) three times; each time removing the pellet containing hepatocytes. These pellets containing hepatocytes were washed twice with ice cold phosphate-buffered saline (PBS) and diluted in PBS to 1×10^7 cells/ml. The remaining cells in the supernatant were collected by centrifugation at 300xg for 15 min at 4°C. The pellet was resuspended in Dulbeccos modified eagle medium (DMEM) and mixed with an equal volume of metrizamide. 3 ml of DMEM was placed over the cell-metrizamide mixture and the tubes were centrifuged at 1400xg for 30 min at 4°C. The Kupffer cells were taken from the interface of the

metrizamide and the media, washed twice with ice cold phosphate-buffered saline (PBS), diluted in PBS to 1 x 10^7 cells/ml, and then used for the β-adrenergic receptor assay. The Kupffer cell isolation procedure required less than 4 hours and produced approximately 5 x 10^7 non-parenchymal liver cells per animal, more than 90% of which ingested India ink. More than 80% of the non-parenchymal liver cells were positive with peroxidase staining. Cell viability was more than 95% as determined by trypan blue exclusion.

KUPFFER CELL AND HEPATOCYTE β-ADRENERGIC RECEPTOR ASSAY

Receptor binding characteristics of [^{125}I]-Iodopindolol binding to Kupffer cells and hepatocytes were determined according to the same method used for the characterization of the binding to hepatic plasma membrane preparations, except cell number (5 x 10^5 cells/tube) was used to standardize the samples, instead of protein concentration.

NOREPINEPHRINE INFUSION

Norepinephrine (Sigma) was infused using Alzet® osmotic pumps (model 2ML1, Alza, Palo Alto, CA) implanted in the peritoneal cavity. The mathematical relationship between the desired mass delivery rate and the proper concentration of drug to be injected into the pump, is as follows:

$$K = Q * C$$

K=mass delivery rate
Q=pumping rate of given model
model 2ML1: 10 μl/hour
C=concentration of drug in pump

The pumps were filled with a 0.6% solution of norepinephrine in normal saline, which results in an infusion rate of 1μg/min. The pumps were incubated in normal saline at 37°C for 24 hours prior to implantation. The rats are anesthetized with ether and given a 2cm midline laparotomy. The pumps are washed with normal saline and implanted in the peritoneal cavity, after which, the abdominal wound is closed in two layers. Following recovery from anesthesia, the rats are returned to their cages and given food and water *ad libitum* for 8 hours. 16 hours prior to sample collection the animals are fasted, but water is continued *ad libitum*. 24 hours following pump implantation, the rats are anesthetized with ether and their right jugular vein and femoral artery are cannulated with PE-50 tubing. Also, a fiberoptic catheter was inserted in the right carotid artery to the level of the aorta, for measurement of cardiac output

and active hepatocellular function. Following surgery the animals are allowed to equilibrate under ether anesthesia for 30 minutes. 1ml of blood is taken via the femoral artery and the plasma is separated by centrifugation, divided into aliquots, and stored at -70°C for later use. After 1ml of blood is taken, 2ml of RL are returned in order to compensate for the fluid loss.

CARDIAC PARAMETERS

Systemic hematocrit (H_{sys}) was measured using microcapilary tube centrifugation with blood sample drawn from the right femoral artery. Mean arterial pressure (MAP) and heart rate (HR) is measured, via the left femoral artery, by a strain gauge pressure transducer (model P23XL; Viggo-Spectramed, Oxnard, CA) coupled to a polygraph (model 7D, Grass Instruments, Quincy, MA).

Cardiac output (CO) was determined using an indocyanine green (ICG) dilution technique. A 2.4-French fiberoptic catheter (Hospex Fiberoptics, Chesnut Hill, MA), attached to an *in vivo* hemoreflectometer (IVH, Hospex Fiberoptics), is inserted into the right carotid artery to the level of the aortic arch. A volume of 0.05ml of ICG solution (1mg/ml) is injected via the right jugular vein to the level of the right atrium. Using computer-assisted data acquisition (Asystant+, Asyst Software Inc., Rochester, NY), 20 data points/seconds of ICG concentration is recorded for

approximately 30 seconds immediately following the injection of ICG. A representative curve of the dilution of ICG from a control rat is illustrated in Figure 2. The area under the under the ICG dilution curve is calculated by Asystant+®, then the cardiac output is determined according to the following equation:

CO = (ICG dose)/(area under the ICG dilution curve)

where the CO is in ml/min, ICG dose is in mg, and the area under the ICG dilution curve is in mg/ml·min.

The CO was transformed into Cardiac Index (CI), which is normalized to body weight (BW) by the following equation:

CI = (CO/BW)

where CO is in ml/min and BW is in 100g.

Stroke volume (SV) is calculated by the following equation:

SV = CI/HR

where SV is in ml/beat/100g (therefore an index of stroke volume), CI is in ml/min/100g, and HR is in beats/min.

Total peripheral resistance (TPR) was calculated by the following degenerate form of poiselle's equation:

$$TPR = MAP/CO$$

Where TPR is in mmHg/ml/min, MAP is in mmHg, and CO is in ml/min.

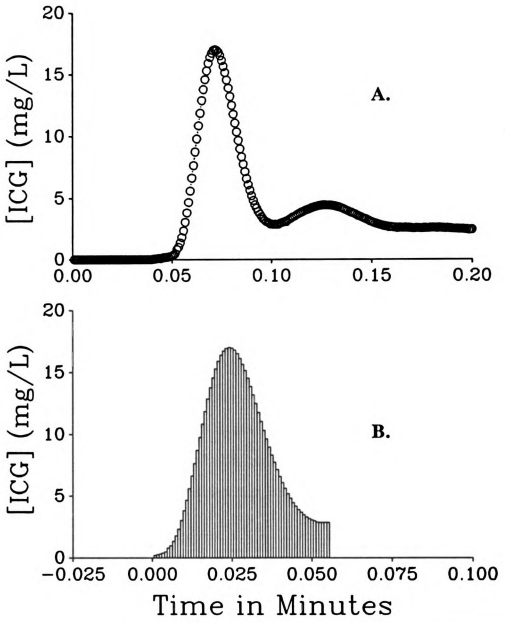

Figure 2. **A)** Representative dilution curve of ICG concentration in blood following injection into the jugular vein. ICG concentration was determined using an *in vivo* hemoreflectometer and 20 data points/second were recorded with the aid of computer assisted data acquisition. **B)** Graph of the area under the representative ICG dilution curve presented in Fig. A. Modified from "Ping Wang and Irshad H. Chaudry. Crystalloid resuscitation restores but does not maintain cardiac output following severe hemorrhage. *J. Surg. Res.* 50:163-169, 1991."

ACTIVE HEPATOCELLULAR FUNCTION

The hardware that was utilized for the measurement of cardiac output was also used for the measurement of active hepatocellular function. A 2.4-French fiberoptic catheter (Hospex Fiberoptics, Chesnut Hill, MA), attached to an *in vivo* hemoreflectometer (IVH, Hospex Fiberoptics), is inserted into the right carotid artery to the level of the aortic arch. Three doses (0.167, 0.333, and 0.833mg/kg BW) of ICG, in a volume of 0.05ml of ICG solution, are injected via the right jugular vein to the level of the right atrium. Using computer-assisted data acquisition (Asystant+, Asyst Software Inc., Rochester, NY), one data point/second of ICG concentration is recorded for approximately 5 minutes following the injection of ICG. An example of the data attained is illustrated in Figure 3. Nonlinear regression is used to determine the relationship between time and concentration of ICG, as shown in the following equation:

$$[ICG] = e^{(a+bt+ct\wedge 2)}$$

where [ICG] is in mg/l, t is in minutes, e^a is [ICG] at time 0, $e^a b$ is the initial velocity of ICG clearance (in $mg \cdot 1^{-1} \cdot min^{-1}$), and c is a coefficient constant.

The initial velocity of clearance (V_o) is determined by the following equation:

$$V_o = e^{-b}(\text{ICG injected in mg}/[ICG_o])/BW$$

where BW is in kg.

The initial clearance of ICG in the circulation follows Michalis-Menten kinetics and can be represented as:

$$1/V_o = 1/V_{max} + K_m/V_{max} \cdot 1/D$$

where V_{max} is the maximal velocity of ICG clearance at infinite [ICG], D is the initial ICG concentration, and K_m represents the efficiency of this carrier mediated active transport process.

The plot of $1/V_o$ versus 1/D, using this equation, gives a straight line, where V_{max} is the reciprocal of the y-intercept and K_m is the negative reciprocal of the x-intercept.

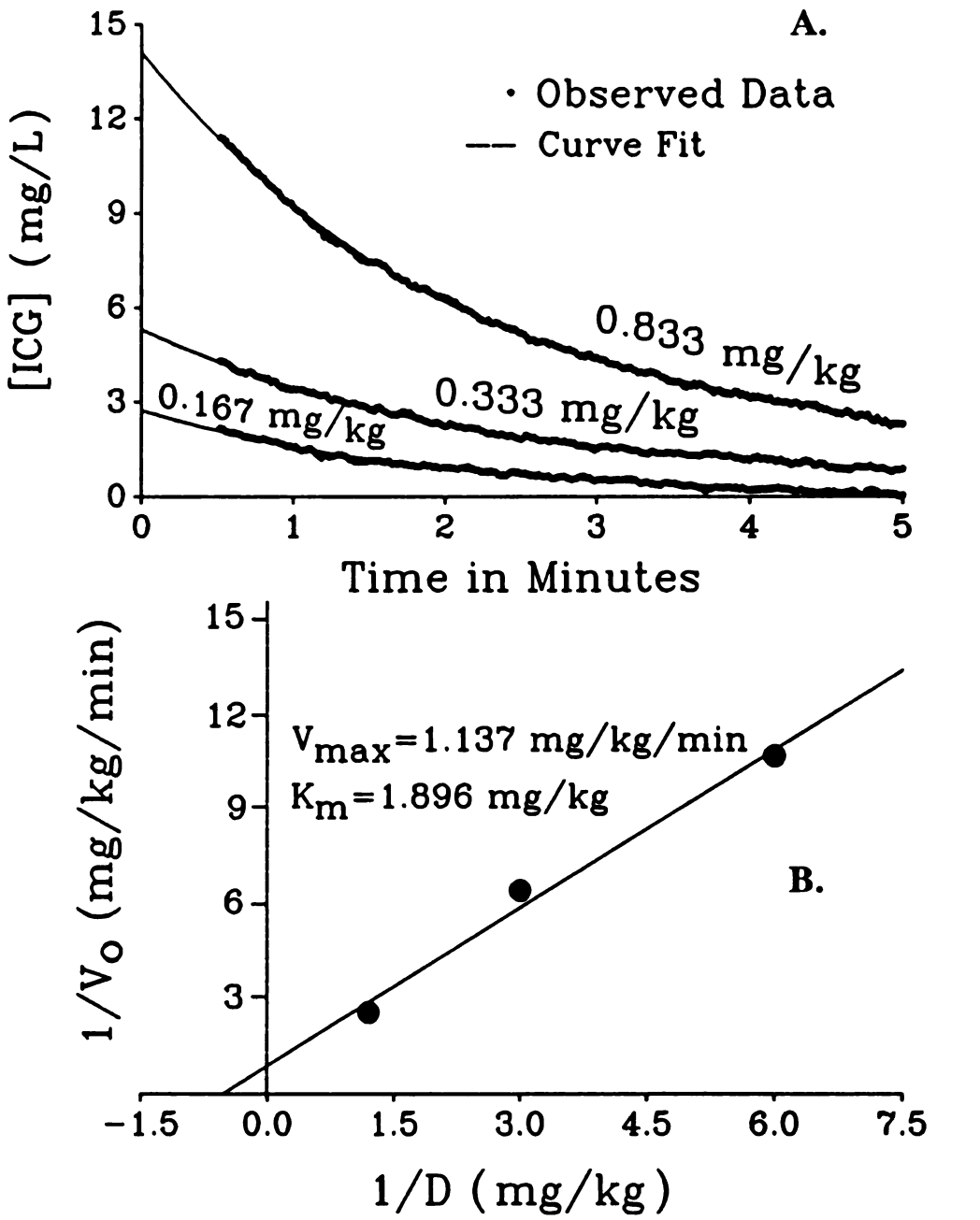

Figure 3. **A)** Representative plot of the blood [ICG] data collected using an *in vivo* hemoreflectometer and computer assisted data acquisition for the determination of active hepatocellular function. **B)** The initial velocity data obtained for each dose (Fig. A.) is plotted as the reciprocal of the initial velocity ($1/V_o$) versus the reciprocal of dose (1/D). Modified from "Wang *et al.* Tumor necrosis factor-α produces hepatocellular dysfunction despite normal cardiac output and hepatic microcirculation. *Am. J. Physiol.* 265 (*Gastrointest. Liver Physiol.* 28): G126-G132, 1993."

MICROVASCULAR BLOOD FLOW

Microvascular blood flow (MBF) is measured using a laser Doppler blood perfusion monitor (Laserflo, model BPM403A, TSI, St. Paul, MN) according to methods used previously in this lab (132,133,141). A flow probe (model P-430, right angle) is placed on the surface of the liver (left lobe), kidney (left renal cortex, lower pole), spleen (middle portion), and small intestine (mid-jejunum). The resultant value, which is averaged over 3 minutes, is obtained using a recorder. The instrument reading is proportional to blood flow in the underlying tissue (in arbitrary units).

MEASUREMENT OF PLASMA GLUCOSE, PLASMA GLUTAMIC PYRUVATE TRANSAMINASE, AND PLASMA GLUTAMIC OXALOACETIC TRANSAMINASE

Plasma levels of glucose are determined enzymatically utilizing commercially available kits (Sigma, Procedure #510). Plasma levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT) were determined as a measure of hepatocellular damage, as GOT and GPT are intracellular enzymes which are released into the circulation during hepatocellular damage. The plasma activities of GOT and GPT were assayed enzymatically with commercially available kits (Sigma, procedure #505).

CYTOKINE ASSAYS

Plasma samples are first filtered through a 0.22μm filter, following which the samples are diluted beginning with 1:10 (plasma:media) in RPMI-1640 (GIBCO-BRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Biologos, Naperville, IL) and by two-fold steps thereafter. TNF activity was determined by assessing plasma samples for WEHI 164 subclone 13 cytotoxicity (36), according to the methods performed previously in this lab (7,8). Briefly, WEHI cells are harvested and incubated with the plasma/media solution for 20 hours at 37°C. The cell solution is then incubated at 37°C for 4 hours with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue, yellow crystals, Sigma, #M-2128), which is a dye that is taken up by the living WEHI cells. The supernatant is aspirated from each well, then the cells are lysed and left overnight at room temperature. The absorbance of each well is read at 595 nm and the samples are compared to a standard curve, which is run along with the samples, using murine TNF-α (Genzyme, Boston, MA). IL-6 activity was determined utilizing the IL-6 dependent 7TD1 B-cell hybridoma, according to the method of Hültner *et al.* (56), as performed previously in our lab (9,137). Briefly, 7TD1 B-cells are harvested and incubated with the plasma/media solution for 72 hours at 37°C. The cell solution is then incubated at 37°C for 4 hours with MTT,

which is also taken up by the living 7TD1 B-cells. The supernatant is aspirated from each well, then the cells are lysed and left overnight at 37°C. The absorbance of each well is read at 595 nm and the samples are compared to a standard curve, which is run along with the samples, using recombinant human IL-6 (Amgen, Thousand Oaks, CA). The assays described above have been shown to react in a specific fashion to TNF and IL-6, respectively (35,56).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA), Tukey's test, Student's t-test, Wilcoxon Rank Sum test, and linear regression were used and the differences were considered significant at $P < 0.05$. Results are presented as means ± SEM.

RESULTS

MEMBRANE MARKER ENZYME ACTIVITIES

Normal animals were used to determine the activities of membrane bound enzymes from samples taken at the beginning and end of the plasma membrane isolation procedure. The data in Table 1 indicate that 5'-nucleotidase activity in purified membrane preparations increased approximately 8-fold as compared to liver homogenate. Since 5'-nucleotidase is incorporated into the plasma membrane, the purity of the membrane preparation is approximately 8-fold as compared with liver homogenate. Glucose-6-phosphatase activity in purified membrane preparation did not significantly change (Table 1), indicating that the endoplasmic reticulum was not purified along with the plasma membrane.

Table 1.

Differential activities of membrane marker enzymes indicative of the plasma membrane (5'-nucleotidase) or the endoplasmic reticulum (glucose-6-phosphatase).

	5'-Nucleotidase	Glucose-6-Phosphatase
Liver homogenate	4.47 ± 0.88	13.75 ± 2.56
Discontinuous Percoll gradient interface	36.62 ± 3.74*	14.45 ± 4.29

The units are μmoles P_i/hour/mg protein for both enzyme activities. Data are given as means ± SEM (n=4 animals). The data was analyzed using a paired Student's t-test, *$P < 0.05$ as compared with liver homogenate.

TIME REQUIRED FOR SATURATION OF LIGAND-RECEPTOR BINDING

Saturation of specific binding occurs between 15 and 20 minutes using 200 μg protein at 37°C. Therefore, 20 minutes was adequate for the binding to reach equilibrium. The results are shown in Figure 4, and the data are given as an average of two experiments. Figure 5 illustrates that an incubation period of 20 minutes for both hepatocytes and Kupffer cells was adequate, as the lowest concentration used (0.0625 nM) showed saturation between 10 and 20 minutes. Data are given as the average of two experiments, using normal rats.

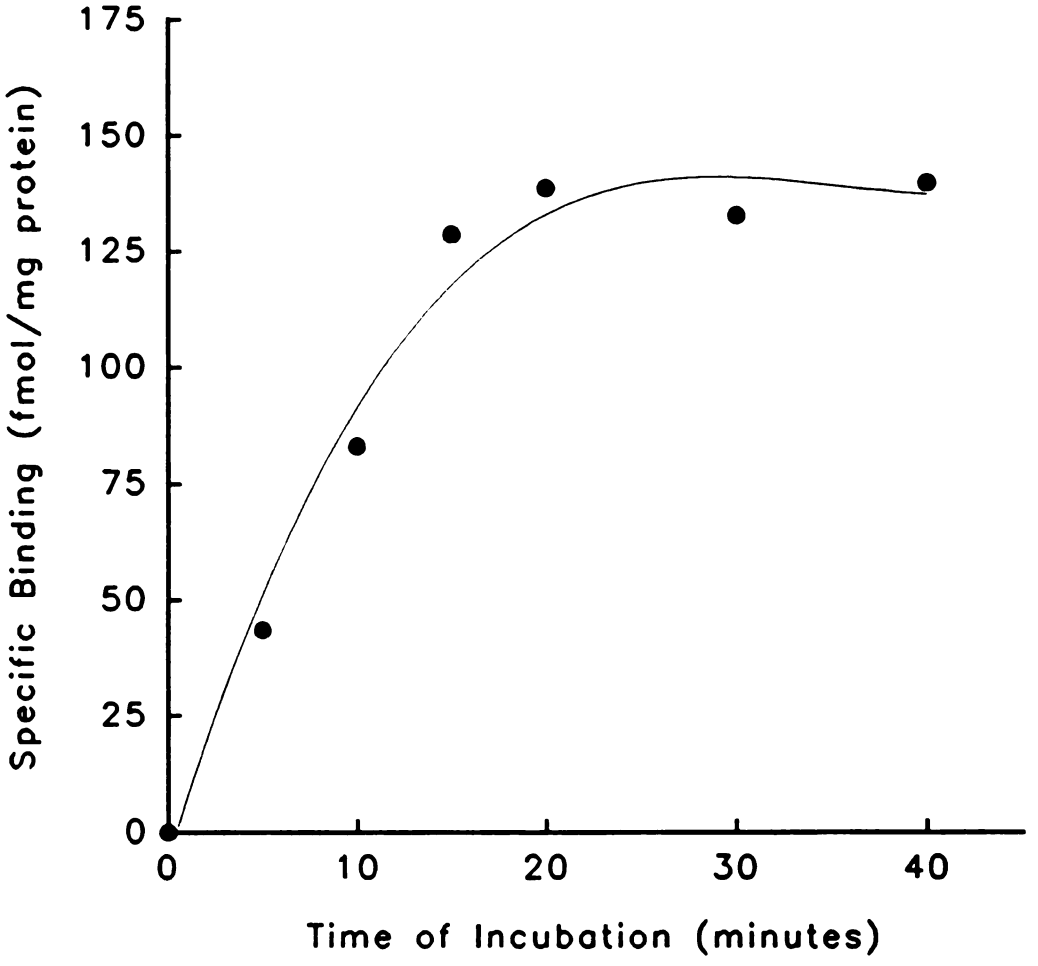

Figure 4. Effects of different incubation times on specific binding activity of ^{125}I-Iodopindolol to liver plasma membrane preparations, obtained from sham-operated animals, using a concentration of 1.5 nM. Saturation of specific binding occurs between 15 and 20 minutes using 200μg protein at 37°C. The data are given as average of two experiments.

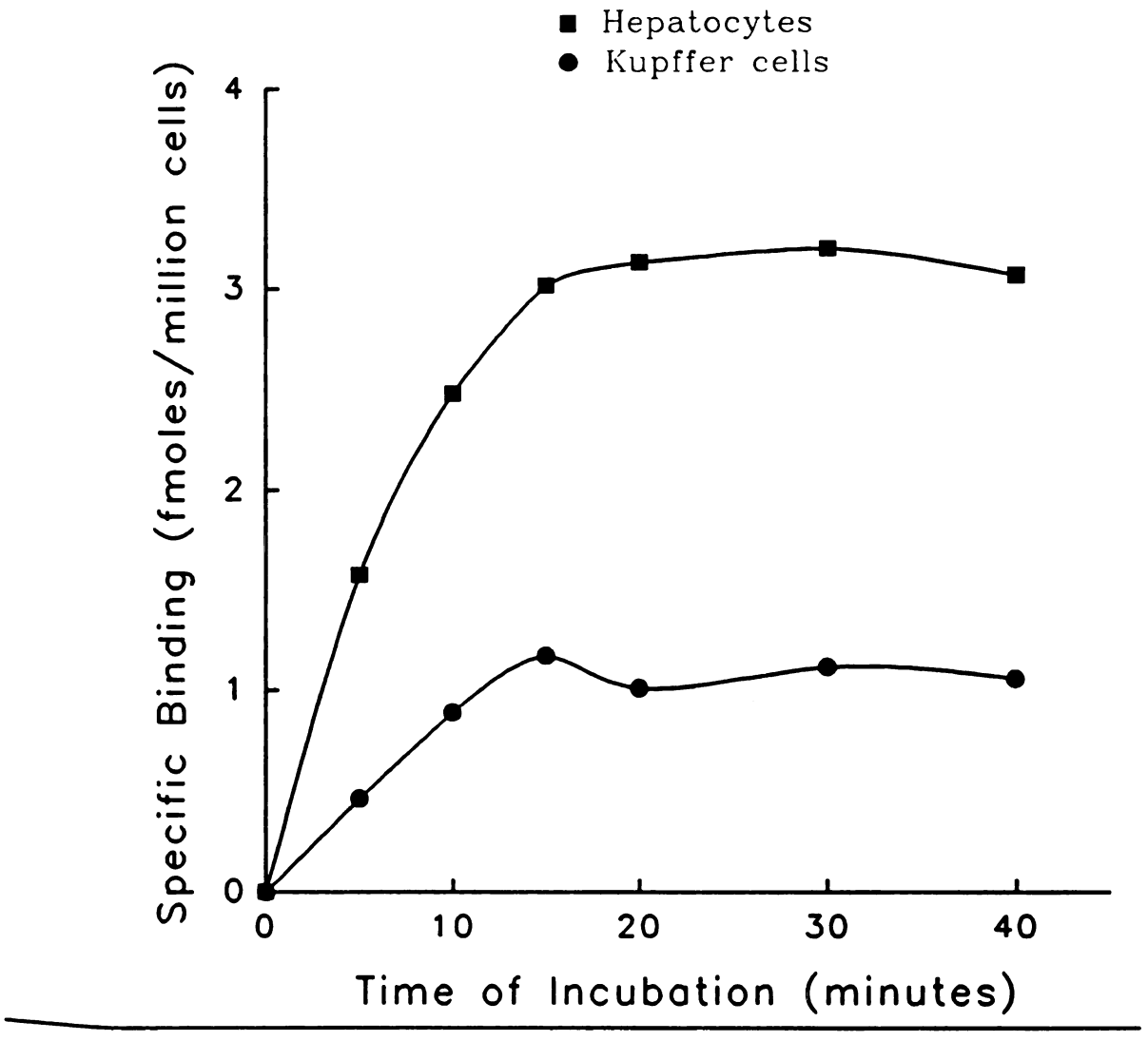

Figure 5. Effects of different incubation times on specific binding activity of ^{125}I-Iodopindolol to hepatocytes and Kupffer cells, obtained from sham-operated animals using a concentration of 0.0625 nM. Saturation of specific binding occurs between 10 and 20 minutes at 37°C. The data are given as the mean of 2 animals per group.

HEMORRHAGE RELATED PARAMETERS

The average maximum bleedout volume in hemorrhaged animals was 3.06 ± 0.17 ml/100g body weight (n=6 animals/group). The average time for maximum bleedout was 45 ± 2 minutes. The total hemorrhage time was 85 ± 2 minutes. During hemorrhage, the MAP was maintained at 40 mmHg (pre-hemorrhage value: 115 ± 2 mmHg). Following crystalloid resuscitation the MAP was increased approximately 2-fold from the hemorrhage value: 85 ± 4 mmHg immediately following resuscitation and 79 ± 8 mmHg at 1.5 hours following resuscitation. The post-resuscitation MAP was still significantly lower than the pre-hemorrhage value of 115 ± 2 mmHg ($P < 0.05$). The systemic hematocrit was decreased by approximately one half following hemorrhage and resuscitation to 19 ± 1 % immediately following resuscitation and 18 ± 1 % at 1.5 hours post-resuscitation from the pre-hemorrhage value of 45 ± 1 %.

PLASMA LEVELS OF NOREPINEPHRINE AND EPINEPHRINE DURING TRAUMA-HEMORRHAGE AND FOLLOWING RESUSCITATION

There was no significant difference in the circulating levels of norepinephrine or epinephrine between the sham animals sampled at 3 hours after surgery, shams sampled at 24 hours after surgery, and normal animals that underwent no surgical procedures. Therefore, the sham animals sampled at 3 hours after surgery were used as the control group.

Figure 6 illustrates the alterations in plasma levels of norepinephrine during hemorrhage (at the time of maximum bleedout) and following resuscitation. Data are presented as means ± SEM and compared using one-way ANOVA and Tukey's test (n = 6 animals/group). These results indicate that during hemorrhage, circulating levels of norepinephrine increase approximately 10-fold. These levels remain elevated up to 24 hours following crystalloid resuscitation. Figure 7 illustrates the alterations in plasma levels of epinephrine during hemorrhage (at the time of maximum bleedout) and following resuscitation. These results indicate that during hemorrhage, circulating levels of epinephrine increase approximately 100-fold as compared to shams. Following resuscitation plasma epinephrine levels decline, however, they are normalized between 4 and 8 hours following resuscitation.

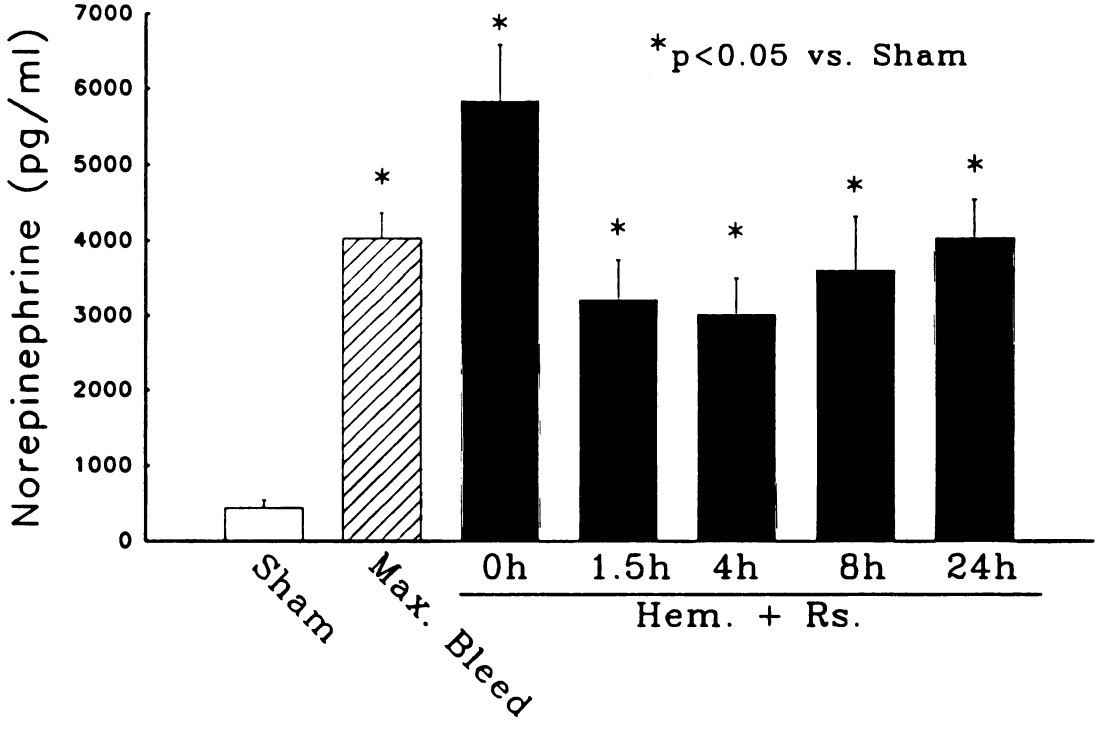

Figure 6. Alterations in plasma levels of norepinephrine during hemorrhage (Max. Bleed) and following crystalloid resuscitation. Sham animals underwent the same surgical procedure but were neither bled nor resuscitated. Samples from the sham animals were taken at 3 hours following surgery, which corresponds to 1.5 hours after hemorrhage and resuscitation. Data are presented as means ± SEM and compared using one-way ANOVA and Tukey's test (n=6 animals/group)

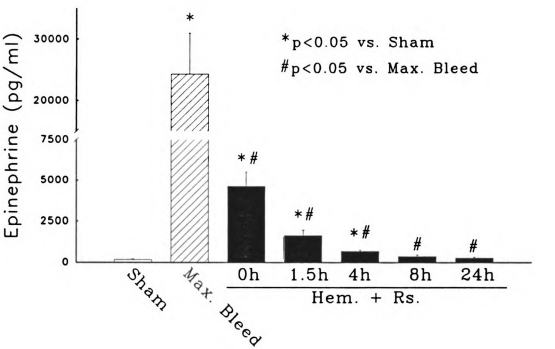

Figure 7. Alterations in plasma levels of epinephrine during hemorrhage (Max. Bleed) and following crystalloid resuscitation. Sham animals underwent the same surgical procedure but were neither bled nor resuscitated. Samples from the sham animals were taken at 3 hours following surgery, which corresponds to 1.5 hours after hemorrhage and resuscitation. Data are presented as means $\pm$ SEM and compared using one-way ANOVA and Tukey's test (n=6 animals/group)

ALTERATIONS IN HEPATIC β-ADRENERGIC RECEPTOR BINDING CHARACTERISTICS DURING HEMORRHAGE AND FOLLOWING RESUSCITATION

Figure 8 illustrates the alterations in β-adrenergic receptor B_{max} on whole liver plasma membrane preparations. The pre-hemorrhage B_{max} of the β-adrenergic receptors on the liver plasma membrane preparation was 119.17 ± 5.14 fmoles/mg protein. During hemorrhage (at maximum bleedout) there is a significant depression in hepatic β-adrenergic receptor B_{max}. The B_{max} at maximum bleedout was 31% of the pre-hemorrhage value. Although insignificant, there is a trend of increasing B_{max} from maximal bleedout to 0 hr and at 1.5 hr after the completion of hemorrhage and resuscitation. The B_{max} values at both 0 and 1.5 hr following hemorrhage and resuscitation were still significantly lower than the pre-hemorrhage values. Figure 9 illustrates the alterations in the dissociation constant (K_d, i.e., 1/affinity) of β-adrenergic receptors on the hepatic plasma membrane preparations. During hemorrhage and following resuscitation there were no significant changes in hepatic β-adrenergic receptor K_d at any time point during this study.

Figure 10 illustrates the differences in β-adrenergic receptor B_{max} on hepatocytes following hemorrhage and crystalloid resuscitation. At 1.5 hours after hemorrhage and resuscitation, the β-adrenergic receptor B_{max} was significantly depressed as compared to sham-operated

animals. This is similar to the alterations observed in the whole liver plasma membrane preparations. Figure 11 illustrates that there were no significant changes in β-adrenergic receptor K_d on hepatocytes at 1.5 hours following hemorrhage and resuscitation.

Figure 12 illustrates the alterations in the B_{max} of Kupffer cell β-adrenergic receptors. At 1.5 hours following hemorrhage and resuscitation there is an increase in Kupffer cell β-adrenergic receptor B_{max}. Figure 13 illustrates the alterations in the K_d of Kupffer cell β-adrenergic receptors. There was no significant difference noted in the K_d of Kupffer cell β-adrenergic receptors following hemorrhage and resuscitation.

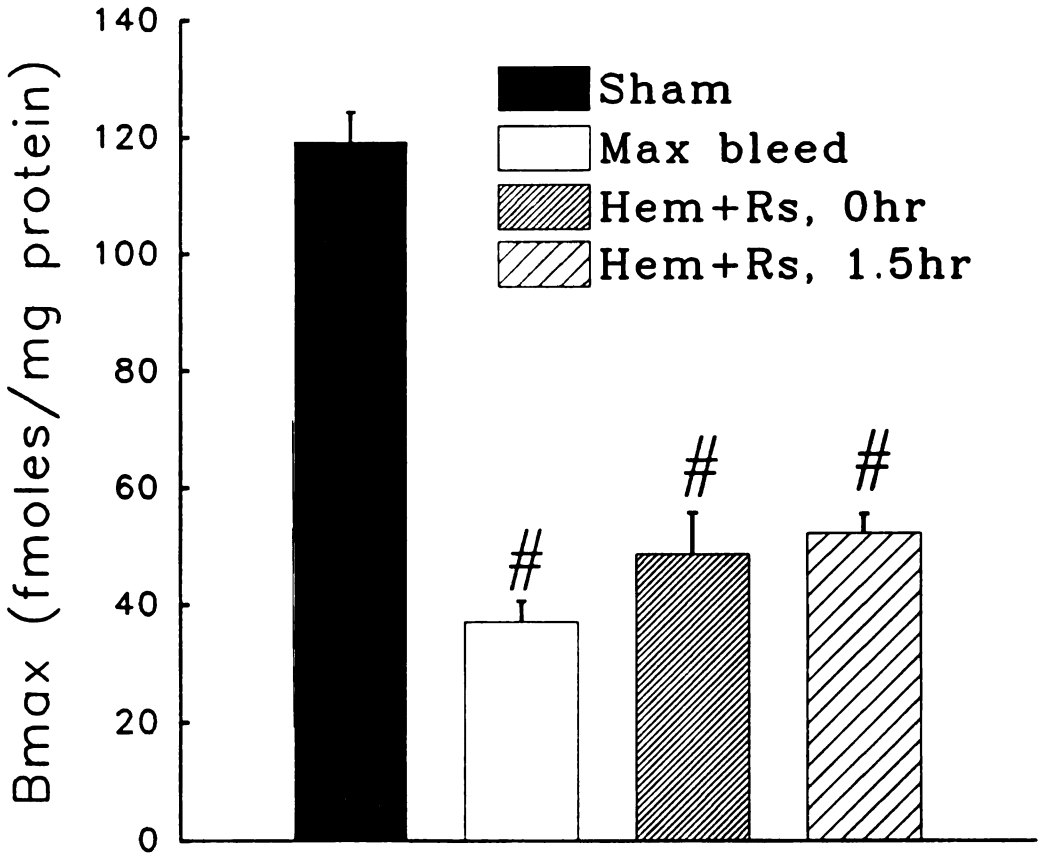

Figure 8. Alterations in the maximal binding capacity of β-adrenergic receptors in the hepatic plasma membrane preparations. The experimental groups are as follows: samples taken from sham-operated animals (Sham), samples taken at the time of maximal bleedout (Max bleed), samples taken immediately after hemorrhage and resuscitation (Hem + Rs, 0hr), and samples taken 1.5 hours following hemorrhage and resuscitation (Hem + Rs, 1.5hr). Data are given as means ± SEM and analyzed using one-way ANOVA and Tukey's test (n=6/group). $^{\#}P < 0.05$ as compared to Sham.

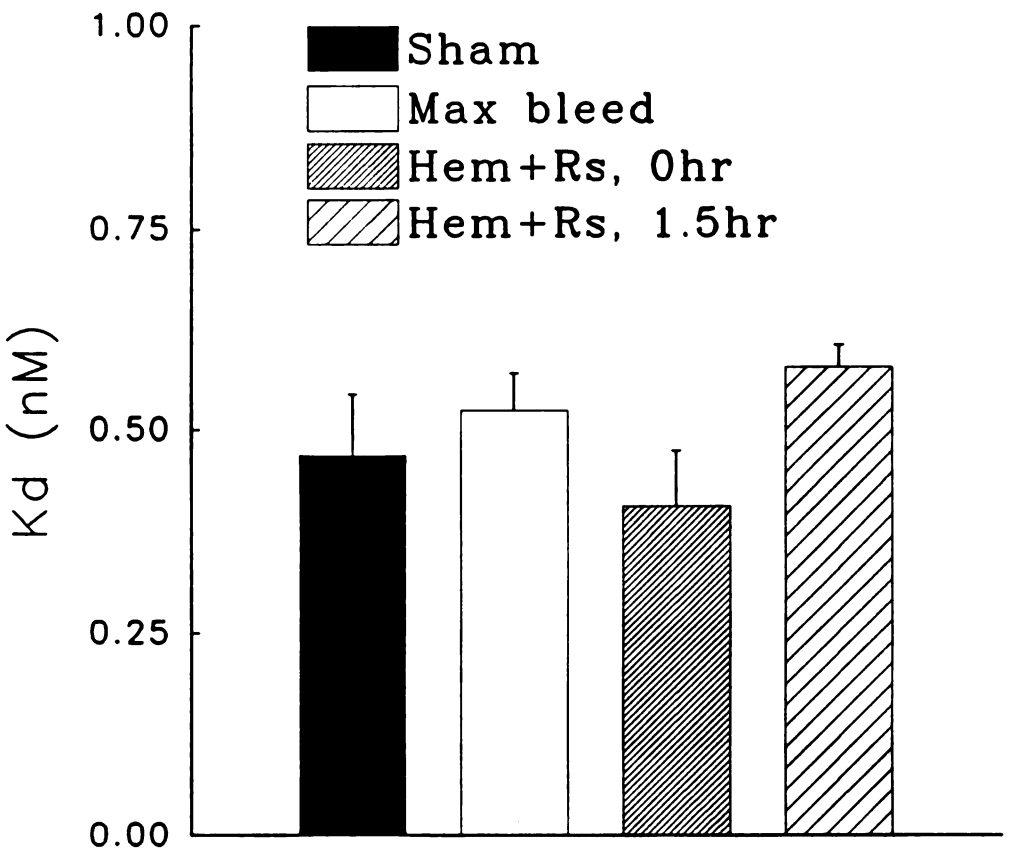

Figure 9. Alterations in the dissociation constant (K_d, i.e., 1/affinity) of β-adrenergic receptors in the hepatic plasma membrane preparations. The experimental groups are as follows: samples taken from sham-operated animals (Sham), samples taken at the time of maximal bleedout (Max bleed), samples taken immediately after hemorrhage and resuscitation (Hem + Rs, 0hr), and samples taken 1.5 hours following hemorrhage and resuscitation (Hem + Rs, 1.5hr). Data are given as means ± SEM and analyzed using one-way ANOVA and Tukey's test (n=6/group). $^{*}P < 0.05$ as compared to Sham.

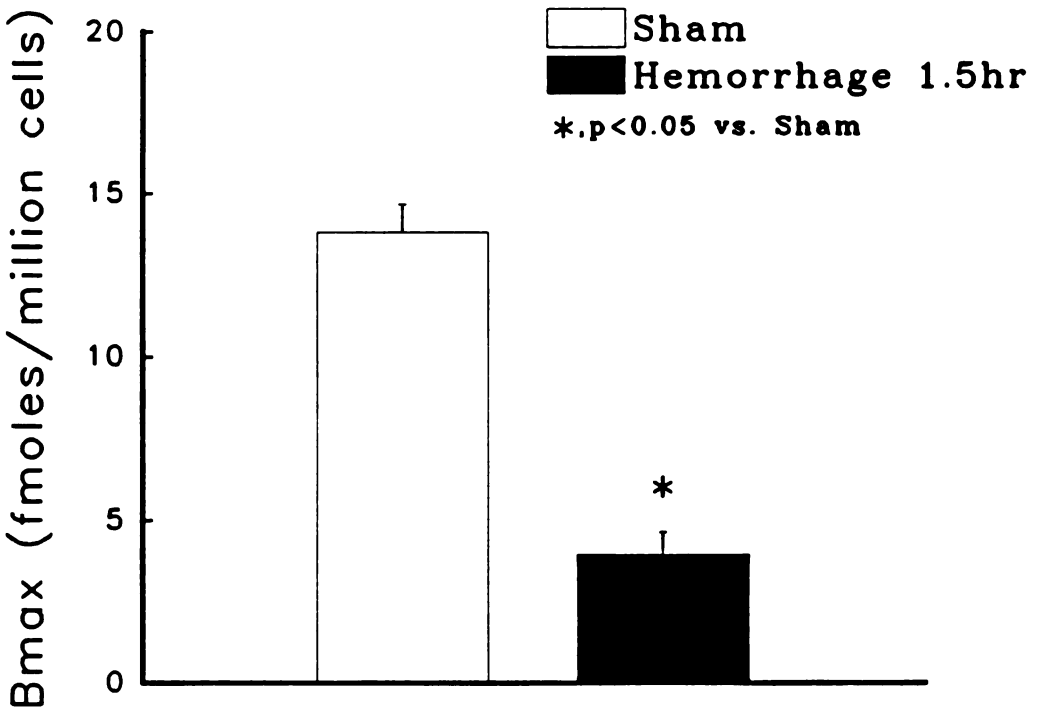

Figure 10. Changes in the maximal binding capacity (B_{max}) of β-adrenergic receptors on hepatocytes isolated from sham-operated animals (Sham) and at 1.5 hours following hemorrhage and resuscitation (Hem. 1.5hr). Data are given as means ± SEM and analyzed using students t-test (n=6/group).

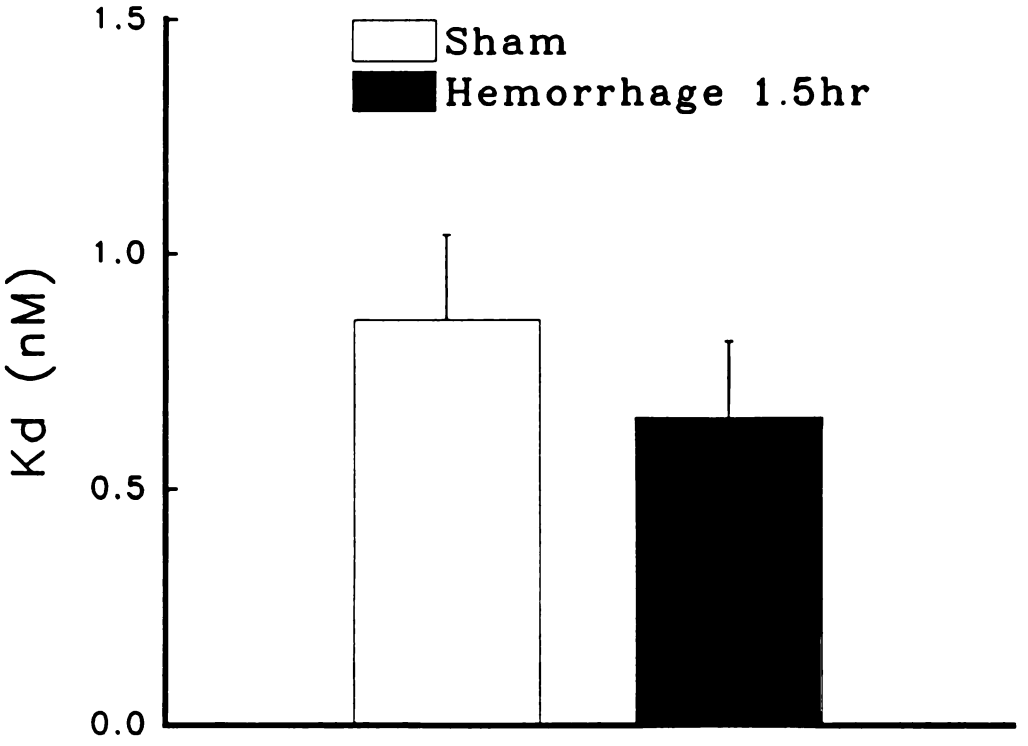

Figure 11. Changes in the dissociation constant (K_d, i.e., 1/affinity) of β-adrenergic receptors on hepatocytes isolated from sham-operated animals (Sham) and at 1.5 hours following hemorrhage and resuscitation (Hem. 1.5hr). Data are given as means $\pm$ SEM and analyzed using students t-test (n=6/group).

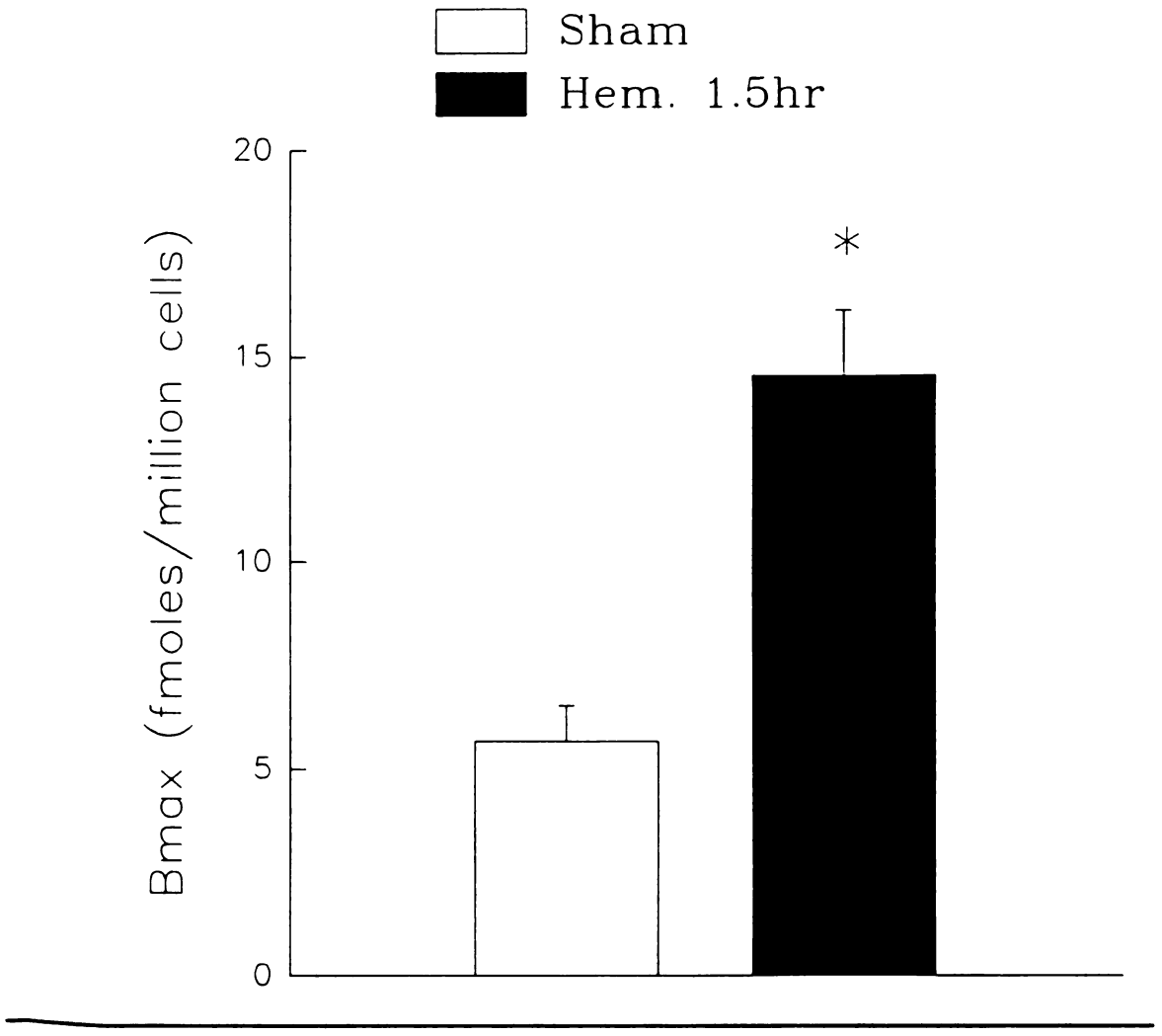

Figure 12. Changes in the maximal binding capacity (B_{max}) of β-adrenergic receptors on Kupffer cells isolated from sham-operated animals (Sham) and at 1.5 hours following hemorrhage and resuscitation (Hem. 1.5hr). Data are given as means ± SEM and analyzed using students t-test (n=6/group). *$P < 0.05$ as compared to Sham.

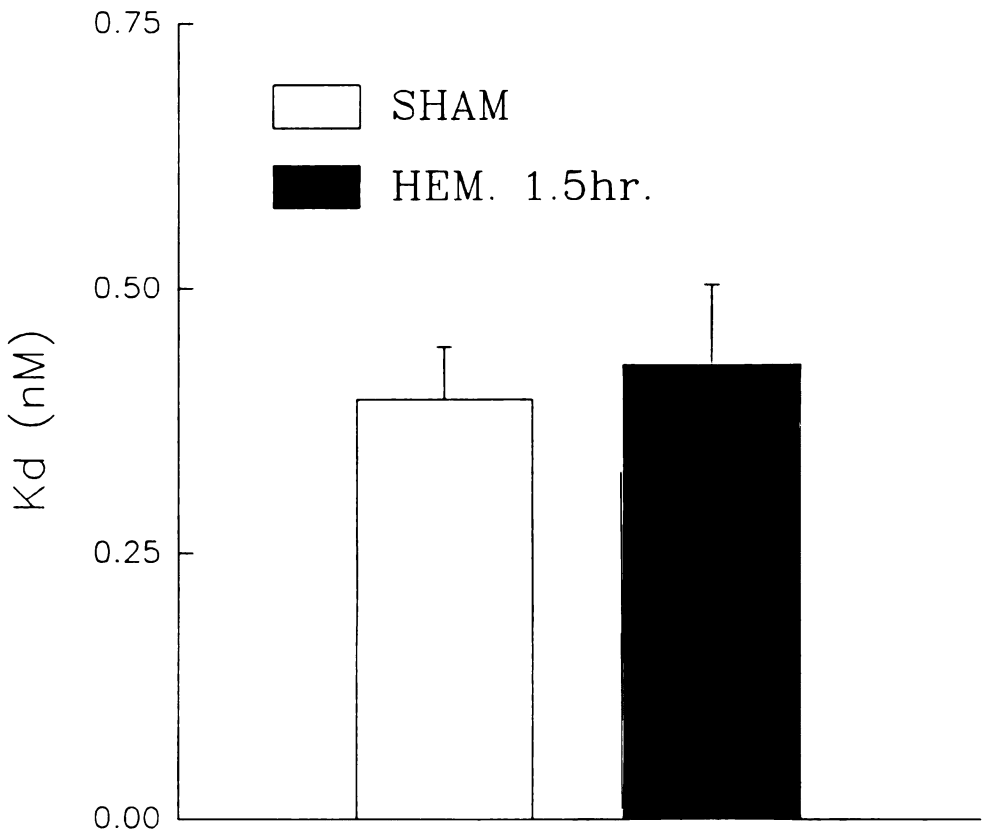

Figure 13. Changes in the dissociation constant (K_d, i.e., 1/affinity) of β-adrenergic receptors on Kupffer cells isolated from sham-operated animals (Sham) and at 1.5 hours following hemorrhage and resuscitation (Hem. 1.5hr). Data are given as means ± SEM and analyzed using students t-test (n=6/group).

PLASMA LEVELS OF CATECHOLAMINES AT 24 HOURS FOLLOWING IMPLANTATION OF PUMPS

The infusion rate of norepinephrine used for the experimental procedure (1μg/minute) increased the plasma levels of norepinephrine ~10-fold at 24 hours without altering the circulating levels of dopamine or epinephrine (Figure 14). The plasma levels of norepinephrine were maintained at levels similar to those observed at 24 hours following hemorrhage and resuscitation (Figure 6 and 14).

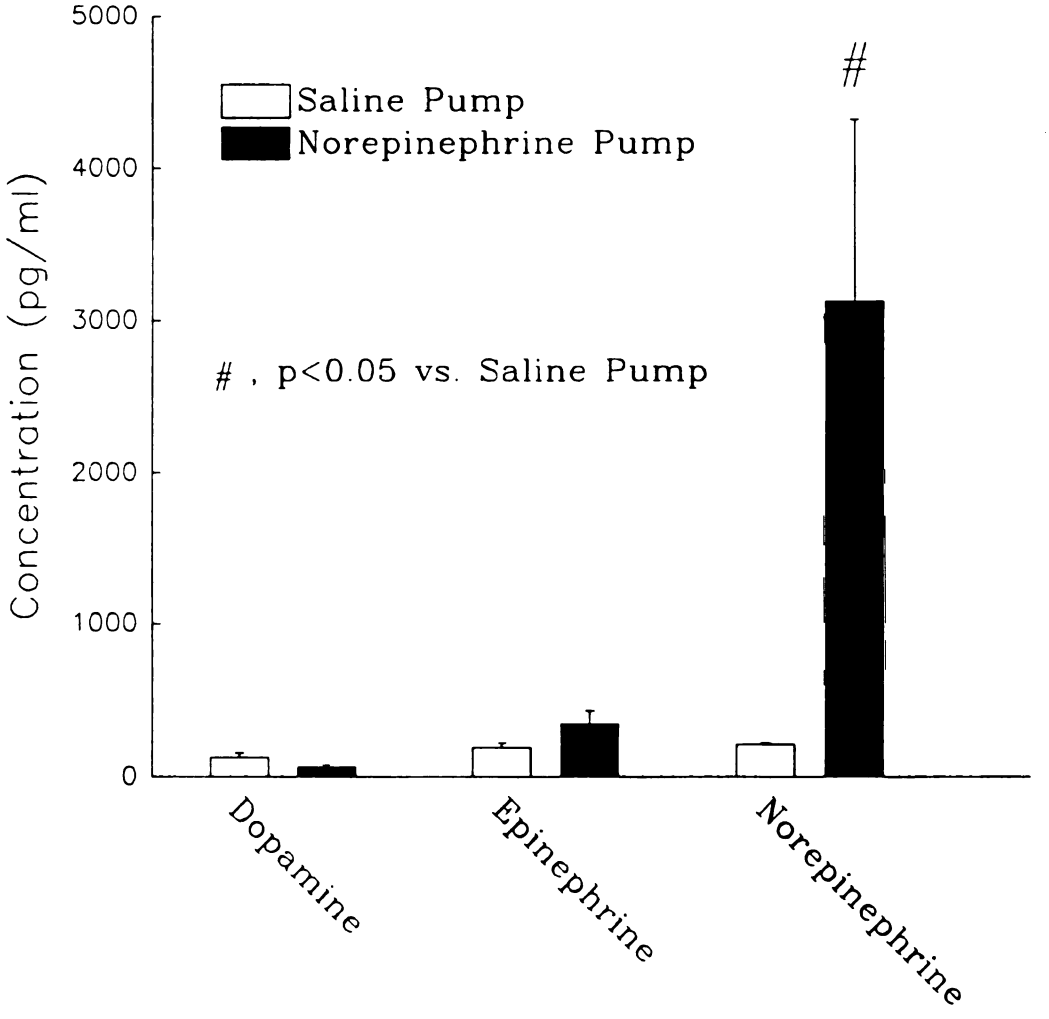

Figure 14. Plasma catecholamine levels at 24 hours following implantation of mini-osmotic pumps filled with either saline (Saline Pump) or norepinephrine (Norepinephrine Pump). Data are given as means ± SEM and analyzed using student's t-test (n=6/group).

ALTERATIONS IN CARDIOVASCULAR PARAMETERS FOLLOWING INFUSION OF NOREPINEPHRINE

Figure 15 illustrates that at 24 hours following continuous infusion of norepinephrine there was a significant depression in cardiac index. Rats given an infusion of saline had a cardiac index of 33.08 ± 1.29 ml/min/100g, while rats given an infusion of norepinephrine had a cardiac index of 22.5 ± .84 ml/min/100g. However, these changes in cardiac output were not due to changes in heart rate, which was approximately 410 beats/min in both groups (Figure 16). A depression in cardiac index with no change in heart rate was caused by a decreased stroke volume in the norepinephrine infused animals (Figure 17). Figure 18 illustrates that there was a significant depression in mean arterial blood pressure in norepinephrine infused animals (95.67 ± 4.65 mmHg), as compared to animals given saline (109.67 ± 3.58 mmHg). There is a significant increase in the TPR of norepinephrine infused animals as compared to shams (Figure 19). Norepinephrine infusion caused a significant depression in the microvascular blood flow in the liver, spleen, and small intestine as compared to shams (Figure 20). However, the decrease in kidney microvascular blood flow was insignificant (Figure 20). Figure 21 shows that systemic hematocrit was significantly increased in norepinephrine infused animals (51 ± 1%) as compared to shams (46 ± 1%).

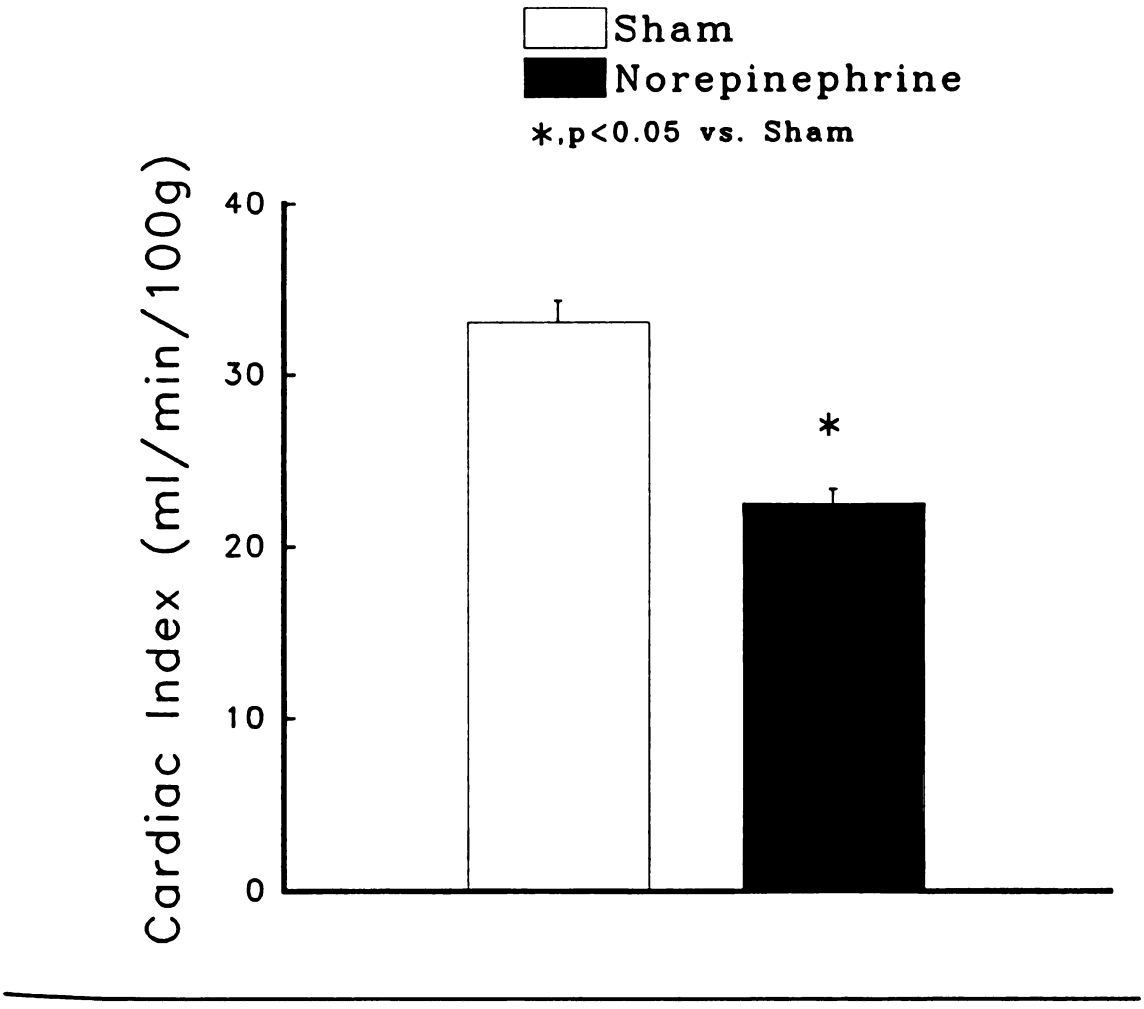

Figure 15. Differences in cardiac index at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

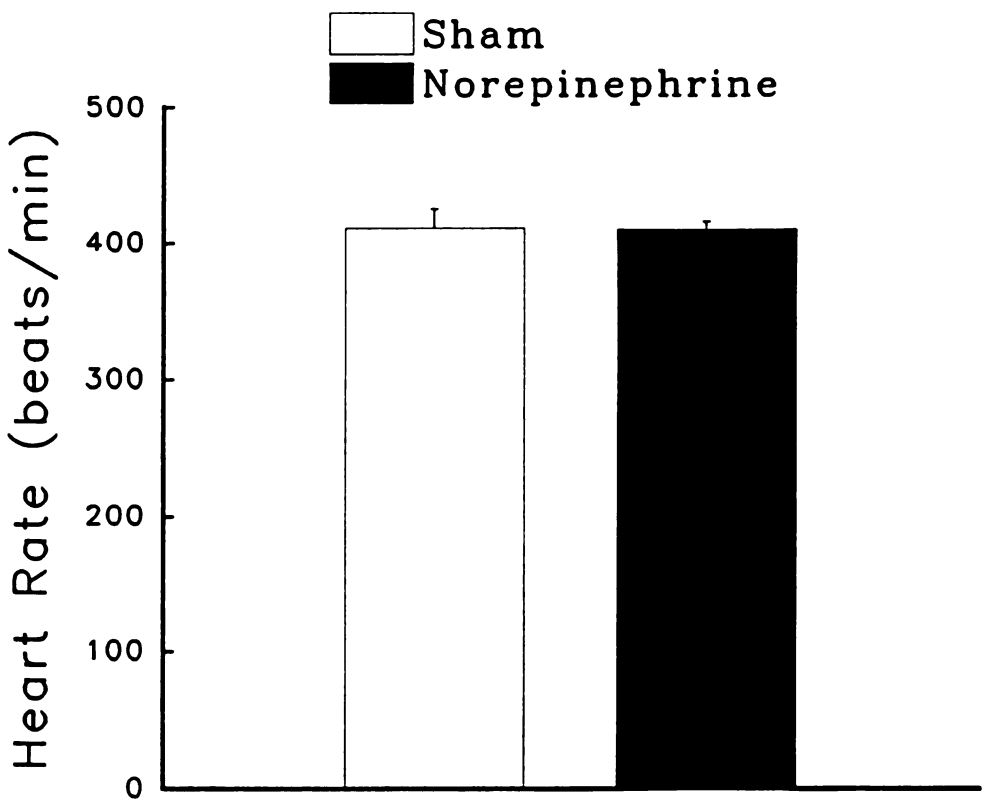

Figure 16. Heart rate at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

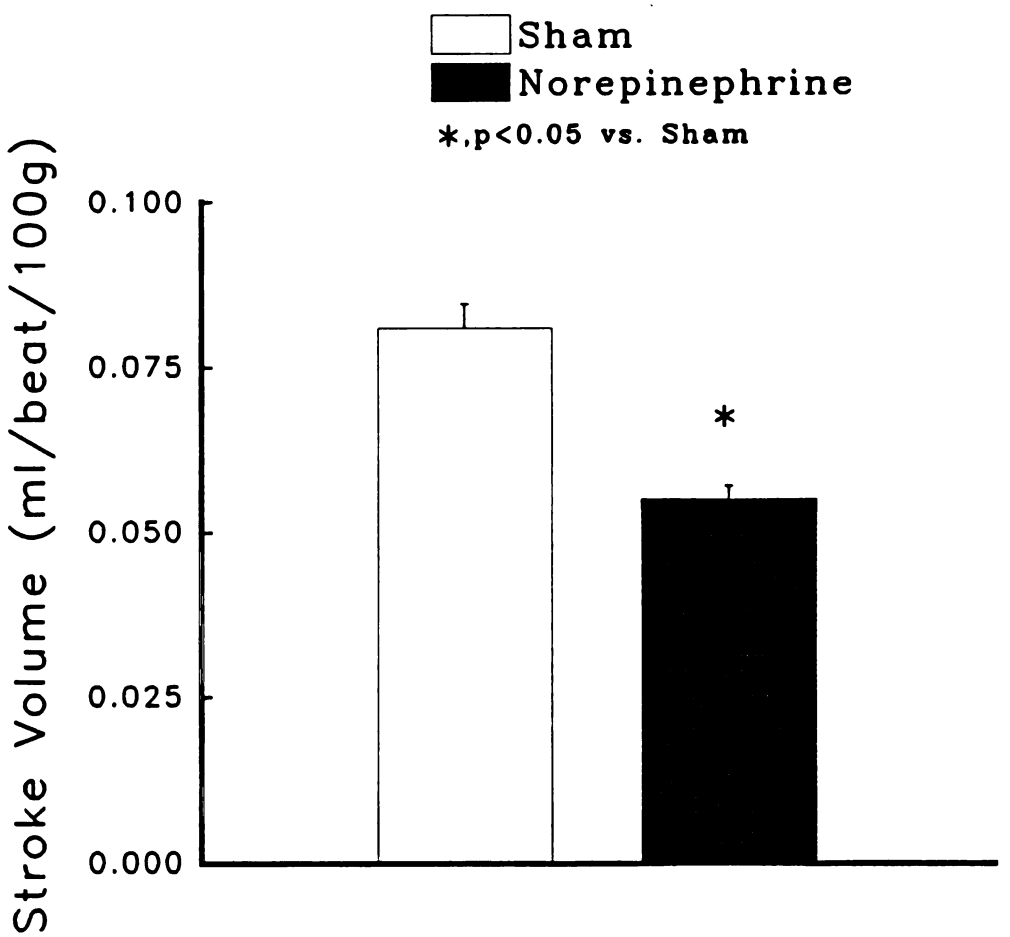

Figure 17. Differences in stroke volume at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

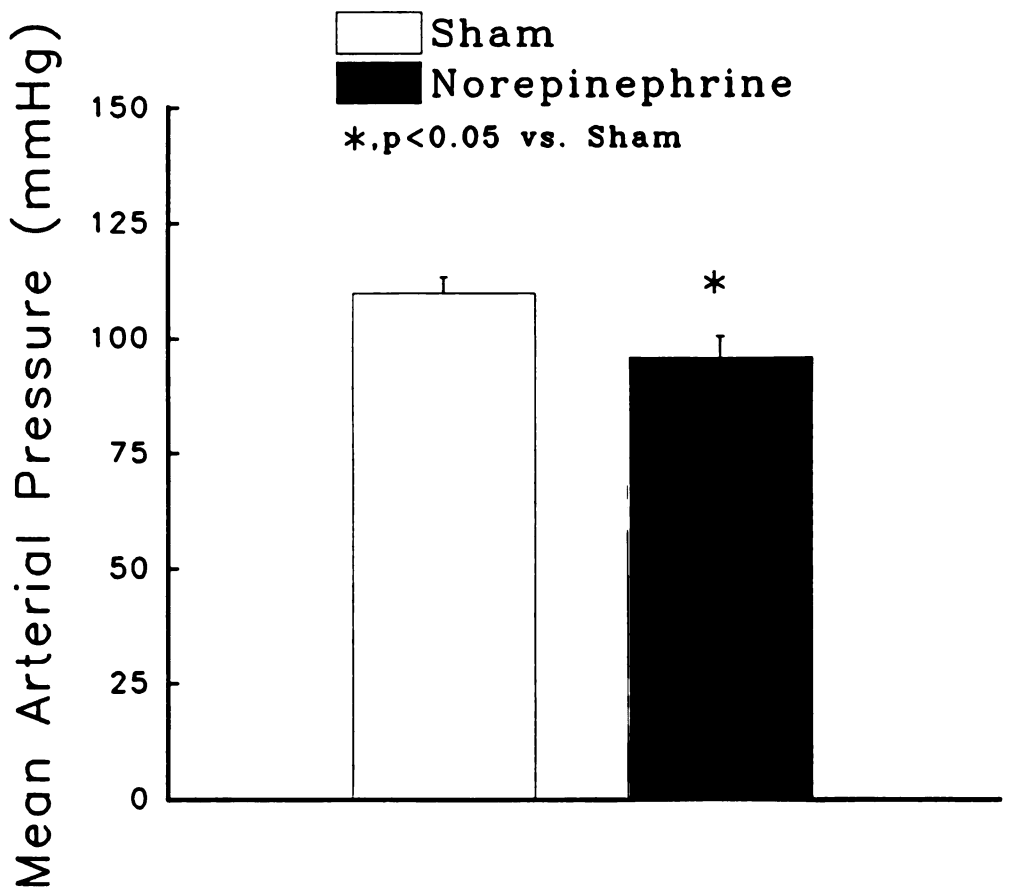

Figure 18. Differences in mean arterial pressure at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

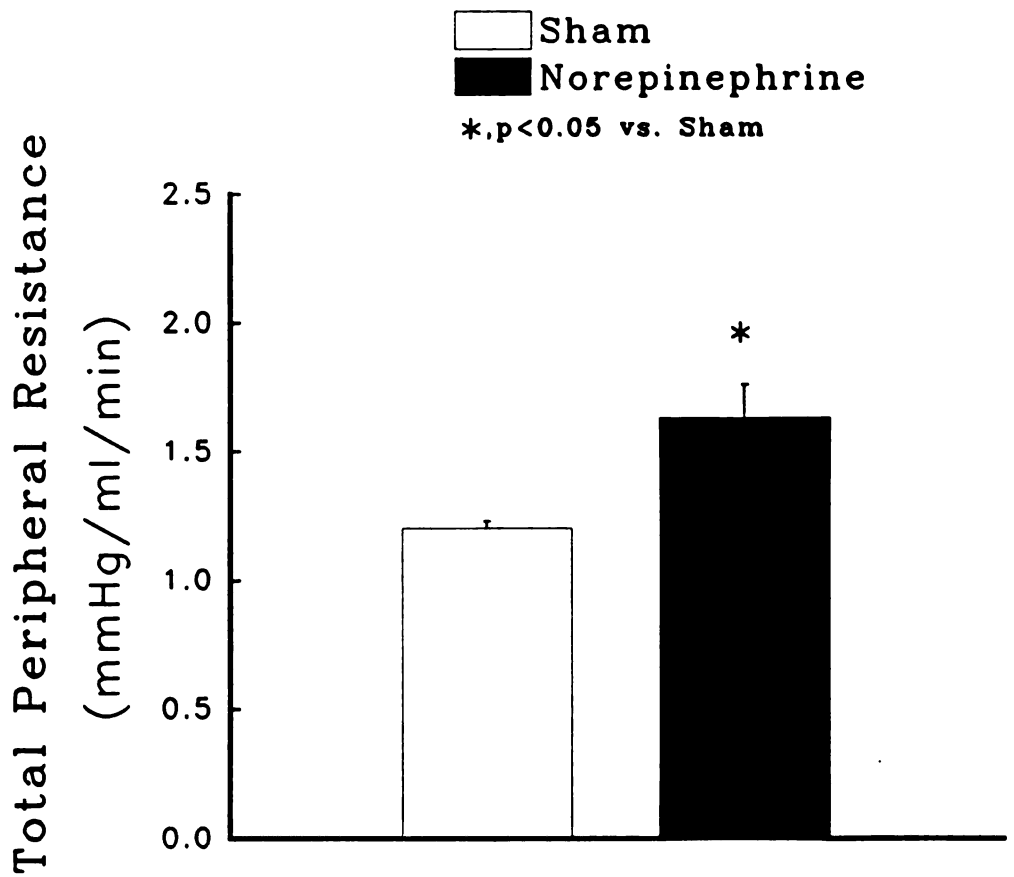

Figure 19. Differences in total peripheral resistance at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

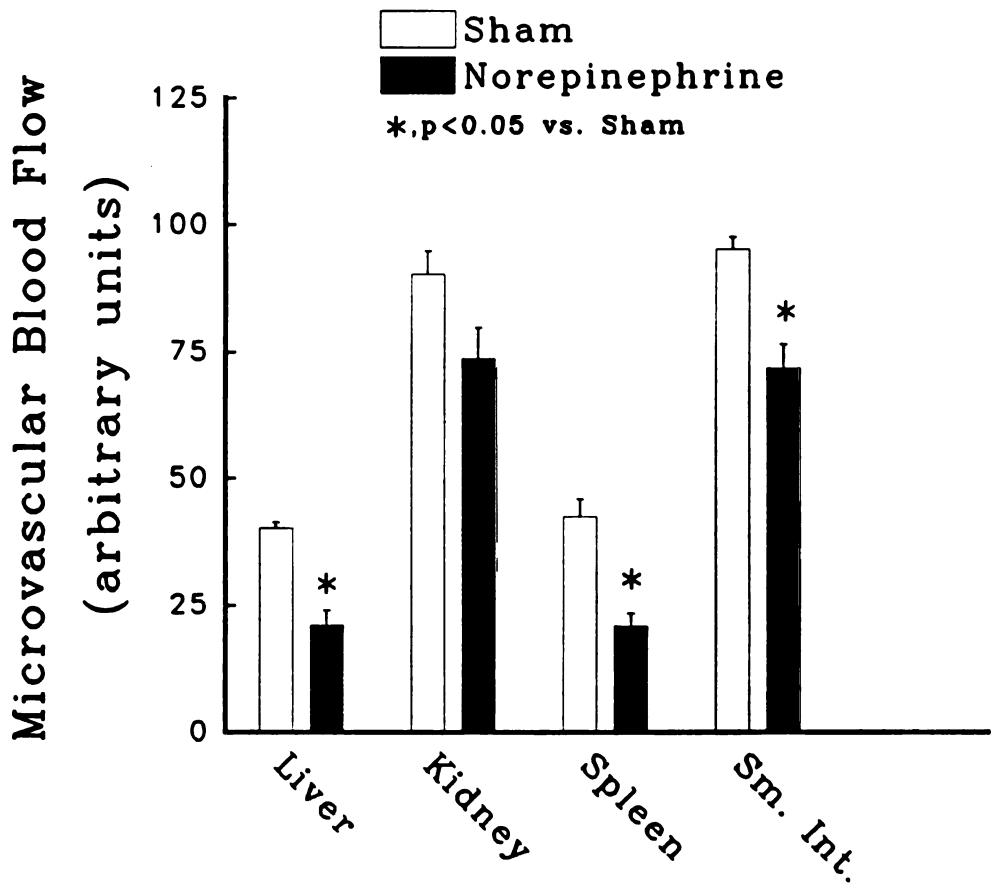

Figure 20. Differences in microvascular blood flow (MBF) in the liver, kidney, spleen, and small intestine (Sm. Int.) at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). MBF was determined using a laser doppler blood perfusion monitor. Data is given as means ± SEM and compared using Student's t-test (n=6/group).

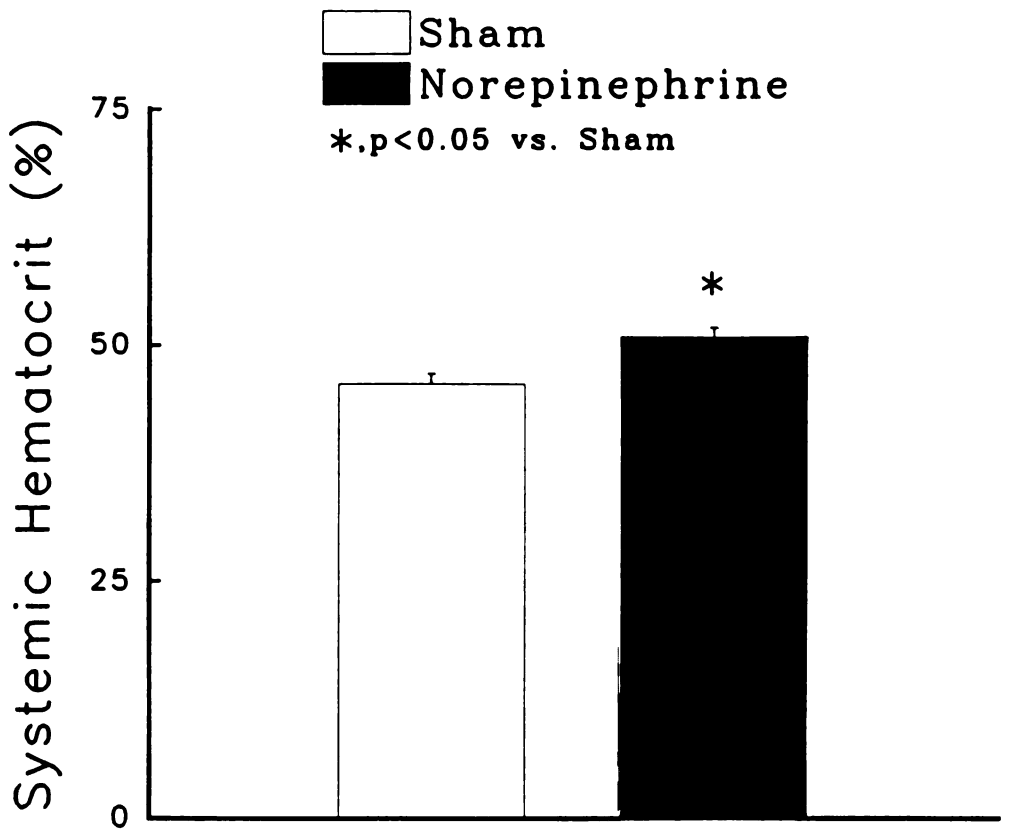

Figure 21. Differences in systemic hematocrit at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Wilcoxon's Rank Sum test (n=6/group).

ALTERATIONS IN BODY WEIGHT DUE TO INFUSION OF NOREPINEPHRINE

Figure 22 illustrates that norepinephrine infused animals showed a significant reduction in body weight, losing approximately 8% of their total body weight during the 24 hour infusion of norepinephrine.

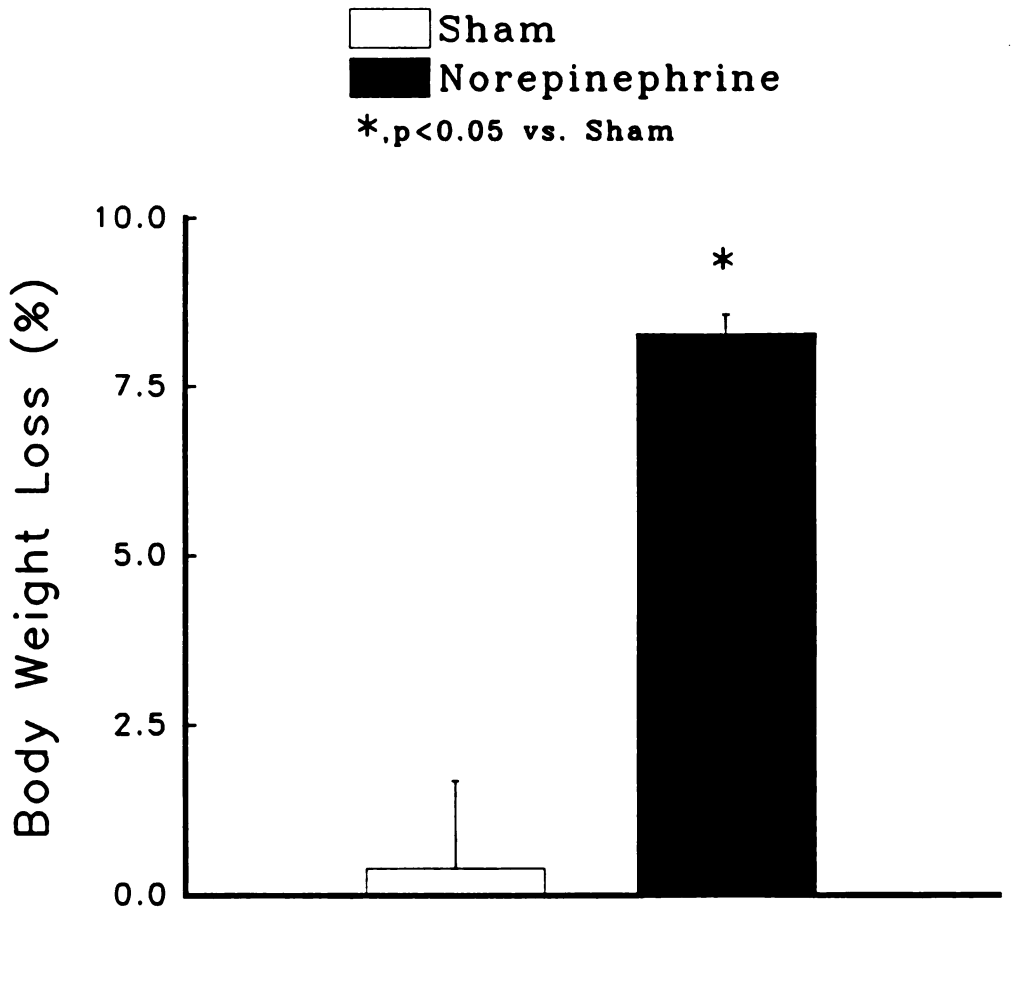

Figure 22. Body weight loss at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM of the % of total body weight lost. Data was compared using Wilcoxon Rank Sum test (n=6/group).

ALTERATIONS IN ACTIVE HEPATOCELLULAR FUNCTION, PLASMA GLUCOSE LEVELS, AND HEPATOCELLULAR DAMAGE FOLLOWING NOREPINEPHRINE INFUSION

Active hepatocellular function, as measured by the V_{max} and K_m of ICG uptake by hepatocytes, was depressed following a 24 hour infusion of norepinephrine (Figure 23). 24 hour infusion of norepinephrine caused an increase in the circulating levels of GOT and GPT, indicating an altered integrity of the hepatocyte plasma membranes (Figure 24). Those animals that were given an infusion of norepinephrine showed an elevated plasma glucose level as compared to shams (Figure 25).

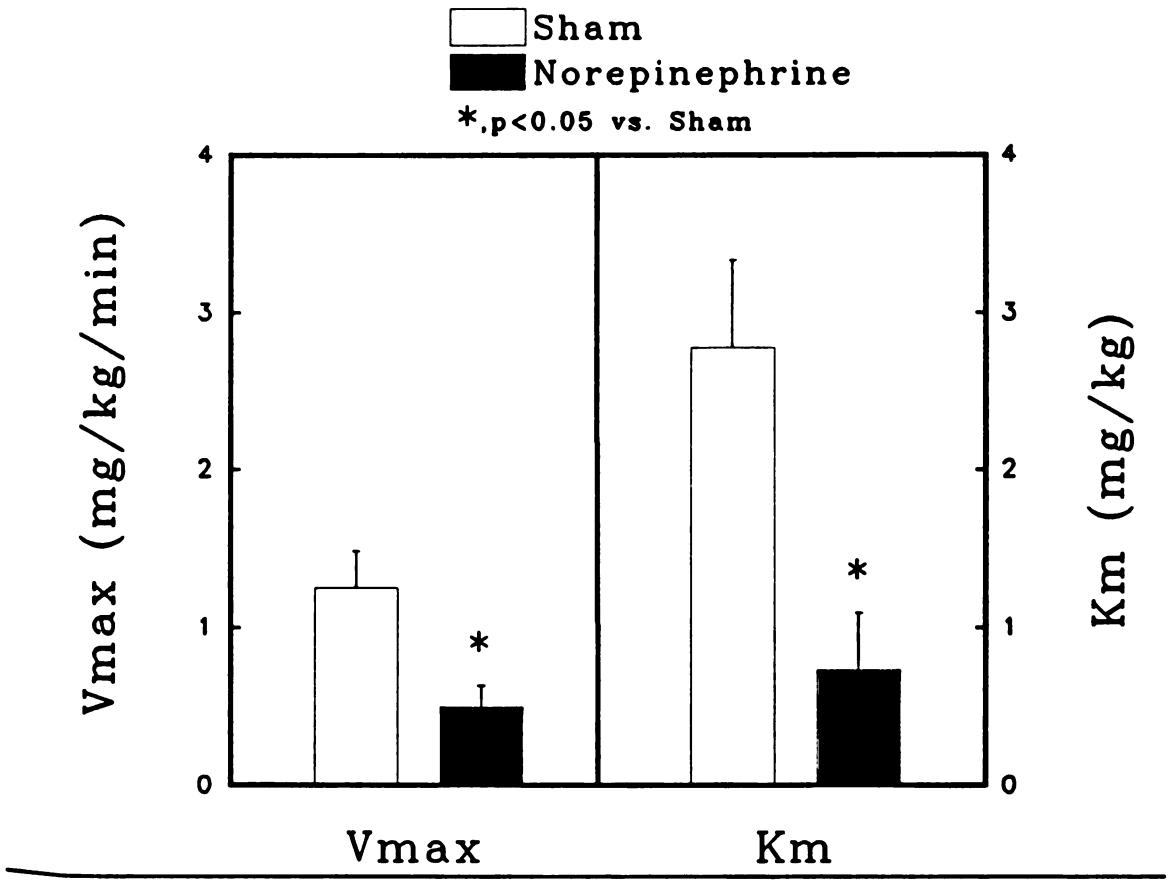

Figure 23. Differences in active hepatocellular function, represented by the V_{max} and K_m of ICG clearance, at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

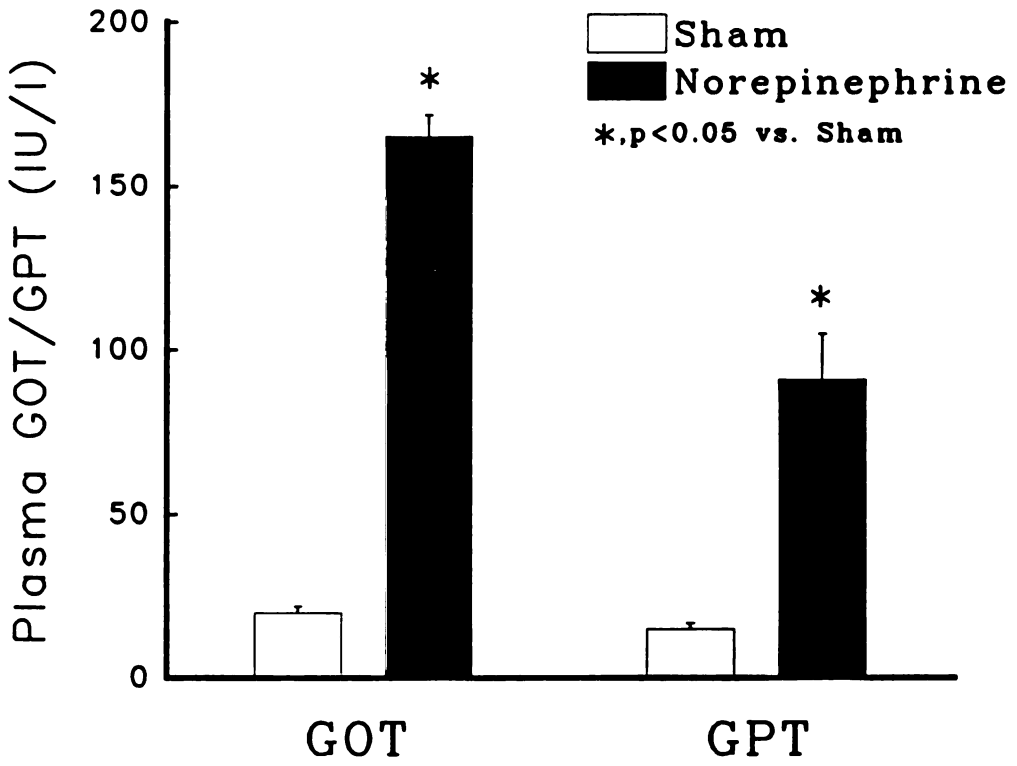

Figure 24. Hepatocellular damage, measured by plasma levels of intrahepatocellular enzymes (GOT/GPT), at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

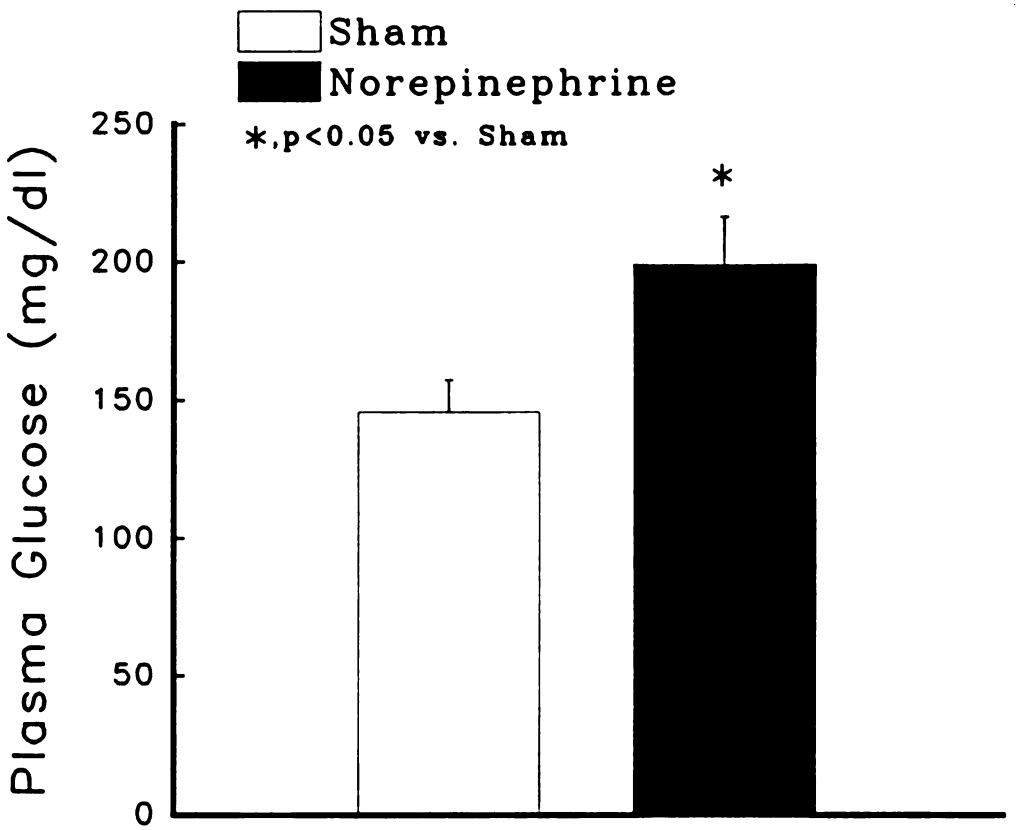

Figure 25. Differences in plasma glucose levels at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

ALTERATIONS IN HEPATOCELLULAR β-ADRENERGIC RECEPTOR BINDING CHARACTERISTICS FOLLOWING INFUSION OF NOREPINEPHRINE

Following 24 hour infusion of norepinephrine there was a significant depression in the β-adrenergic receptor B_{max} of hepatocytes (Figure 26). However, there was no significant depression in the K_d of these receptors (Figure 27).

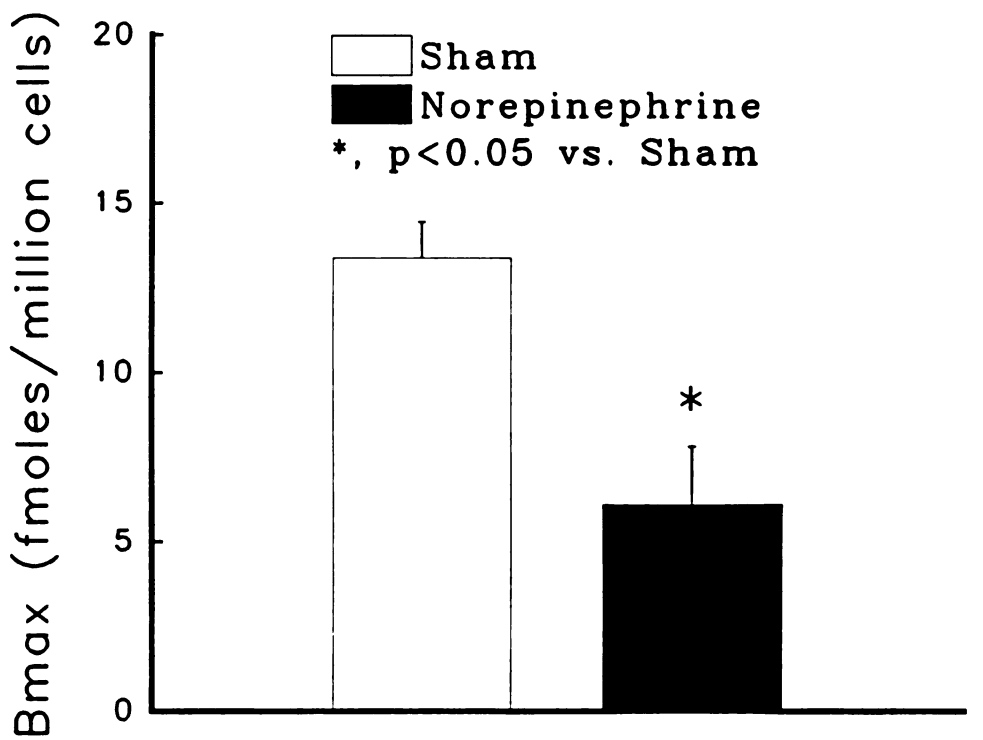

Figure 26. Differences in the maximal binding capacity (B_{max}) of β-adrenergic receptors on hepatocytes at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

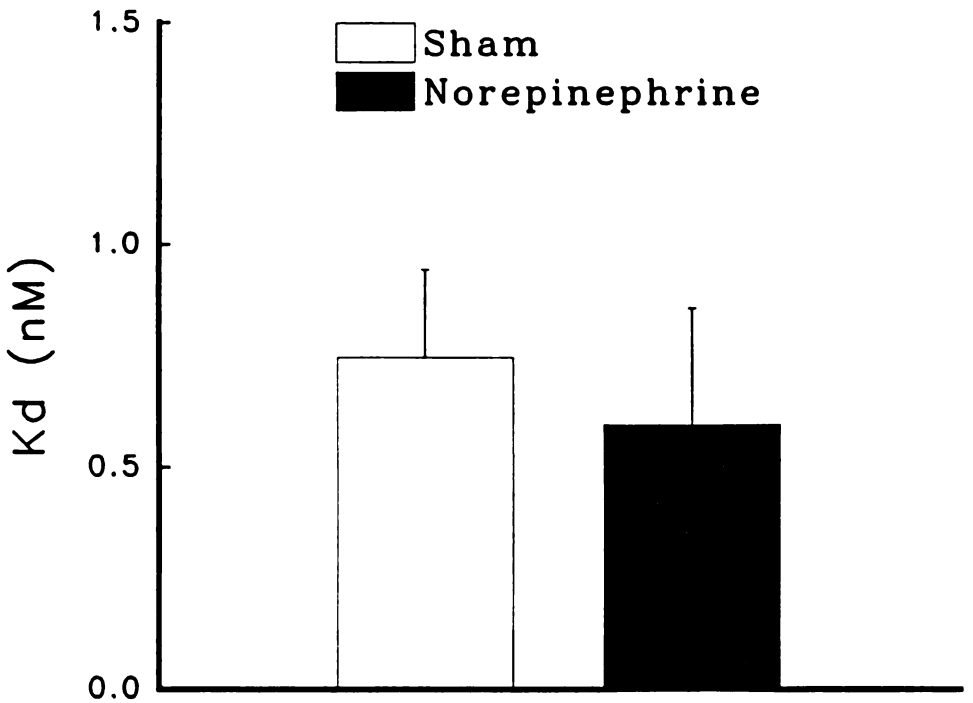

Figure 27. Differences in the dissociation constant (K_d, i.e., 1/affinity) of β-adrenergic receptors on hepatocytes at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

CHANGES IN CIRCULATING LEVELS OF TNF AND IL-6 FOLLOWING INFUSION OF NOREPINEPHRINE

Figure 26 illustrates that infusion of norepinephrine caused no significant difference in circulating levels of TNF. However, norepinephrine infusion caused a significant increase in the circulating level of IL-6 (Figure 27).

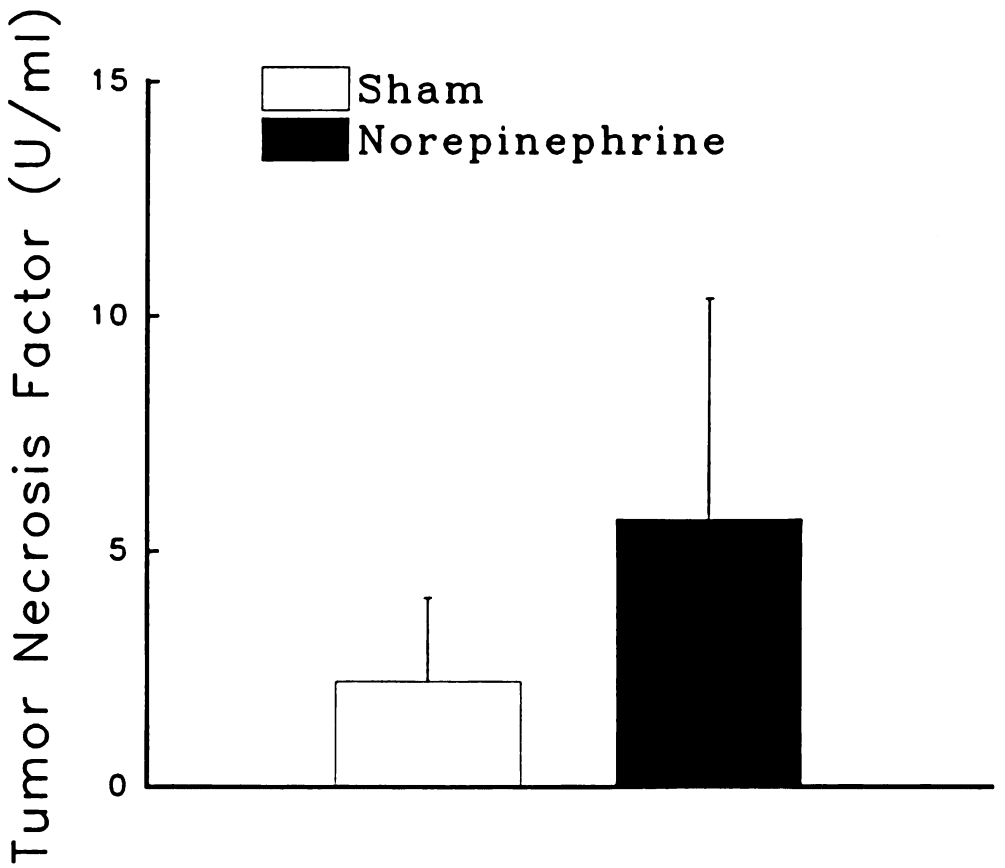

Figure 28. Differences in plasma levels of tumor necrosis factor at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

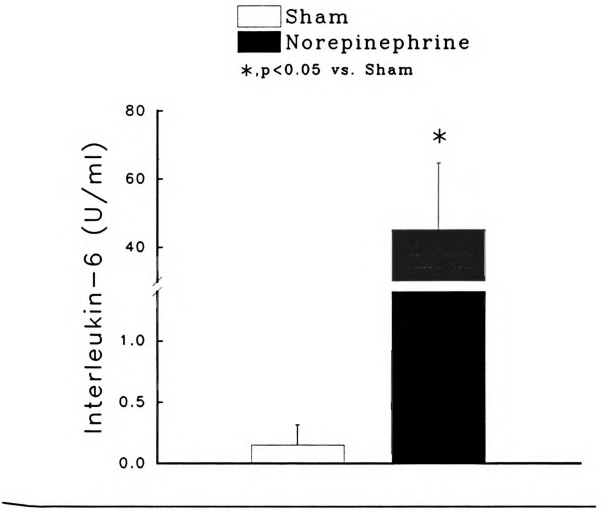

Figure 29. Differences in plasma levels of interleukin-6 at 24 hours following implantation of mini-osmotic pumps infusing either norepinephrine (Norepinephrine) or saline (Sham). Data is given as means ± SEM and compared using Student's t-test (n=6/group).

DISCUSSION

To study the alterations which occur following trauma and hemorrhage, a fixed pressure model of trauma and severe hemorrhage in the rat was utilized. Although other fixed pressure hemorrhage models use preheparinization, this is a nonheparinized model of hemorrhage as pre-heparinization has been shown to have beneficial effects following hemorrhage and resuscitation (143). I used crystalloid resuscitation instead of blood in order to control for any effect of soluble mediators present in the blood. Also, it has been shown that, following trauma-hemorrhage in rats, crystalloid resuscitation is as effective as blood resuscitation in terms of survival (116). In this model I have looked at the effects of trauma and hemorrhage, mimicking the clinical situation as closely as possible with tissue trauma in conjunction with hemorrhage.

Alterations in both cardiac and hepatic function occur during hemorrhage and following resuscitation. It has been shown, using the fixed-pressure model of trauma-hemorrhage and crystalloid resuscitation, that there is a depression in cardiac output (CO) during hemorrhage, and immediately following crystalloid resuscitation the CO is normalized (140). However, soon after the completion of resuscitation the CO is diminished, which persists up to at least 8 hours following completion of resuscitation (140). There is

significant damage to the integrity of the hepatocellular plasma membrane at 1.5 and 4 hours following hemorrhage and resuscitation, as indicated by high circulating plasma levels of GOT and GPT (intrahepatocellular proteins) (139). Elevations in plasma GOT/GPT indicate alterations in the integrity of the hepatocyte plasma membrane. However a more subtle derangement in the function of the intact hepatocytes was observed via changes in the active uptake of ICG, used as an indication of active hepatocellular function. During hemorrhage there is a significant depression in the active hepatocellular function and immediately following resuscitation the active hepatocellular function is restored (130). However, it begins to deteriorate as soon as 1.5 hours following resuscitation and remains depressed up to at least 8 hours post-resuscitation (130).

Derangements in cardiac and hepatic function occur not only during hemorrhage and early following resuscitation, but continue late (24 hours) after hemorrhage and resuscitation. Using the same model of hemorrhage and resuscitation in the rat as this study, Kepros *et al.* showed that at 24 hours after hemorrhage and resuscitation the animals displayed a depressed MAP, due to decreased cardiac index and stroke volume, in spite of an elevated total peripheral resistance (65). In addition, these animals exhibited elevated levels of GOT, hepatic edema, and a depressed hepatic microvascular blood flow (65).

Using a fixed pressure model of trauma-hemorrhage and crystalloid resuscitation, this study has shown that during hemorrhage the plasma levels of epinephrine were elevated approximately 100-fold. However, these levels were normalized as early as 8 hours following resuscitation. Plasma levels of norepinephrine were elevated approximately 10-fold during hemorrhage and remained elevated up to at least 24 hours following resuscitation.

Elevations in plasma levels of catecholamines have been observed in a variety of conditions involving low blood flow (32,57,59,151). It has been observed that circulating levels of norepinephrine and epinephrine are higher in patients that are critically injured as compared to those that are moderately injured (57). Using a fixed pressure model of hemorrhage in dogs, Jakschik *et al.* showed that total catecholamine levels increased approximately 100-fold during hemorrhage, then as blood was returned, catecholamine levels fell but remained 10-fold higher than control levels (59). During this hemorrhage in dogs, it is possible that epinephrine is the catecholamine elevated ~100-fold during hemorrhage, and norepinephrine remains elevated ~10-fold following the return of fluids while epinephrine levels fall dramatically. Woolf *et al.* observed that in patients with multisystem trauma with head injury, plasma norepinephrine levels were highly correlated with patient mortality (151). Although epinephrine levels were initially elevated in these

patients with multisystem trauma, they decreased with time (151). Davies *et al.* also observed that following injury in man, there are rapid increases in plasma levels of norepinephrine and epinephrine in a way that is proportional to the severity of the injury. (32). Using a model of hind limb ischemia and reperfusion in rats, in which a tourniquet is applied to one hind limb for a period of 240 minutes, Vujnov *et al.* observed that during ischemia and following reperfusion the levels of epinephrine in the myocardium increased, while the levels of norepinephrine decreased (127). This model causes a significant decrease in circulating blood volume due to fluid loss into the ischemic limb. Since the levels of catecholamines were determined in the myocardial tissue (not in plasma), it seems that this model causes a release of norepinephrine from the sympathetic nerve endings within the myocardium, while there is an increase in the plasma epinephrine levels circulating through the myocardium. Therefore, one can speculate that if the plasma levels of norepinephrine and epinephrine had been determined in this model, both would have been elevated.

Derangements in plasma levels of insulin and glucagon can be attributed to catecholamines following injury. Plasma levels of insulin have been observed to be low early after injury despite hyperglycemia, which is a result of suppression of insulin secretion from pancreatic β-cells due

to the effects of catecholamines acting via α-adrenergic receptors (41). In contrast, the secretion of glucagon from pancreatic α-cells is stimulated due to elevated catecholamine concentrations acting via β-adrenergic receptors (102). Therefore, despite the hyperglycemia which is occurring following injury, plasma insulin levels are reduced while plasma levels of glucagon are elevated. This response is opposite of that which would occur in normal animals in due to hyperglycemia.

Catecholamines are released following trauma-hemorrhage due to neural reflexes involving the sympathetic nervous system. Normally, the afferent information associated with the realization of danger, fluid loss, and tissue damage leads to an increase in the activity of the sympathetic preganglionic nerve fibers. This increase in sympathetic nervous system activity causes release of norepinephrine from postganglionic neurons and release of epinephrine from the adrenal medullary cells, which are analogous to postganglionic neurons.

The elevated levels of norepinephrine which remain following resuscitated hemorrhagic shock may have a variety of deleterious cardiovascular and hepatic effects. Norepinephrine has been shown to decrease the blood flow through the liver, thus depressing oxygen delivery, by increasing hepatic arteriolar and venous resistance via α-adrenergic receptors (47,72). Normal rat livers given a

perfusion of either α- or β-adrenergic agonists exhibit an increase in glycogenolysis, gluconeogenesis, lactate production, and respiration (58). Norepinephrine is known to cause moderate increases in cardiac contractility and heart rate (95). However, it also causes pronounced global vasoconstriction and no vasodilation. The overall results are slight decreases in cardiac output, increased total peripheral resistance, and increased blood pressure (95). Moderate increases in force of cardiac contractility and heart rate would seem to be beneficial to animals in shock. However, the pronounced vasoconstriction to the visceral organs may be quite detrimental to the survivability of the animal, as depression in visceral organ perfusion can cause regional hypoxia, thereby causing alterations in tissue function and initiating inflammatory responses.

The effects of norepinephrine on the metabolism of the hepatocytes may be detrimental to efficient use of metabolic energy. The increased catecholamine levels may increase energy expenditure by stimulating substrate cycling. This is a process in which, although there is no change in the amount of either substrate or product, ATP is broken down and metabolic energy has to be used up for its resynthesis.

The elevation in catecholamines during hemorrhage and after resuscitation affect the liver via both α and β-adrenergic receptors. The present study does not examine alterations in α-adrenergic receptors; it focuses on

alterations in hepatic β-adrenergic receptors. This does not mean that the α-adrenergic receptors do not play an important role in the development of the physiological derangements following hemorrhage. Instead, β-adrenergic receptors were chosen due to the wealth of information available on the characterization and alteration of these receptors following various adverse circulatory conditions in several tissues.

Alterations in β-adrenergic receptors have been described in liver and other tissues following various adverse circulatory conditions (40,62,79,92,103,127). Studies have shown that at two hours following endotoxin administration, both in vivo and in vitro, there is a decrease in both the affinity and number of β-adrenergic receptors in plasma membrane preparations of dog livers (79). Forse *et al.* examined the response of β-adrenergic agonists on lipolysis in adipose tissue from trauma patients and from patients in late stages of sepsis (40). Their results indicated that following trauma, there is a decrease in both responsiveness and number of β-adrenergic receptors in adipocytes (40). Similarly, there is a decrease in responsiveness and an increase in number of β-adrenergic receptors in adipocytes during the late stages of sepsis (40). In contrast, β-adrenergic receptor numbers in canine cardiac myocytes have been shown to increase following cardiac ischemia and reperfusion (92). In a model of

tourniquet trauma (hind limb ischemia lasting 4 hours) in the rat, the cardiac β-adrenergic receptor numbers decreased while their affinity increased (127). Following burn injury in guinea pigs (third degree scald burns to 45% total body surface area), there was a decrease in the affinity of the β-adrenergic receptors in the heart with no change in receptor number (62). Depending upon the tissue and/or the particular adverse circulatory condition, there is significant variation in the alteration of β-adrenergic receptors.

My results indicate that following trauma-hemorrhage and crystalloid resuscitation, there is a depression in the number of available β-adrenergic receptors on hepatocytes. Furthermore, these receptors do not appear to be internalized into intracellular vesicular stores, as the β-adrenergic receptor B_{max} of isolated membrane vesicles was also lower following resuscitated hemorrhagic shock. I found that during hemorrhage (at maximal bleedout) there is a significant decrease in the B_{max} of hepatic plasma membrane β-adrenergic receptors, however, there was no change in their affinity. Following crystalloid resuscitation there was a trend towards increasing B_{max}. This, however, was not statistically different from the B_{max} values observed at maximal bleedout. Following hemorrhage and resuscitation I observed a depression in hepatocyte B_{max} with no change in K_d. The downregulation of hepatic β-

adrenergic receptor B_{max}, with no change in affinity, is different from that observed in dog liver following endotoxin administration, in which both B_{max} and affinity decreased significantly (79). Endotoxin *in vitro* was shown to decrease both the number and affinity of the β-adrenergic receptors by a mechanism that has yet to be determined (79). Since endotoxin is not detected during hemorrhage (7), it would be supported that endotoxin is not the cause of changes in hepatic β-adrenergic binding characteristics following hemorrhage.

The downregulation of hepatic β-adrenergic receptor B_{max} observed during hemorrhage and up to 1.5 hours after resuscitation may be due to elevated levels of circulating catecholamines. This study has shown that during hemorrhage the circulating epinephrine levels are elevated about 100-fold, and following crystalloid resuscitation the norepinephrine levels remain elevated 10-fold as compared to shams. In addition, this study has shown that a 24 hour elevation in plasma norepinephrine, similar to that which is seen after resuscitated hemorrhagic shock, causes a depression in hepatocellular β-adrenergic receptor B_{max} of normal rats. Strasser *et al.* observed that while cardiac ischemia causes an increase in the number of β-adrenergic receptors in myocytes, perfusion of normal hearts with the perfusate collected from ischemic hearts caused a decrease in the number of β-adrenergic receptors (103). This implies

that while there is a downregulatory influence of catecholamines in the perfusate taken from ischemic hearts, hypoxia induces an increase in the number of cardiac β-adrenergic receptors. Desensitization of β-adrenergic receptor B_{max} in response to high levels of catecholamines has been observed in many tissues and cell populations (147).

Due to the different time frames, the observation that 24 hour infusion of norepinephrine causes downregulation of hepatocyte β-adrenergic receptor B_{max} has limitations as evidence that the downregulation of hepatocyte β-adrenergic receptor B_{max} during hemorrhage and up to 1.5 hours following resuscitation is due to increased circulating catecholamines. Further studies looking at the response of hepatocyte β-adrenergic receptors to 3 hours of norepinephrine infusion are needed to help elucidate if there is a connection between increased catecholamines and decreased hepatocyte β-adrenergic receptor B_{max} at 1.5 hours following hemorrhage and resuscitation.

The desensitization of hepatic β-adrenergic receptors that occurs following resuscitated hemorrhagic shock appears to be due to changes in the number of available receptors rather than changes in there affinity. In our model there were no changes in the K_d of the hepatic β-adrenergic receptors. A decrease in K_d would indicate a change in receptor affinity which can occur by phosphorylation of

amino acid residues (51,81).

The results of this study show that crystalloid resuscitation is not adequate to normalize the depression in hepatic β-adrenergic receptor B_{max} which occurs during hemorrhage and up to at least 1.5 hours after resuscitation. Early (up to at least 4 hours) following crystalloid resuscitation the circulating levels of both epinephrine and norepinephrine remained elevated despite an increased MAP due to crystalloid resuscitation. Also, the elevated levels of plasma norepinephrine which remain up to at least 24 hours after resuscitation may perpetuate the depressions in β-adrenergic receptor B_{max}, as this study has shown that infusion of norepinephrine for 24 hours causes a depression in hepatocyte B_{max}. However, the exact mechanism of the downregulation of hepatic β-adrenergic receptor B_{max} remains to be determined.

The interaction of catecholamines with β-adrenergic receptors on the hepatocytes causes a cAMP dependent activation of phosphorylase A, stimulating glycogenolysis (25), in addition to inhibiting glycogen synthase, thereby decreasing the amount of glycogen synthesis (37). β-adrenergic stimulation of perfused rat liver causes K^+ release (depolarization), lactate production, and an increase in O_2 uptake (58). β-adrenergic receptor agonists have been shown to stimulate gluconeogenesis in isolated rat hepatocytes (38). Studies have shown that following

hemorrhage in rats, the hepatic microvascular blood flow and total hepatic blood flow are both depressed (132). Any changes in hepatocellular β-adrenergic receptors would alter the responsiveness of hepatocytes to epinephrine and norepinephrine.

Following hemorrhage and crystalloid resuscitation, the increased sensitivity (elevated B_{max}) of Kupffer cells to β-adrenergic receptor agonists may be detrimental to the survivability of the animal by causing depressions in Kupffer cell immune function. It has previously been shown that hemorrhagic shock produces depressions of immune function and increases the susceptibility to sepsis (8,21,118). Previous publications from our lab have indicated that circulating levels of TNF and IL-6 are elevated following hemorrhage and resuscitation (137). Kupffer cells have been shown to be a major source of inflammatory cytokine release following hemorrhage (94). We have observed that Kupffer cell cAMP levels are more than doubled at 1.5 hours following hemorrhage and crystalloid resuscitation (144). The elevation of cAMP in those cells following hemorrhage may be due to the upregulation of β-adrenergic receptor B_{max} that this study has shown. These increases in β-adrenergic receptor B_{max} and cAMP levels in Kupffer cells may be responsible for the depressions in phagocytosis observed following hemorrhage and resuscitation (8). β-adrenergic receptor stimulation has been shown to

depress Kupffer cell complement receptor clearance function (80). Thermal injury (immersion in 90°C water for 30 sec.) in rats causes depressions in Kupffer cell complement receptor clearance function, and this depression is minimized by β-adrenergic receptor blockade (propanolol) (80). Administration of ATP-$MgCl_2$ (12), which has been shown to have various beneficial effects following hemorrhage, has been shown to normalize the elevated Kupffer cell β-adrenergic receptor B_{max} at 1.5 hours following hemorrhage and crystalloid resuscitation (144).

It is probable that the mechanism of this increase in Kupffer cell β-adrenergic receptor B_{max} is due to some mediator other than catecholamines. The increase in Kupffer cell β-adrenergic receptor B_{max} is contrary to the typical response of β-adrenergic receptors to high levels of catecholamines, which is that of downregulation of B_{max}, K_d, or both (51,81,147). Desensitization of β-adrenergic receptors in response to high levels of catecholamines has been demonstrated in numerous cell types and animals (147). In spite of the fact that some cells have been shown to increase their sensitivity to a given ligand in response to that ligand, especially in immune cells (69), there is no example of cells increasing β-adrenergic receptor B_{max} in response to elevated catecholamine levels.

The increase in β-adrenergic receptor B_{max} in Kupffer cells may be due to hypoxia, cytokines, or some unknown

mediator which is elevated following shock (9,34,115,144). It is well known that some ligands have a permissive effect on the sensitivity of cells to a particular ligand. For example, increased levels of thyroid hormone have been shown to cause an increase in the β-adrenergic receptor B_{max} on myocytes by stimulating the production of more β-adrenergic receptors (147). The mechanism of this increase in Kupffer cell β-adrenergic receptor B_{max} has yet to be determined.

The elevated plasma levels of norepinephrine that remain up to at least 24 hours following resuscitation may play a significant role in the development of some of the physiological derangements which occur following hemorrhage. Norepinephrine is well known to have profound cardiovascular effects resulting in heightened vasoconstriction to the visceral organs (95). It has previously been shown that there is depressed hepatic blood flow following hemorrhage and resuscitation (132). Following low flow conditions, a deficit in energy substrate delivery to the liver is proposed to be an initiating factor in the development of hepatic dysfunction leading to multiple organ failure (129). Elevations in plasma levels of norepinephrine during hemorrhage and following resuscitation may be responsible for most of the depression in hepatic blood flow and for the associated hepatic dysfunction. Therefore, I have examined several effects of chronic infusion of norepinephrine resulting in plasma levels similar to those observed

following resuscitated hemorrhagic shock.

I have observed that following chronic infusion of norepinephrine the cardiac output is significantly depressed with no changes in heart rate. Since the cardiac output is equal to the heart rate multiplied by the stroke volume, the depression in cardiac output was due to a decreased stroke volume. Three principle components influence the stroke volume, including: preload (end-diastolic volume or passive load), which establishes the initial muscle length of the cardiac fibers prior to contraction; afterload (loads that the myocardial fibers encounter during systole); and the inotropic state of the heart (capacity for myocardial shortening). One or more of these parameters may be altered following chronic exposure to high circulating levels of norepinephrine. An increase in the systemic hematocrit, observed due to norepinephrine, would be expected to increase the amount of afterload by making the blood more viscous. Also, the increase in TPR that was observed in norepinephrine infused animals would be expected to increase afterload. However, mean arterial pressure, which is the parameter that I measured which most directly approximates afterload, was decreased due to infusion of norepinephrine. The mean arterial pressure is a function of TPR and cardiac output. Although an increase in TPR was observed, it was not adequate to maintain the MAP due to an overwhelming depression in cardiac output. Although one would expect an

increase in the inotropic state of the myocytes due to norepinephrine (15,127), it appears that this inotropic effect was not evident at the time of measurement. Preload (end diastolic volume) appears to have been reduced by norepinephrine.

The increase in systemic hematocrit was likely due to a loss of water from the blood. This is supported by the observation that the norepinephrine infused animals had lost about 8% (−20g) of their body weight during the infusion, which is approximately 1g of body weight per hour. This loss of mass can't be accounted for by metabolism alone. It is unlikely that the increased hematocrit was due to an increase in the number of circulating blood cells, as increased blood volume would tend to raise the mean arterial pressure and cardiac output, both of which are depressed following chronic norepinephrine infusion.

Following chronic infusion of norepinephrine, there are derangements in hepatic function and a significant amount of hepatocellular damage. This is evident by the depressions in the V_{max} and K_m of active hepatocellular clearance of ICG and the presence of elevated levels of GOT/GPT in the plasma, respectively, at 24 hours after norepinephrine infusion. It has been shown that both the V_{max} and K_m of ICG clearance are depressed during hemorrhage and at 4 to 8 hours following resuscitation, even though these values are normalized early following hemorrhage (130). The presence

of GOT and GPT in the plasma is a manifestation of deteriorations in the integrity of the hepatocellular plasma membrane. Elevated levels of GOT and GPT have been observed following hemorrhage and resuscitation (139). The elevation in plasma norepinephrine, observed following resuscitated hemorrhagic shock, may play an important role in the development of the derangements in active hepatocellular function and the occurrence of hepatocellular damage.

The present study shows that following chronic infusion of norepinephrine, there is a significant increase in the circulating levels of IL-6 while there is no increase in the circulating levels of TNF. TNF levels in the plasma have been shown to be transiently increased early following hemorrhage (9). Also, α-adrenergic receptor stimulation augments macrophage production of TNF (117). It is possible that there was a transient increase in circulating levels of TNF early during norepinephrine infusion. The elevation in plasma IL-6 levels following norepinephrine infusion correlates with the observation that IL-6 is elevated during hemorrhage and following tissue trauma, both of which are conditions in which one would expect elevated catecholamine levels (9). IL-6 plays a role in the development of the immunosuppression observed following resuscitated hemorrhagic shock; and this study shows that an elevation in plasma levels of norepinephrine, similar to that which is observed following resuscitated hemorrhagic shock, causes

plasma levels of IL-6 to be elevated.

The use of α-adrenergic blockers along with a vasopressor which has pronounced effects on the heart (inotropic and chronotropic) but does not cause peripheral vasoconstriction via α-adrenergic receptors, may prove to be a beneficial adjuvant to fluid resuscitation in patients who have suffered hemorrhagic shock. Several investigators have demonstrated protective effects of α-adrenergic blockade following low blood flow conditions (3,39,86).

SUMMARY OF RESULTS AND CONCLUSIONS

There are differential alterations in the circulating levels of norepinephrine and epinephrine during trauma-hemorrhage and following crystalloid resuscitation. During hemorrhage, plasma norepinephrine levels increased by ~10-fold, while there is a dramatic increase in circulating epinephrine, ~100-fold. As early as 8 hours following crystalloid resuscitation, epinephrine levels are normalized, while norepinephrine remains elevated up to at least 24 hours. While crystalloid resuscitation alone can normalize the plasma levels of epinephrine, it is not adequate to normalize the elevated levels of norepinephrine following hemorrhage.

Both hepatocyte and whole liver plasma membrane preparations exhibited a decrease in B_{max} during hemorrhage, although there are no changes in β-adrenergic receptor K_d. The depression in B_{max} persisted up to at least 1.5 hours after crystalloid resuscitation. The desensitization of hepatocyte β-adrenergic receptors during hemorrhage and following resuscitation is due to a decrease in the number of β-adrenergic receptors available to bind ligand on the plasma membranes. Elevations in plasma catecholamines may contribute to the depression in hepatocellular β-adrenergic receptor B_{max} without significantly affecting K_d.

Kupffer cell β-adrenergic receptors exhibit a different trend following hemorrhage and crystalloid resuscitation.

There is an increase in the β-adrenergic receptor B_{max} while the K_d remains unchanged on Kupffer cells following resuscitated hemorrhagic shock. Therefore, the sensitivity of Kupffer cells to β-adrenergic agonists is increased following hemorrhage and resuscitation. It is unlikely that this is due to the direct effect of catecholamines; the mediators and mechanisms remain unknown.

With this study, we now know the differential alterations in plasma levels of catecholamines and hepatic β-adrenergic receptors that occur in this fixed pressure model of trauma-hemorrhage in the rat. Other data collected in this model can now be interpreted with the understanding of such alterations.

Chronic infusion (24 hours) of norepinephrine, resulting in plasma levels similar to those observed up to 24 hours following resuscitated hemorrhagic shock, caused various physiologic derangements similar to those that are observed following hemorrhage. Chronic infusion of norepinephrine caused a significant depression in cardiac output, which was due to a depression in stroke volume as there was no difference in heart rate. Mean arterial pressure was depressed in spite of an overall increase in the total peripheral resistance as seen in the decreased microvascular blood flow to several visceral organs (liver, spleen, small intestine). This depression in mean arterial pressure was therefore due to a decreased stroke volume.

Norepinephrine infusion caused a significant amount of weight loss (~8% of total body weight). Some of this weight loss is most likely due to water loss, as systemic hematocrit was significantly elevated. At 24 hours following the initiation of norepinephrine infusion there were significant alterations in the hepatocellular plasma membrane integrity, as indicated by elevated circulating levels of intrahepatocellular enzymes. In addition, there were depressions in active hepatocellular function as seen in the V_{max} and K_m of ICG clearance. At 24 hours following the initiation of norepinephrine infusion, the plasma glucose levels were elevated. While norepinephrine did not cause a significant elevation in the circulating levels of TNF, it did elevate the circulating levels of IL-6 by approximately 200-fold.

Norepinephrine may play an important role in the development of many of the physiological derangements that occur following trauma-hemorrhage and crystalloid resuscitation. These derangements may lead to hepatic failure and subsequent multiple organ failure following resuscitated hemorrhagic shock.

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