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## Mechanisms Mediating Sympathoadrenal Activation During Endotoxycosis in the Rat

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MECHANISMS MEDIATING SYMPATHOADRENAL ACTIVATION  
DURING ENDOTOXICOSIS IN THE RAT

by

ZHENGZHENG ZHOU

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University  
of Chicago in Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

May

1992

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## VITA

Zhengzheng Zhou, daughter of Qiaoxian Chang and Jingzhang Zhou, was born on November 13, 1960 in Jilin, Jilin province, People's Republic of China (P.R.C.). She attended elementary and secondary schools in Jilin, Jilin Province and graduated from The Fourth High School in Xuzhou, Jiangsu Province in June, 1978.

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1. Zhou, Z.Z. and S.B. Jones. The vascular response to adrenergic stimulation in pithed rats following endotoxin in the rat. *Circ Shock* 32(1):55-66, 1990.
2. Zhou Z.Z., R.D. Wurster, M. Qi and S.B. Jones. Sympathoadrenal activation in sinoaortic denervated rats following endotoxin. *Am J Physiol.* 260: R739-R746, 1991.
3. Zhou Z.Z., R.D. Wurster and S.B. Jones. Arterial baroreceptors are not dominant in mediating sympathoadrenal activation in conscious, endotoxic rats. *J Auton Nerv Syst*, 1992. (in press).
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5. Zhou Z.Z., R.D. Wurster and S.B. Jones. Sympathoadrenal response to central administration of endotoxin, IL-1, IL-6, and TNF in conscious rats. *J Auton Nerv Syst.* (submitted).
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## LIST OF ABBREVIATIONS

NE	Norepinephrine
EPI	Epinephrine
MBP	Mean Blood Pressure
HR	Heart Rate
RSNA	Renal Sympathetic Nerve Activity
SAD	Sinoaortic Denervation
ADR DENERV	Adrenal Denervation
ICV	Intracerebroventricular
PVN	Paraventricular Nucleus of the Hypothalamus
IL-1	Interleukin-1
IL-6	Interleukin-6
TNF	Tumor Necrosis Factor

## CHAPTER I

### INTRODUCTION

Septicemia is a leading cause of morbidity and mortality among hospitalized patients. There are approximately 400,000 cases each year in the United States, and the incidence continues to increase (1). It has been estimated that gram-negative bacteremia occurs in about 30 percent of patients with septicemia (32). Despite the use of potent antibiotics and intensive supportive care, the mortality among patients with gram-negative bacteremia remains high, varying from 20 to 60 percent (32,132,231). The common fatal cause of overwhelming gram-negative bacterial infection is septic shock, a syndrome characterized by inadequate tissue perfusion which ultimately leads to organ failure and cellular death (3,97). Pathophysiological changes involve hemodynamic, metabolic, neuroendocrine and immunologic alterations (3,252). Clinical manifestations include chills, fever, hypotension, decreased vascular resistance, glucose dyshomeostasis, lactacidemia, and the acute phase response.

Endotoxin, which is composed of lipopolysaccharide and associated proteins from the gram-negative bacterial wall, has long been considered to be the principal causative factor in the pathogenesis of gram-negative septic shock (103,167). Striking parallels exist between the effects of endotoxin in experimental animals and those observed in



patients with septic shock (104,122,168,171). Thus, endotoxin-treated animals have been extensively used as an experimental septic model. Alternatively, recent experimental findings suggest that endotoxin elicits most (if not all) of its pathophysiological effects not directly but via stimulating different cell populations to release mediators. These mediators, such as prostaglandins (230), leukotrienes (230), platelet activating factor (45) and cytokines (78,79,171), have been linked to the pathogenesis of endotoxic shock.

Among many pathophysiological changes which occur during endotoxic shock is markedly increased sympathoadrenal activity which has been viewed as a compensatory response of the body to septic challenge (51). Evidence for this activation includes an elevation of plasma catecholamines in septic patients and animal models (17,94,113,115), a depletion of tissue catecholamine content in the terminal state (187), and an enhanced norepinephrine turnover in certain tissues during endotoxic shock (188). These findings have been interpreted as an increased release of transmitters from sympathetic nerves and the adrenal medulla. In addition, direct nerve recording has shown augmented sympathetic nerve discharges in selective organs of endotoxic animals (92,185), although some conflicting results have been reported (127,128). The sympathoadrenal activation has been shown to have both detrimental and beneficial effects during the development of septic shock (148). In the early stages, plasma catecholamines may support the cardiovascular and metabolic adjustments to septic insult. Overwhelming and sustained sympathetic activation, however, may contribute to the irreversibility of septic shock by restricting nutritional organ blood flow. Even in light of these detrimental effects, adrenergic agents have been widely used in patients with septic shock as a treatment to

support the cardiovascular system (33,50,141). Although concerns were raised regarding local ischemia of vital organs, particularly the kidney as a result of increased vascular resistance (33), several recent studies report that norepinephrine therapy improves the blood pressure and urine output (43,62,197). The dual-sided effects of sympathoadrenal stimulation in septic shock imply that a better understanding of the mechanisms of the sympathoadrenal activation may allow prompt manipulations of the system and facilitate the management of sepsis.

The mechanisms responsible for mediating the sympathoadrenal activation during sepsis and septic shock are not clear and remain basically unexplored. The critical role of centrally mediated mechanisms and neural dependence in the sympathetic activation during developing endotoxic shock has been demonstrated in previous studies (71,149,176,253). Recent studies using more sensitive catecholamine assay, however, suggest the occurrence of peripheral modulations of catecholamine secretion from the adrenal medulla during endotoxic shock (112,211). Evidence of non-neurogenic stimulation of adrenal epinephrine secretion was provided by experiments showing that ganglionic blockade could not prevent catecholamine release from the adrenal medulla in septic animals (211). *In vitro* incubation of adrenal chromaffin cells with endotoxin or other endotoxin-elaborated agents enhanced catecholamine secretion (177,225).

Another consideration emphasizes that the adrenergic discharge may be primarily stimulated by endotoxin-induced systemic hypotension and associated baroreceptor reflex deactivation (93,148). However, certain experimental findings challenge this concept and suggest that the sympathoadrenal system can still be markedly activated by endotoxin or

gram-negative bacteria in the absence of significant hypotension (89,115) and remain activated after restoration of blood pressure (185).

It is now clear that cytokines, a family of protein molecules produced by macrophages and other cells, are the key mediators in the pathogenesis of sepsis and septic shock. Currently, growing evidence also suggests that cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor may mediate interactions between the immune and neuroendocrine systems. On one hand, cytokines are produced mostly by cells of the immune system and exert many actions during immune responses. On the other hand, cytokines have a broad spectrum of actions including influences on neuroendocrine functions. Among the cytokine actions, some involve the central nervous system and may be initiated in the brain side of the blood brain barrier (107,118,194). Brain tissues have receptors for and can respond directly to certain cytokines (87,107,117,194,250). Furthermore, interleukin-1 immunoreactive innervation of brain areas was recently reported (34,136). *In vivo* production of interleukin-1, tumor necrosis factor and interleukin-6 in the brain has been demonstrated in infectious and various other pathological states (54,82,95,135). *In vitro*, brain cells have also been shown to produce interleukin-1, tumor necrosis factor and interleukin-6 in response to endotoxin or virus challenge (138,200). Thus, it is logical to propose that cytokines might be plausible factors in mediating the sympathoadrenal activation with endotoxin via their central actions.

Considering the clinical importance of gram-negative bacteremia and septic shock, the involvement of the sympathoadrenal system in septic pathogenesis, the reappraisal of

norepinephrine therapy in septic patients, and unknown mechanisms for the sympathetic activation during sepsis, this dissertation was designed to explore some potential mechanisms involved in mediating the sympathetic activation, using endotoxin-treated rats as an experimental septic model. The specific aims of the following studies include: 1) the examination of the overall contributions of central versus peripheral mechanisms to the sympathoadrenal response during endotoxemia, 2) the evaluation of the role of afferent neural inputs from arterial baroreceptors in mediating the sympathoadrenal activation in endotoxic animals, and 3) the assessment of the effects of centrally-administered cytokines, interleukin-1, interleukin-6 and tumor necrosis factor, on central sympathetic outflow.

## CHAPTER II

### REVIEW OF RELATED LITERATURE

#### A. SYMPATHOADRENAL ACTIVATION DURING DEVELOPING SEPTIC SHOCK

##### I. Evidence for Sympathoadrenal Activation during Septic Shock

Plasma catecholamines are shown to be elevated in human patients with septic shock or serious infection in early stages (17,19,89). In most non-surviving patients, high plasma norepinephrine and, to a lesser extent, epinephrine concentrations were sustained but fell rapidly just before death (19). This fall is presumably due to either the depletion of catecholamines from the adrenal medulla and sympathetic nerve endings or the overall failure of the central nervous system. Plasma catecholamines have also been measured in a variety of septic animal models (endotoxic, bacteremic, cecal-ligation and puncture (CLP)) (112,113,115) and different species (dog, rabbit, cat, and rat) (94,99,113,218). Although the patterns and magnitude of the catecholamine response during septic/endotoxic shock varied in these different models, the increases in plasma concentrations were frequently demonstrated. For example, endotoxin administration in rats resulted in a rapid (within 30 min) and dose-dependent elevation of plasma catecholamines, in which norepinephrine elevation was sustained while epinephrine

elevation declined with time (114). In contrast, the rat model of CLP had a prolonged time course for the increment of plasma norepinephrine which developed hours after CLP and remained elevated for 40 hours with only modest increase in epinephrine (126).

Depletion of tissue catecholamine content has been observed in the terminal state of septic shock. Hanquet et al. (96) measured the catecholamine content of the adrenal medulla removed from patients who had died of shock and reported a marked depletion of catecholamine stores. In different animal species, endotoxic shock was also associated with the depletion of adrenergic neurotransmitters in tissues such as heart, spleen and adrenal medulla (98,187,254). Pohorecky et al.(195) indicated that such tissue depletion was dose- and time-dependent following endotoxin. The depletion is speculated to be due to a sustained release combined with decreased synthesis possibly caused by hypoxia and acidosis.

Studies using radio-labeled norepinephrine and subsequent incorporation into the existing transmitter pools showed that endotoxin induced a time-dependent decay in specific activity of heart and spleen (188). This result indicates an increased norepinephrine turnover and, hence, increased sympathetic activity during endotoxicosis.

Direct recording of sympathetic nerve discharge, in most cases, also revealed an enhanced sympathetic nerve activity. In studies with anesthetized dogs and cats, Halinen (92,93) demonstrated that endotoxin induced increased discharge of cardiac sympathetic efferent nerves 15 min following intravenous administration. Cervical sympathetic nerve activity was also found markedly elevated in anesthetized rats following endotoxin (159). Using conscious, unstrained rats, Palsson et al. (185) showed that intravenous injection

of either endotoxin or bacteria significantly enhanced renal sympathetic nerve activity. In contrast to the above findings, Koyoma et al. (127,128) reported that preganglionic splanchnic nerve activity and renal sympathetic nerve activity were decreased after endotoxin injection in anesthetized cats and rabbits, respectively. It was concluded in their studies that endotoxin treatment inhibited the central sympathetic outflow.

Collectively, a large body of literature can be found regarding the sympathoadrenal activity during the development of septic shock and the demonstrations of its activation in septic processes have been convincing and generally accepted. More thorough review of this topic can be found elsewhere (186).

## II. Effects of Sympathoadrenal Activation during Septic Shock

The sympathoadrenal system appears to be critically important, if not essential, in maintaining life in the early stage of endotoxic shock in animals. Endotoxin treatment in rats with adrenal demedullation or/and chemical sympathectomy are usually associated with rapid onset of death and markedly increased mortality (131,156). Those animals often have a very rapid and more profound fall in blood pressure and attenuated tachycardia. This result suggests that both sympathetic and adrenal catecholamines contribute importantly to the maintenance of arterial blood pressure and cardiac output during endotoxemia. Catecholamines are known to increase ventricular contractility, which improves cardiac output, and mobilize liver glycogen to support increased tissue metabolism during endotoxin shock (77,125).

On the other hand, the detrimental effects of the sympathoadrenal activation during

the development of endotoxic shock have also been suggested. Blunting of the excessive sympathetic activation has been shown to protect against the shock pathogenesis (31,88). For example, Lillehei and Maclean (140) pretreated dogs with alpha adrenergic antagonist to block the vasoconstrictor effects of catecholamines released during endotoxemia. They found that this pretreatment significantly improved the survival rate (84% lethality with endotoxin alone, 0% lethality with endotoxin and alpha blockade, measured 72 hours after endotoxin) and alleviated the pathological changes. Filkins also reported that alpha adrenergic blockade (phentolamine) protected rats against endotoxin shock lethality and blunted the development of endotoxic hypoglycemia. In isolated hepatocytes, alpha blockade protected against endotoxin-induced depression of hepatic gluconeogenesis (76).

Exogenous catecholamines have been widely used in patients with septic shock to support the failing cardiovascular system (33,50,139). Dopamine is particularly indicated for treatment of patients with oliguria and decreased peripheral resistance. The use of norepinephrine in septic patients is controversial due to the concerns regarding organ ischemia as a consequence of diffusive vasoconstriction (33). However, interest in the therapeutic use of norepinephrine has recently been reported (43,62,197). Particularly in septic patients with high cardiac output and low peripheral resistance, norepinephrine treatment improved blood pressure and renal function without deleterious effects on cardiac index, oxygen delivery and oxygen consumption (197).

A generally accepted view (148) is that in early phases of septic shock, the sympathoadrenal activation may be beneficial by virtue of vasoconstriction in non-vital



organs which favors the perfusion of more vital organs. Excessive sympathetic activation may be detrimental in the long run by restricting nutritional blood flow which ultimately leads to severe metabolic and circulatory failure. Unfortunately, we still are unable to determine with adequate precision when an increased sympathetic tone is no longer beneficial during the developing septic shock.

## B. REGULATION OF THE SYMPATHETIC NERVOUS SYSTEM

### I. Spinal Cord Control

The traditional concept that the spinal cord serves solely as a relay station for transmitting information from the medulla to postganglionic neurons innervating the cardiovascular system is no longer tenable (14). Several observations indicate that information processing in spinal neural circuits can be an important determinant of the level of sympathetic control of the cardiovascular system. In animals with maximal reduction of blood pressure and sympathetic nerve discharge by medullary transection at the level of the obex, secondary section of cervical cord induced increases in sympathetic nerve discharge (7). This implies that the final output from the central nervous system to the circulation can be modulated by neurons in the spinal locus. Activation of somatic and visceral afferent fibers produces reflex responses in sympathetic nerves which do not require the integrity of bulbospinal pathways (207). Furthermore, blood pressure can gradually return towards control levels and prominent cardiovascular reflexes can be elicited in chronic spinal animals and man (14). Local interneurons have been shown to

exist in the intermediate gray matter of the spinal cord which innervate the sympathetic preganglionic neurons at all levels of the sympathetic outflow (14). The presence of local interneurons implies that there are additional descending and intrinsic spinal pathways that indirectly control the sympathetic outflow, but relatively little is known about these pathways.

## II. Supraspinal Control

Several interconnected central networks regulate the sympathetic outflow. The incoming afferent fibers carrying visceral sensation from all the major organs of the body including the cardiovascular system primarily terminate in the nucleus tractus solitarius (NTS), the most important visceral sensory relay cell group in the brain stem. From here, information is sent via interneurons either to a number of key nuclei in the brain stem which project directly to autonomic preganglionic neurons of the sympathetic and the vagal systems or to the forebrain nuclei in the central autonomic network that are capable of regulating both autonomic and neuroendocrine functions (143). Reciprocal pathways between the NTS, the brain stem nuclei (ventrolateral medulla, the A5 cell group, the parabrachial nucleus) and forebrain areas (the paraventricular hypothalamic nucleus, the bed nucleus of the stria terminalis, and the central nucleus of the amygdala) are the anatomical substrates for sympathetic regulation (143). Direct projections to the intermediolateral cell column, where the sympathetic preganglionic neurons are located, have been demonstrated to originate from seven areas of the brain (147,223): 1. the rostral ventrolateral medulla, 2. the caudal raphe nuclei (raphe pallidus and raphe

obscurus), 3. the A5 noradrenergic cell group, 4. the Kolliker-Fuse nucleus, 5. the paraventricular hypothalamic nucleus, 6. the lateral hypothalamic areas, and 7. the central gray matter.

### III. Central vs. Peripheral Involvement in Sympathoadrenal Activation during Endotoxic Shock

Lutherer et al. (149) showed that acute lesion of the cerebellar fastigial nuclei in anesthetized dogs prevented the recovery and maintenance of blood pressure following induction of hypotension either by hemorrhage or by administration of endotoxin. This implies that the normal sympathetic response to endotoxin challenge requires intact central regulatory mechanisms.

Intact spinal cord also seems critical to endotoxin-evoked sympathetic response. Spinal transection at the C-7 level in dogs abolished the catecholamine release in response to endotoxin, suggesting that endotoxin-elicited sympathoadrenal response was presumably dependent on descending spinal pathways (71).

Additionally, endotoxin-induced elevation of plasma epinephrine was eliminated by section of splanchnic nerves in the dog (176). Fine and associates (253) also demonstrated that acute denervation of hemi-spleens protected against the norepinephrine depletion whereas the intact spleen was depleted of norepinephrine, typical of the end stage of endotoxic shock. This result suggests that increased catecholamine release during septic shock depends on nerve activation.

Recent studies, using more sensitive catecholamine assay, show that peripheral

modulation of catecholamine secretion from the adrenal medulla might be present during endotoxin shock. In pithed rats with no centrally-mediated reflexes, endotoxin did not elevate plasma norepinephrine but epinephrine was still augmented (112), although this elevation was greatly attenuated compared to central nervous system (CNS) intact animals. These results indicate that norepinephrine release depends on intact CNS while epinephrine release may involve both CNS and peripheral (either neurogenic or non-neurogenic) mechanisms. Evidence of the non-neurogenic stimulation of adrenal epinephrine secretion was provided by experiments in newborn rats showing that ganglionic blockade could not prevent catecholamine release from the adrenal medulla (211). Furthermore, *in vitro* incubation of adrenal chromaffin cells with endotoxin or other endotoxin-elaborated agents enhanced catecholamine secretion (177,225).

Collectively, only a few studies have been conducted to evaluate central versus peripheral involvements in mediating sympathetic activation during sepsis or septic shock. The results were not consistent between studies, although it seems that the major sympathoadrenal activation during sepsis is dependent on the intact CNS and peripheral adrenal modulation may also be present. Further studies are needed to clarify whether both central and peripheral mechanisms are involved in this event during septic states. Thus, one of the present dissertation projects focuses on examining the central dependence of the sympathoadrenal activation during septic shock as well as the contribution of peripheral modulation to such activation.

## C. INVOLVEMENT OF ARTERIAL BARORECEPTORS IN MEDIATING SYMPATHOADRENAL ACTIVATION DURING SEPTIC SHOCK

### I. Baroreceptor Reflex Control of the Circulation

Reflex regulation of the cardiovascular system is one of the important mechanisms in maintaining the homeostasis of the internal environment. Two main types of receptors, mechanoreceptors and chemoreceptors, exist in the cardiovascular system and are involved in the major reflex control of the circulation. Arterial baroreceptors are the mechanoreceptors located in the aortic arch and the carotid sinuses, which monitor arterial blood pressure. Afferent sensory fibers from baroreceptors project to the nucleus tractus solitarius in the medulla. From there, information is sent to the brain stem nuclei which may project directly to the ambiguous nucleus of vagal nerve and the sympathetic preganglionic neurons in the intermediolateral column of the spinal cord (220). Studies have demonstrated that baroreceptor reflexes can be modified by peripheral as well as central mechanisms (47).

### II. Afferent Neural Input from the Arterial Baroreceptors in Mediating Sympathoadrenal Activation during Developing Septic Shock

Since hypotension typically occurs along with increases in heart rate, sympathetic nerve discharge and plasma catecholamines during endotoxic shock (93,113,185), it is suggested that endotoxin induced-hypotension may be the primary cause of the sympathoadrenal activation by unloading baroreceptors and associated deactivation of

baroreflexes. In anesthetized dogs and cats, Halinen et al. (92,93) demonstrated that endotoxin administration induced a rapid drop of blood pressure which was associated with cessation of the aortic arch baroreceptor afferent impulses and increased cardiac sympathetic efferent discharges during their 15 min protocol. The author concluded that the sympathetic pathways were primarily activated through cardiovascular receptor reflexes to maintain blood pressure. Baroreceptor reflex participation in mediating sympathetic activation during endotoxic shock has also been suggested by several other investigators (159,185).

However, certain experimental findings in the literature challenge the concept that baroreceptor reflexes are the dominant factor in mediating the sympathoadrenal activation following endotoxicosis. In rats treated with *E. coli* bacteria, plasma catecholamines were markedly increased while blood pressure was not significantly altered (115). In experiments involving bolus administration of endotoxin, plasma catecholamines were also markedly enhanced well before hypotension occurred (114). Complete blockade of early endotoxin-induced hypotension with platelet activating factor antagonist did not prevent catecholamine elevations following endotoxin (196). Direct nerve recording in endotoxin and *E. coli* bacteria treated-animals indicated that sympathetic nerve discharge markedly increased with transient decrease in mean blood pressure and remained elevated when blood pressure returned to the control levels (185). Mills (159) studied the sympathetic response to endotoxicosis in pre-weanling rats before maturation of baroreflex and adult rats with matured baroreflex. He reported that sympathetic nerve discharge was augmented prior to the onset of hypotension in both groups of rats and

additional increases in nerve activity occurred following the fall in blood pressure in adults rats but not in pre-weanling rats. The increased activity in both groups persisted after blood pressure was restored to basal levels with volume infusion. The author concluded that although there was baroreflex participation in the sympathetic activation during the hypotensive phase following endotoxicosis, the initiation and continuous activation were mediated by non-baroreflexogenic mechanisms.

Sympathetic activation independent of hypotension was also observed in septic patients. Groves et al. (89) reported significantly elevated plasma catecholamines in patients with serious postoperative infection. Although some patients in their studies had low blood pressures, most were normotensive and none were hypovolemic when plasma catecholamines were elevated. They suggest that factors other than the baroreceptor reflexes are important causes of increased plasma catecholamines in septic patients. Failure to demonstrate a direct relationship between the degree of hypotension and the sympathetic activation has been reported elsewhere (96).

### III. Resetting of Baroreceptor Reflexes during Endotoxin Shock

The function of the carotid sinus baroreceptors during endotoxemia was investigated by Trank and Visscher (238) in pentobarbital anesthetized dogs with cut sinus nerves. Endotoxin was shown to produce a left shift of baroreceptor discharge frequency versus intrasinus pressure curve. This resetting resulted in post-endotoxin baroreceptor discharge frequencies always being higher for a given pressure stimulus compared to control levels. The authors speculated that the effect of endotoxin on the carotid sinus baroreceptor

activity was not a direct receptor stimulation but an indirect effect produced through a chemical mediator released during endotoxemia. This mediator may sensitize the receptors or modify the physical properties of the muscular or elastic components of the carotid sinus wall, but the nature of the mediator was not known.

Recent studies have shown that baroreceptor reflexes can be modulated by certain circulating factors. Prostanoids, substance P, atrial natriuretic factor, and vasopressin have all been demonstrated to augment the baroreflex control of the circulation (35,44,74,249). In other words, at a given carotid sinus pressure those mediators either decrease the afferent nerve activity of baroreceptors (peripheral resetting) or increase the central efferent sympathetic outflow (central resetting). In contrast, PGI<sub>2</sub>, opiates, endothelial factor, and neurotensin have been linked to the suppression of baroreceptor reflexes (48,49,228,256). This suppression was also achieved either by peripheral baroreceptor resetting (i.e. increased baroreceptor afferent activity) or by central resetting of the coupling between the baroreceptor afferent and nerve efferent activities (i.e. decreased central efferent sympathetic activity). These findings may have implications in the sympathetic response during septic shock, such that the augmentation of baroreceptor reflexes may be involved in reducing sympathetic activity whereas suppression of baroreceptor reflex may contribute to enhanced sympathetic outflow.

As can be summarized from the evidence reviewed in the above two sections (II and III), hypotension does not necessarily initiate or maintain the increased sympathoadrenal activity which occurs with septic insult despite baroreflex participation for such activation during hypotensive state. Modulation of baroreflexes by certain mediators may also



influence the sympathetic activation during sepsis, but the nature of such mediator is unknown. Regardless of the causes of baroreflex deactivation, the role of baroreceptors in mediating sympathetic activation during sepsis/endotoxemia has never been directly evaluated. This will be another focus of the present dissertation studies.

#### D. POTENTIAL INVOLVEMENT OF CYTOKINES IN MEDIATING SYMPATHOADRENAL ACTIVATION DURING SEPTIC SHOCK

Cytokines are a family of closely related proteins produced primarily by monocytes/macrophages and lymphocytes in response to microbial infection, injury, physiological stress, or antigenic challenge (9). Although not released by a specialized gland, cytokines, like hormones, can act in autocrine, paracrine, and/or endocrine fashions to stimulate their producing cells, local vicinity tissues, or distant sites (65,198). Cytokines have a wide range of biological effects on target cells and are responsible for mediating a variety of processes in host defense, inflammation, and responses to injury. Extensive studies have demonstrated that cytokines are the principal mediators for the pathogenesis of endotoxin/septic shock (37,79,174). Particularly, interleukin-1 (IL-1), tumor necrosis factor (TNF), and interleukin-6 (IL-6) may be grouped together on the basis of related patterns of production and several overlapping activities to mediate common effects in the development of septic shock (198). Growing evidence suggests that there are interactions between immune and neuroendocrine systems with those cytokines as likely mediators.

## I. Biochemistry of Cytokines

### 1. Interleukin-1 (IL-1)

Although activated macrophages are the major source of IL-1, this cytokine is also produced by a variety of other cells including fibroblasts, endothelial cells, T and B lymphocytes, as well as brain astrocytes and microglial cells etc. (162). IL-1 is initially synthesized as a 33,000 dalton precursor, which is subsequently processed during or after secretion to molecular weight forms in the range of 13,000-17,000 daltons (86). Two distinct genes, IL-1  $\alpha$  and IL-1  $\beta$ , have been identified and localized to the long arm of chromosome 2. The mature forms of IL-1  $\alpha$  and IL-1  $\beta$  exhibit limited amino acid homology (22-26%) and different isoelectric points (pI5 for IL-1  $\alpha$  and pI7 for IL-1  $\beta$ ) (2). In most human tissues IL-1  $\beta$  mRNA predominates over IL-1  $\alpha$  (9). Both molecules interact with the same membrane-associated receptors and their biological activities appear to be identical (68,198). The human and murine IL-1  $\beta$  or  $\alpha$  share approximately 60-70% homology (198).

### 2. Tumor Necrosis Factor (TNF)

TNF  $\alpha$ , also known as cachectin, is a single polypeptide chain of 156-158 amino acid residues produced primarily by activated monocytes/macrophages in response to a variety of agents including endotoxin, Bacille Calmette Guerin (BCG), and phorbol esters (191,233). Natural killer cells, T lymphocytes, astrocytes and some macrophages or tumor cell lines can also produce TNF (52,138,192,200,219). TNF is produced as a

prohormone which undergoes extensive cleavage to generate the mature protein. Studies have suggested that human TNF  $\alpha$  exists as a dimer or trimer which dissociates into a monomer with molecular weight of 17,000 daltons under denaturing condition (4). TNF  $\alpha$  is pH and temperature sensitive. The gene for TNF  $\alpha$  has been found on chromosome 6 in man (217), which is linked to the gene encoding lymphotoxin that is also called TNF  $\beta$ . TNF  $\alpha$  and TNF  $\beta$  have a 28% sequence homology as well as they bind to the same receptor and evoke similar biological responses (173). TNF is a highly conserved protein, as illustrated by approximately 80% homology between mouse, rabbit and human TNF (109,154).

### 3. Interleukin-6 (IL-6)

IL-6 is a glycoprotein with a molecular weight of 23,000-30,000 daltons (heterogeneity in size being a function of different glycosylation) (162). The peptide is stable at 56 °C but is rapidly inactivated at 100 °C (198). IL-6 can be produced by diverse cell types including fibroblasts, monocytes/macrophages, endothelial cells, T and B cells, mesangial cells and astrocytes upon appropriate stimulations (162). Those stimuli include endotoxin, IL-1, TNF alpha, platelet-derived growth factor and virus infection. The human IL-6 gene is located on chromosome 7 (210). About 60% homology between human and mouse has been shown at the DNA level and 42% at the protein level (123).

## II. General Biological Effects of Cytokines

IL-1, TNF, and IL-6 are the most typical examples of multifunctional cytokines.

Their functions are widely overlapping, but each shows its own characteristic properties.

IL-1 was originally discovered as an endogenous pyrogen which induced fever (198). IL-1 has a broad spectrum of target cells that share a common involvement in immune or inflammatory responses. For example, IL-1 aids in the development of specific immune responses by regulating the activation of T and B cells and by stimulating the release of growth and differentiation factors that act on T and B cells (5,63,174). IL-1 has proinflammatory effects on a variety of cells including fibroblasts, synovial cells, chondrocytes, endothelial cells, hepatocytes, and osteoclasts (63,162). IL-1 also increases the hepatic acute phase protein synthesis and mediates many other metabolic alterations (64,78). A key feature of IL-1 action is to stimulate the arachidonic acid metabolism which may be involved in many responses in different organs or systems such as cardiovascular, pulmonary, and neuroendocrine systems (63,140,198).

TNF was first identified as an endotoxin-induced factor causing hemorrhagic necrosis in certain tumors *in vivo* (42). It is now evident that both TNF  $\alpha$  and  $\beta$  possess cytolytic and cytostatic properties for certain solid tumors *in vivo* and specific tumor lines *in vitro* (9). TNF  $\alpha$  appears to play a major role in trapping neutrophils in a localized inflammatory area by increasing the expression of adhesion molecules on the surface of both endothelial cells and neutrophils (36,124). TNF  $\alpha$  could further stimulate neutrophil phagocytosis and superoxide anion production as well as be involved in driving the anticoagulant status of the endothelium to a procoagulant state (124). TNF  $\alpha$  can also induce some cells such as endothelial cells and fibroblasts to secrete a number of important immune mediators (198). These mediators, including IL-1, platelet-activating

factor, prostaglandins, interferons, colony-stimulating factor (CSF) and collagenase, may actively participate in local and systemic immune responses. TNF  $\alpha$  has been demonstrated to induce the differentiation and proliferation of B lymphocytes (110). TNF  $\alpha$  also has endogenous properties to cause fever (66) and is involved in acute phase response (24). Suppression of lipoprotein lipase production may be one of the mechanisms for cachexia associated with TNF (163).

IL-6 was initially described as T cell derived-lymphokine that induced antibody production in B cells (123). IL-6 can markedly influence the growth and differentiation of T and B lymphocytes, especially in the presence of other stimuli such as IL-1 (5). IL-6 can also stimulate the growth of hematopoietic colonies composed of granulocytes and macrophage and modify the effects of other hematopoietic factors (123). Nerve-growth factor-like activity of IL-6 has been suggested. IL-6 can act as a pyrogen (100) and is involved in mediating the acute phase responses by liver cells (83).

A cytokine network exists, as indicated by the induction of these cytokines (5). For example, TNF induces IL-1 expression and vice versa. IL-1 and TNF both induce IL-6. Unlike IL-1 and TNF, IL-6 does not induce IL-1 or TNF. IL-6 suppresses endotoxin- or TNF-induced IL-1 production as well as endotoxin-induced TNF production. Moreover, IL-1, IL-6 and TNF are each capable of inducing their own production. Thus, this network implies complex functional links between those cytokines.

### III. Involvement of Cytokines in the Pathogenesis of Septic/Endotoxin Shock

The involvement of IL-1, TNF and IL-6 as key mediators in the pathogenesis of

septic/endotoxic shock are mainly demonstrated by three separate lines of evidence. First, a marked elevation of production and secretion of each of the cytokines have been demonstrated during the development of septic/endotoxin shock. Second, administration of exogenous cytokines can duplicate most of the manifestations and pathophysiological changes associated with septic/endotoxic shock. Third, treatment of the septic animals with specific antibodies against those cytokines shows protective effects and decreases lethality.

### 1. Circulating Cytokines Levels in Septic Patients and Animal Models

Several investigators have demonstrated that in septic patients, there are elevations of plasma TNF levels, either alone or along with increased IL-1 or IL-6 (41,57,60,178,245). High level of TNF or IL-6 has been associated with severity and high mortality in some studies (178,245). Michie et al. (158) reported that endotoxin administration (4 ng/kg) to healthy human volunteers induced a 7 fold single peak elevation of plasma TNF levels within 90 -180 min while IL-1 level did not change. However, detectable elevations of IL-1 at 120 min and IL-6 at 2 to 4 hr following endotoxin in human volunteers have been demonstrated in other studies (80,101).

Increases in circulating TNF, IL-1, and IL-6 have been substantiated in septic models with endotoxin or bacterial injections in different animal species (73,81,121,155,244). Mathison et al. (155) reported that elevation of plasma TNF was detected within 30 min and peaked between 45 to 120 min after endotoxin challenge in rabbits. In studies with baboons, Fong et al. (81) demonstrated that circulating TNF was

increased with peak elevation at 1.5 hr after *E. coli* injection. IL-1 was detectable by 2 hr and peaked 3 hr after bacterial infusion. IL-6 was detectable within 3 hr and continued to rise throughout 8 hrs of the protocol.

## 2. Duplication of Septic Syndromes by Cytokine Administration

Extensive studies have shown that TNF administration in different animal models precipitates a syndrome strikingly similar to that of human septic shock (18,53,234,236). Tracey et al. (234) reported that recombinant human TNF infusion induced dose-related endotoxin-like syndrome, tissue injury, and death. Endotoxic shock-like syndrome was also observed in TNF-treated rats, including hypotension, tachycardia, hyperglycemia, lactacidemia, hemoconcentration. Pathological analysis revealed organ-specific changes indistinguishable from those seen during human septic shock such as adrenal necrosis, pulmonary inflammation and hemorrhage. Cardiovascular collapse along with tissue necrosis and elevation of stress hormones was also observed in canine models after TNF infusion (236). The modulation effects of TNF on endotoxin-induced hemodynamic, metabolic and endocrine responses have been demonstrated (53).

Relatively few studies have examined the pathophysiological alterations associated with IL-1 administration. Okusawa et al. (179) reported that intravenous treatment of IL-1  $\beta$  (5 mg/kg) to rabbits induced hypotension, decreased vascular resistance and decreased central venous pressure. In studies with mice, Beutler et al. (40) demonstrated that IL-1  $\alpha$  and  $\beta$  induced dose-dependent lethality and pathological changes similar to septic shock. Using nontoxic doses of IL-1  $\beta$  and TNF in combination in rabbits, a

profound shock-like state was induced with evidence of pulmonary edema and hemorrhage (179). It is suggested that the combination of IL-1 and TNF together is much more potent than either agent alone.

### 3. Protective Effects of Cytokine Antibodies

Using polyclonal rabbit antiserum and the derived IgG against TNF to passively immunize mice, Beutler et al. (25) reported that endotoxin induced lethal effects were significantly attenuated in a dose-dependent manner. The protective effects were most effective when antiserum was given prior to endotoxin injection. Tracy et al. (235) reported that pretreatment of baboons with monoclonal anti-TNF antibody fragments (Fab2) 2 hr before LD 100 of *E. coli* bacteria infusion provided complete protection against shock, vital organ dysfunction, and persistent stress hormone release. Anti-TNF antibodies also attenuated the *E. coli* bacteria-induced elevation of TNF, IL-1  $\beta$  and IL-6 (81), suggesting that TNF may be essential for the initiation and amplification of IL-1 and IL-6 release during septic shock.

Wallach et al. (246) demonstrated that treatment with IL-1 (0.4  $\mu$ g, ip) 12 hr before endotoxin injection (100  $\mu$ g, iv) to BCG-primed mice prevented the lethal effects of endotoxin, suggesting that IL-1 was an important mediator in endotoxin lethal effects.

In recent studies by Starnes et al. (221) the effects of *in vivo* anti-mouse IL-6 antibody treatment were evaluated in a mouse model of septic shock. Anti-IL-6 antibodies significantly attenuated the lethality caused by *E. coli* (ip) or murine recombinant TNF  $\alpha$ , suggesting that IL-6 is an important mediator in lethal *E. coli*



infection. Anti-IL-6 also led to an increase in *E. coli* induced elevation of serum TNF levels, implicating that IL-6 was a negative modulator of TNF *in vivo*.

Clearly, extensive studies have demonstrated that TNF, IL-1 and IL-6 are the principle mediators in the pathogenesis of sepsis and septic shock. More exhaustive review of this topic can be found in a recent monograph (79).

#### IV. Centrally-Mediated Effects of Cytokines

##### 1. Fever

Fever is well known to be a centrally-regulated response. The temperature regulation center is primarily located in the anterior hypothalamus (10). When endotoxin, the exogenous pyrogen was administered intravenously (i.v.) to rabbits, it caused monophasic fever at small doses but biphasic fever at larger doses (164). It is now recognized that the exogenous pyrogens exert their pyrogenic effects by releasing several endogenous pyrogens such as TNF  $\alpha$ , IL-1, and IL-6. These systemically released cytokines are presumably transported to the brain by the blood and initiate fever by increasing prostaglandin PGE<sub>2</sub> synthesis in the hypothalamus. It is not known how the cytokine signals in the circulation get into the hypothalamus (26,222).

Intravenous injection of human recombinant IL-1 into rabbits resulted in a monophasic fever which peaked approximately 50 min after injection (66). This fever induction was more rapid than endotoxin-induced fever which peaked approximately 90 min post administration. TNF  $\alpha$  is also pyrogenic and has dose-dependent fever-inducing

properties. Small i.v. dose of TNF induced a brisk, monophasic fever which peaked coincidentally with IL-1 induced fever (66). High doses of TNF  $\alpha$  resulted in a biphasic fever profile. The initial peak, which was correspondent to the monophasic peak observed with small dose of TNF, was followed by a second peak at 3.5 hr post injection. Pretreatment with a cyclooxygenase inhibitor could block IL-1 induced fever as well as the initial rise in temperature evoked by TNF. The second peak has been demonstrated to be mediated by TNF-induced IL-1 release (66,243). Intravenous injection of IL-6 to rabbits also produced fever response which was monophasic with peak elevation around 60 min (100).

## 2. Neuro-Endocrine System

Extensive studies have been conducted to investigate the cytokine effects on the neuro-endocrine systems, particularly the hypothalamus-pituitary-adrenal axis.

IL-1 has been reported to increase serum levels of adrenocorticotrophic hormone (ACTH) and corticosterone in different species (22,182). Such elevations are primarily mediated through central mechanisms. Intracerebroventricular (ICV) administration of IL-1-stimulated secretion of ACTH and corticosterone, whereas the same dose was not effective when injected peripherally (119,182). It has been shown that IL-1 stimulates ACTH and subsequent corticosterone secretion by triggering release of corticotropin-releasing factor (CRF) from the hypothalamus, as measured from both systemic (22) and hypothalamic-pituitary portal circulations (206). Pretreatment with CRF antibodies abolished the IL-1-induced increase in ACTH (206). Complete neural dissociation of the

medial basal hypothalamus prevented the adrenocortical activation produced by IL-1 (182). Additional effects of IL-1 at the level of the pituitary and the adrenal gland has also been proposed (16).

Similarly, TNF as well as IL-6 have also been demonstrated to stimulate the hypothalamic-pituitary-adrenal axis (53,59,72,170,213,236,247). *In vitro* incubation of the pituitary with IL-6 induced ACTH secretion (150). Intravenous administration of IL-6 into rats increased the plasma level of ACTH 30 min after the injection (170). Pretreatment with anti-CRF completely abolished the IL-6 induced increase of ACTH (170), suggesting that IL-6 stimulated the secretion of ACTH through CRF.

Recent evidence indicates that IL-1 and TNF modulate the secretion of various other hypothalamic-pituitary peptides, including prolactin, growth hormone, thyrotropin-releasing hormone, and luteinizing hormone-releasing hormone (26,199).

### 3. Sympathetic Nervous System

Cytokine-induced activation of the sympathetic nervous system has been reported by several authors. In 1987, Tracey et al. (236) investigated the stress hormone responses to recombinant human TNF in anesthetized dogs. Intra-arterial infusion of sublethal dose of TNF (10  $\mu\text{g}/\text{kg}$ ) induced no significant changes in plasma catecholamines. Lethal dose of TNF (100  $\mu\text{g}/\text{kg}$ ), however, precipitated significant increases of plasma catecholamines. Within 15 min after TNF infusion, plasma epinephrine and norepinephrine increased from a pre-injection value of 58 and 282 pg/ml to 1148 and 1400 pg/ml, respectively. By 3 hrs, plasma epinephrine and norepinephrine were elevated

to 1623 and 1982 pg/ml, respectively. Since these elevations occurred prior to the onset of diminished mean blood pressure or cardiac output, the authors proposed that circulating TNF may stimulate the sympathetic response. Sharp et al. (213) showed that ICV injection of TNF was more effective than intravenous (iv) injection, which may suggest a central site of action for TNF.

In 1988 Bagby et al.(11) demonstrated that non-lethal dose of TNF (150  $\mu$ g/kg, iv) induced about 2-3 fold increase in plasma epinephrine and norepinephrine in conscious rats, which was not associated with hypotension. Similar findings were also observed by Darling et al. (59), except that the increment of epinephrine did not reach statistical significance. In recent studies with conscious rats, Ciancio et al. (53) reported that significant elevation in plasma catecholamine were induced within 90 min after high dose of TNF injection (1 mg/kg), with peak elevation of approximately 20 fold.

In anesthetized dogs Evans et al. (72) reported that 6-hr infusion of sublethal dose of TNF increased stress hormone levels including plasma catecholamines, which were associated with hemodynamic and metabolic changes. Pretreatment with cyclooxygenase inhibitors abolished most of the hemodynamic changes and attenuated elevation of plasma catecholamines. In contrast to the plasma catecholamine response to TNF, Holt et al. (106) reported that ICV injection of TNF  $\alpha$  resulted in decreased sympathetic firing rate to interscapular adipose tissue, whereas lymphotoxin (TNF  $\beta$ ) increased the sympathetic nerve activity to that tissue. The authors proposed that both cytokines acted directly in the CNS to modulate sympathetic outflow, possibly via separate receptor systems.

Recombinant IL-1  $\alpha$  and IL-1  $\beta$  have also been evaluated for their ability to

stimulate catecholamine secretion in the rat (202). Intravenous injection of either IL-1  $\alpha$  or IL-1  $\beta$  (1-1000 ng) caused dose-related but modest increases in plasma catecholamines at 15 min post injection. Similarly, ICV administration of same doses of IL-1 induced 2-3 fold elevation of plasma catecholamines. In both cases no alteration in blood pressure occurred, suggesting that this elevation does not result from a cardiovascular reflex.

A recent study assessed norepinephrine turnover in various organs of rats after intraperitoneal injection of recombinant human IL-1  $\beta$  (6). IL-1 administration increased norepinephrine turnover in the spleen, lung and hypothalamus. Modest elevation of plasma catecholamine levels was also observed 30 min and 1 hr following intraperitoneal IL-1 injection (21).

Saigusa (204) studied the regional sympathetic response to IL-1  $\beta$  and TNF  $\alpha$  using anesthetized rabbits. He found that each of the cytokines typically induced a decrease in ear temperature, indicative of cutaneous sympathetic activation, and simultaneous inhibition of renal sympathetic nerve activity at the initial phase (30 min) which returned to control levels around 60 min post treatment.

#### 4. Others

In addition to the above-mentioned central effects of cytokines, IL-1 and TNF have also been shown to induce many other centrally-mediated host defense responses to infectious pathogens. Among those are acute-phase glycoproteinemia (214), hyperinsulinemia (55), increased counts of white blood cells, enhanced slow-wave sleep (30) and suppression of food intake (194).

## V. Passages of Cytokines To the Brain

A prerequisite for a direct CNS effects of systemic endotoxin or cytokines is that the specific molecule contacts CNS elements. Controversial results have been reported in the literature regarding the possibility of systemic endotoxin and cytokines entering the CNS and interacting with the brain parenchyma.

Prior to the understanding of cytokines, endotoxin was believed to be the causative factor for the pathogenesis of gram-negative septic shock. At that time, some evidence suggested direct actions of endotoxin on the CNS which might be responsible for some of its effects. Experiments were then conducted attempting to establish the basis of direct contact of endotoxin with CNS components. Bennett et al. (20) presented evidence that endotoxin was found in cerebrospinal fluid (CSF) of dogs within 15-30 min after intravenous injection of *Shigella* endotoxin, using the pyrogenic effects in rabbits as an assay. In contrast, most other studies reported negative results in general. When radioactive-labelled endotoxin was given systemically, no endotoxin was found in the brain parenchyma although radioactivity was detected throughout peripheral organs (109). In another experiment involving intravenous endotoxin administration, no endotoxin was detected in the CSF during the period when plasma endotoxin was at its highest concentration (240). Although in a few cases of this experiment, a low level of endotoxin was found in the CSF, it was usually correlated with high red blood cell count in the CSF, and it was considered to be due to contamination from cerebral bleeding during the CSF sampling. From these studies, the generally accepted view is that endotoxin cannot cross the blood brain barrier.

It is now recognized that host-released factors, primarily cytokines, are responsible for most of endotoxin effects, including those involved in the CNS. However, whether cytokines have direct physical contact with the brain tissues is not clear. Experiments in the past several years have focused on three major aspects: 1) whether cytokines can cross the blood brain barrier, 2) whether circulating cytokines enter the brain at the circumventricular organs where the blood brain barrier is absent, and 3) whether cytokines are produced locally in the brain and, thereby, exert their actions.

In 1987 Dinarello et al. (67) reported that intravenous injection of  $^{125}\text{I}$  labelled endogenous pyrogen (EP), prepared from monocytes, did not induce any radioactive detection in the anterior hypothalamus. In 1987 the same group (54) reported that when endotoxin or IL-1 was given intravenously, CSF IL-1 concentration did not increase at any stages of the sustained fever, suggesting that IL-1 did not cross the blood brain barrier. In contrast, when pyrogenic doses of endotoxin or IL-1  $\beta$  was administered ICV, elevation of IL-1 in the CSF was detected, indicating the ability of brain tissues to produce IL-1 *in vivo*. Bank et al. (12) reported bidirectional transports of IL-1  $\alpha$  across the blood brain barrier. In their studies  $^{125}\text{I}$ -radiolabelled IL-1  $\alpha$ , which retains full biological activity, was injected intravenously into mice along with  $^{99}\text{Te}$ -radiolabelled albumin. When the brain/serum radioactivity ratio was counted at different times post injection, the ratio for  $^{125}\text{I}$  increased significantly over time whereas the ratio for  $^{99}\text{Te}$  stayed constant, suggesting that there was a blood to brain transport of IL-1 but not albumin. Many regions of the brain contained IL-1 radioactivity. The brain to blood transport of IL-1 was also demonstrated in the same studies.

In another line of investigation, the possible role of organum vasculosum laminae terminalis (OVLT) in connection with the pathogenesis of fever and activation of hypothalamic-pituitary-adrenal axis was extensively studied. OVLT is one of the seven or eight so called "circumventricular organs" as it is adjacent to the cerebral ventricles. The OVLT is highly vascularized and lacks a blood brain barrier (111). Some big molecules such as horseradish peroxidase (HRP) (45,00 dalton) can not cross the blood brain barrier but can reach interstitial space of the OVLT (222). The ependyma of the OVLT faces toward the third ventricle and the parenchyma of the OVLT is comprised of neuronal perikarya, glial and neuronal processes. The OVLT is close to the hypothalamus and has direct projections to the hypothalamus which is known to be an important area in autonomic, endocrine and fever regulations (111).

In studies with guinea pigs, Blatteis reported that an extensive lesion of the anteroventral third ventricular wall including OVLT resulted in suppression of the febrile response evoked by systemically administered endotoxin, IL-1 and TNF (27-29). Direct injection of endogenous pyrogen into the preoptic region of the hypothalamus in the lesioned rats, however, induced fever similar to that seen in sham rats (27). They proposed that the OVLT may be the passage site of systemic endogenous pyrogens to the preoptic region of the hypothalamus which directly responds to pyrogens to induce fever. Thus, lesions of the OVLT subsequently prevent the entry of pyrogens into the brain.

In contrast, Stitt et al. (222) reported different findings. Small perhaps incomplete lesion of the OVLT region in rabbits greatly augmented the febrile response to endogenous pyrogen with maximum responses 3-6 days post lesions which gradually



diminished over 3-4 weeks. They suggested that this enhanced response may be due to increased phagocytosis in the remaining OVLT cells since zymosan, a phagocytotic stimulating agent, produced similar enhancement of the febrile response. Similarly, Katsuura et al. (116) demonstrated that OVLT lesion enhanced the intravenous IL-1  $\beta$ -induced ACTH response whereas lesions of preoptic area of the hypothalamus suppressed the response. They proposed that the OVLT may be the entry site of blood-borne IL-1  $\beta$  into the brain leading to the preoptic area of the hypothalamus, which may contain the neurons required for the ACTH response.

Finally, using antiserum directed against human IL-1  $\beta$  to stain the human brain immunohistologically (34), IL-1  $\beta$  immunoreactive fibers were found in many areas of the brain with a heavy innervation to the hypothalamus. Similarly, immunoreactive IL-1  $\beta$  also present in the hypothalamus and the extrahypothalamic regions of the rat brain (136). The authors speculated that IL-1 may be an intrinsic neuromodulator in the central pathways that may mediate neuroendocrine, febrile and many other responses. *In vitro* production of IL-1, IL-6 and TNF by certain brain cells such as astrocytes and glial cells in response to endotoxin challenge or viral infection have also been demonstrated (138,200,215). Furthermore, *in vivo* production of those three cytokines in the brain or CSF has also been demonstrated in a variety of infectious or inflammatory states (82,95,105,135). For example, Fontana et al. (82) studied the *in vivo* synthesis of IL-1/endogenous pyrogen within the brain of endotoxin treated mice. They found that IL-1 activity of brain extract, as determined by stimulating phytohemagglutinin (PHA)-initiated proliferation of thymocytes, increased in a dose-dependent manner 5 hr after the injection

of 50  $\mu\text{g}$  endotoxin (i.p.). The enhanced IL-1 activity was first detectable 3 hr post endotoxin. The febrile response induced by the brain extract was in accord with temperature changes observed in purified IL-1 treated mice. A recent study by Hallenbeck et al. (95) showed that the ICV injection of endotoxin (at doses of 1.8 and 3.6 mg/kg) induced a dose-dependent TNF production, as detected in the CSF. Elevated mRNA expressions of IL-1, IL-6, and/or TNF in the rat brain particularly the hypothalamus have also been observed *in vivo* following the central administration of gamma-interferon and endotoxin, systemic injection of kainic acid, and immobilization stress (102,160,161). Immobilization induced IL-1 mRNA was detected as early as 30 min after the start of the stress and reached a maximum at 60 min (161).

## VI. Evidence for the Hypothalamus as the Potential Action Sites of Cytokines in the Brain

Several authors have reported changes, either increases or decreases, in the electrophysiological activity of hypothalamic neurons during the immune response (23,205,216). Electrophoretically applied recombinant human IL-1  $\beta$  (rhIL-1 $\beta$ ) and rhTNF have also been shown to suppress the activity of glucose-sensitive neurons in the rat lateral hypothalamic area and increase the neuronal activity of glucoreceptor neurons in the rat ventromedial hypothalamic nucleus (180,194). Hypothalamic neurons could be excited by interleukin-1 *in vitro* (107). Enhanced hypothalamic norepinephrine metabolism following the IL-1 treatment, either *in vivo* or *in vitro*, has also been demonstrated (69,183,184,248). Direct injection of cytokines to different areas of the

hypothalamus induces a febrile response (27), hyperinsulinemia (55) and suppression of food intake (194). *In vitro* incubation of the hypothalamus with either IL-1 or IL-6 stimulated CRF secretion (172,241). Therefore, the hypothalamus has been considered to be one of the most important sites for cytokine actions.

As can be summarized from the cytokine review, many host defense responses to infectious and inflammatory processes are mediated by cytokines via their central actions. Some of the cytokine effects may be initiated in the brain side of the blood brain barrier, particularly the hypothalamus. Relevant to the interest of the present dissertation study, cytokines may act in the CNS, possibly the hypothalamus, to mediate the sympathoadrenal activation during developing septic shock. However, these possibilities have not been examined in a systematic manner, which will be the focus of another project of the dissertation study.

## THE RATIONALE

Despite extensive demonstrations of the sympathoadrenal activation during developing septic shock, mechanisms responsible for this activation are apparently unknown and basically unexplored. After review of the limited and indirect data in the literature, three themes might be suggested. First, major sympathoadrenal activation in response to endotoxin challenge may be dependent on an intact CNS. Peripheral modulation of epinephrine release from the adrenal gland might also make minor contribution to the overall sympathoadrenal activation during endotoxemia. Second, afferent neural input from arterial baroreceptors may not have a dominant role in mediating the sympathoadrenal activation in endotoxin shock. Third, interactions between the immune-neuroendocrine systems exist with cytokines as likely mediators. This interaction may also be applicable to the sympathetic response to endotoxin challenge such that cytokines may mediate the sympathoadrenal activation during septic shock via their central actions. However, these three aspects have never been studied directly, or in a systematic manner. Thus, the presently proposed dissertation study will focus on these potential mechanisms of sympathoadrenal activation in endotoxin shock. The specific aims include: 1) to examine the overall contributions of central nervous system as well as peripheral modulation (either neurogenic or non-neurogenic) to the sympathoadrenal response in endotoxin shock, 2) to evaluate the role of afferent neural input from the arterial baroreceptors in mediating sympathoadrenal activation during endotoxin shock, and 3) to assess the central roles of three major cytokines, IL-1, IL-6

and TNF, as mediators of the sympathoadrenal activation.

## CHAPTER III

### GENERAL MATERIALS AND METHODS

#### A. ANIMALS

Male, Holtzman rats, obtained from Harlan Sprague Dawley Inc. (Indianapolis), weighing 300-500 g, were used in all experiments. The rats were housed in shoe box cages with filter tops at least 1 week before use in order to recover from the stress of shipping and to adjust to the new environment. Ambient temperature was approximately 22 °C to 25°C, and the illumination was controlled on 12 hr light-dark cycle. Wayne Lab. Blox (full nutrient meal) and tap water were provided *ad libitum* except where indicated prior to some experimental protocols.

#### B. CARDIOVASCULAR INSTRUMENTATION AND MEASUREMENTS

Arterial and venous cannulae were implanted either on the day of experiments for acute preparations or the day prior to experiments for chronic preparations. These cannulae permitted monitoring the hemodynamic changes (e.g. arterial blood pressure, heart rate), collecting blood samples, or administering intravenous drugs throughout the time course of experiments. In experiments with sinoaortic baroreceptor denervation, femoral vessels rather than carotid arteries were cannulated to avoid any potential

disturbances in the carotid sinus regions. In all other studies, carotid artery and jugular vein cannulae were used.

For femoral vessel cannulation, a 1 cm long incision was made in the left inguinal area. After exposure of the femoral vessels by blunt dissection, the arterial and venous cannulae (PE-50 polyethylene tubing, Clay-Adams, NY), filled with heparinized saline (100 U/ml), were inserted approximately 5 cm to the level of abdominal aorta and inferior vena cava, respectively.

For carotid artery and jugular vein cannulation, a 2 cm ventral midline incision was made in the neck and blunt dissection was employed to expose the vessels. The arterial and venous cannulae were advanced approximately 3 cm to the level of the ascending aorta and superior vena cava-right atrial junction, respectively.

In conscious rat experiments the ends of the cannulae were heat sealed, tunneled under the skin, and were exteriorized through the back of the neck in the interscapular region. The cannulated animals were then placed one per cage to prevent damage to the cannulae and fasted for about 24 hrs with free access to water.

Prior to the onset of experimentation, the arterial cannula was extended to a saline-filled Statham pressure transducer (model P23) via a tygon tubing adaptor and additional PE-50 tubing. Pulsatile as well as mean blood pressures (MBP) were monitored continuously using a Grass oscillograph (model 7). The heart rate (HR) was determined from the pressure pulses. Extension of the venous line provided a convenient means for intravenous drug administration and replacing volume for blood sampling. Care was taken to minimize heparin administration to each rat during experiments by withdrawing

the heparinized saline before flushing the cannula with non-heparinized saline. Before beginning each experiment, rats were allowed time (approximately 30 min) to become acclimatized to their environment as determined by normal, steady mean arterial blood pressures (approximately 90-120 mmHg) and heart rates (approximately 300-400 beats/min) as well as by quiet behavior.

### C. Sinoaortic Baroreceptor Denervation

Sinoaortic baroreceptor denervation (SAD) was performed by a modification of the method originally described by Krieger (133). Anesthetized animals were placed in a supine position on a warm pad. Using aseptic technique, a midline incision (2.5-3 cm) was made in the neck and the neurovascular sheath enclosing the common carotid arteries, the vagi, and the cervical sympathetic nerve trunks were exposed by retracting the sternohyoid and sternomastoid muscles. The vagi were carefully separated from the neurovascular sheath with the aid of a dissecting microscope (0.7x-3x, Bausch & Lomb Inc.). For aortic baroreceptor denervation, the superior laryngeal nerves were cut near the vagi and the superior cervical ganglia including a small segment of the sympathetic chain were removed to prevent the possibility of reconnection. Aortic depressor nerves, usually found associated with the sympathetic trunk, were sectioned when evident. For carotid sinus denervation, the area of the carotid bifurcation was widely exposed. All connective tissue and nerve fibers were stripped from the thyroid, occipital, internal, external and common carotid arteries. Extra care was taken to prevent injury of vagal nerves. Sham operations were performed by exposing the carotid sinuses bilaterally



without dissection of the region. Bilateral sinoaortic denervation was normally performed in a one-stage operation lasting approximately 30 min. In chronic SAD and sham preparations, animals received 40,000 IU penicillin (i.m.) following the surgery and were allowed 2-4 weeks for recovery.

Completeness of the baroreceptor denervation was tested by determining the magnitude of the reflex bradycardia in response to phenylephrine-induced hypertension ( $4 \mu\text{g}/\text{kg}$ , i.v.) and the reflex tachycardia in response to nitroglycerine-induced hypotension ( $0.5 \mu\text{g}/\text{kg}$ , i.v.). Such observations have been routinely used as the criteria of the effectiveness of the SAD (8,13,169,239). Only those animals which exhibited a reflex bradycardia of  $< 20$  beats/minute and a reflex tachycardia of  $< 20$  beats/minute in response to 40-50 mmHg change in MBP were accepted as SAD rats (8). The bradycardia or tachycardia typically observed in sham controls was 60-120 beats/min after the same dose of phenylephrine or nitroglycerine.

#### D. SYMPATHETIC NERVE RECORDINGS

Renal sympathetic nerve activity (RSNA) was recorded from a renal branch of the left greater splanchnic nerve (61,185). Using aseptic techniques, the left kidney was exposed retroperitoneally through a flank incision. The renal nerve branch was usually found in the aortic-renal artery angle, coursing to the renal vascular pedicle through the perirenal fat and into the hilus of the kidney. Under a dissection microscope, the renal nerve was gently separated from fat and connective tissues, and carefully placed on a thin, bipolar gold electrode hook. The gold electrodes were teflon-coated with one end

bared, bent to a hook, and the other end connected to extension cables. When an optimal nerve signal was obtained, the electrode and the nerve were fixed with a mixture of equal parts of dental elastomeric impression material base, catalyst (both from Sybron-Kerr, Romulus, MI), and RTV silicon gel base (Petrarch Systems Inc., Bristol, PA). After closure of the incision in layers, the electrode cables were tunnelled subcutaneously and exteriorized on the back of the neck.

On the next day, the electrode cables were extended to an amplifier while the conscious rats were in cages. The nerve signal was amplified 1,000-10,000 fold and filtered (low-frequency cutoff at 35 Hz and high-frequency cutoff at 2,000 Hz) by a Grass model HIP511A pre-amplifier and a Grass model P511R amplifier. The amplified signal was continuously displayed on an oscilloscope (Tektronix Inc., Type 561A) and led to an audio amplifier/loud speaker (Grass AM7). Representative oscilloscope displays of amplified raw renal sympathetic nerve activity from unrestrained, conscious rats are shown in figure 3-1. For estimation of frequencies of RSNA, the signal was full-wave rectified and integrated by a Grass model 7P3A integrator with a time constant 0.02 sec and the spikes/min were measured. For simultaneous estimation of overall RSNA, which involves both frequencies of RSNA and amplitudes of RSNA spikes, the amplified signal was also rectified and integrated by a Grass model 7P10E integrator and the slopes of integration were determined. In both cases, the rectified-integrated signal was continuously recorded using a Grass oscillograph (model 7).

The background noise level for the nerve recording was determined 30 min after each animal was euthanized with i.v. pentobarbital overdose. For RSNA spike

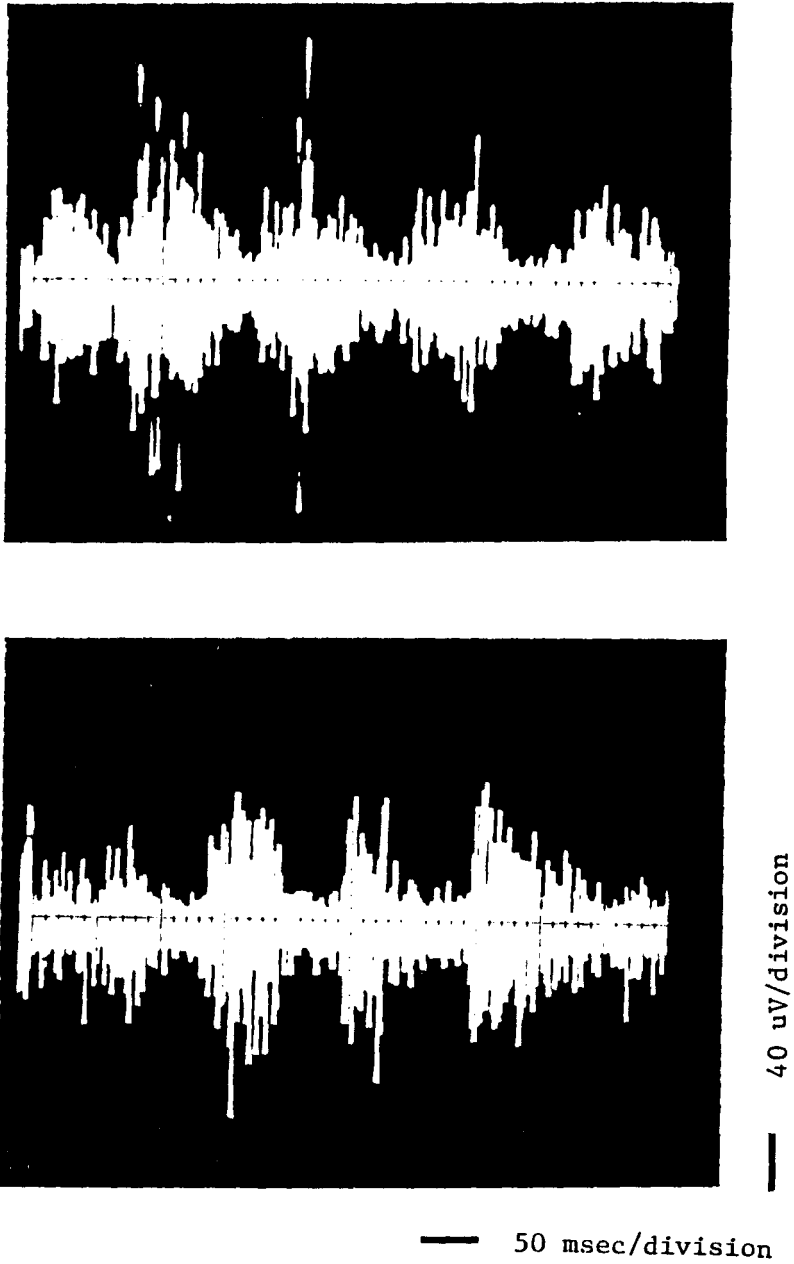


Figure 3-1. Oscilloscope display of amplified raw renal sympathetic nerve activity from unrestrained, conscious rats.

measurement, only spikes with amplitudes greater than background noise level were counted. For slope measurement, background noise slope was subtracted from the mean rectified nerve signal slope obtained in the living animal. All RSNA were sampled for 20 sec at indicated sampling times and expressed as percent change from baseline control. Percent changes of RSNA were used because the absolute value of multiunit activity is influenced by recording conditions that vary between different preparations. Such variations include the spatial relationship of the nerve fibers to the electrodes and the amount of tissue fluid around each nerve.

#### E. IMPLANTATION OF INTRACEREBROVENTRICULAR (ICV) CANNULA

Anesthetized rats were placed in a stereotaxic apparatus (Trent H. Wells Jr. Mechanical Developments Co. South Gate, CA) in a prone position. A 2 cm midline incision was made on the skull and the bregma was exposed. After determining the coordinates for the right lateral ventricle using the bregma as a reference point, a small hole (0.5 mm diameter) was made with an electric drill. A 30 gauge stainless steel cannula was inserted through the hole in the skull into the lateral ventricle with the tip coordinates: 0.5 mm caudal to bregma, 1.5 mm lateral to the midline, and 4.5 mm below the surface of the skull (10). The end of the cannula was pre-bent to a right angle and connected to a piece of PE-10 tubing (5 cm long), filled with saline (3-4  $\mu$ l) and heat sealed. Two stainless steel anchoring screws were fixed on the skull, and the cannula secured in place by dental acrylic cement. The incision was sutured with the end of the PE-10 tubing exteriorized. On the day of experiment, the cannula was connected to a 10

$\mu$ l Hamilton microsyringe filled with test solution. A 10  $\mu$ l test solution (plus 3-4  $\mu$ l saline in the cannula) was infused over a period of 5 min.

At the end of each experiment, methylene blue dye solution (15  $\mu$ l) was injected via the cannula. The animals were sacrificed with pentobarbital overdose (i.v.), and the brains were removed. The exact location of the injection was verified. Only those animals with successful ICV injections, as indicated by staining of the lateral ventricles, were included in the data analysis.

#### F. IMPLANTATION OF CANNULAE INTO THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS (PVN)

Cannulae for injections into the paraventricular nucleus of the hypothalamus (PVN) were implanted using a stereotaxic apparatus. After exposure of the bregma under anesthesia, a 3 mm x 1 mm slot was prepared across the midsagittal suture on the skull with a electric drill. A bilateral guide cannula (Plastic One Inc., Roanoke, VA), constructed of two 26-gauge stainless steel tubes with a 1.0 mm distance between the centers, was implanted in the bilateral PVN through the slot in the skull. The coordinates for this position were: 1.7 mm caudal to the bregma, 0.5 mm lateral to the midsagittal suture, and 8.0 mm ventral to the skull surface with the incisor bar set at 4.0 mm below the interaural line (190). Four stainless steel anchoring screws were fixed on the skull, and the cannula secured in place with dental acrylic cement. The incision was closed with a suture. A dummy wire with two leads was inserted into the guide cannula to prevent clotting in the cannula and also prevent dust from getting into the cannula. On the day

of experiments, the dummy wires were removed and a double injector cannula (33 gauge, Plastic One Inc.), which was 2 mm longer than the guide cannula, was inserted into the guide cannula. The injector cannula, filled with test solution, was connected to a 10  $\mu$ l Hamilton microsyringe via a flexible tygon tubing (same size as PE-10, Norton Tubing and Molded Products, Akron, OH). Testing solution (500 nl) was injected bilaterally over a 5 min period. The volume of an injection was monitored by observing the movement of a small bubble through a calibrated distance in the tubing.

For histological verification of the injection site, pentobarbital-treated animals were perfused transcardially with 10% buffered formalin, and their brains were removed and preserved in buffered formalin for at least 48 hrs. The brains were then cut on a cryostat (40  $\mu$ m sections) and stained by the cresyl violet method to verify the position of the cannula tip. Only those animals in which the cannulae terminated in the dorsal border of the PVN without damage to the PVN neurons were included in the statistical evaluation.

## G. PITHING PROCEDURES

Pithed rats were prepared according to the method of Gillespie and Muir (85). The animals were anesthetized with ether, and the trachea cannulated. An aluminum pithing rod (2 mm diameter) was passed into the brain through the right orbit and advanced down the spinal cord to the sacral vertebrae for a total distance of 15 cm. The rats were immediately ventilated with Harvard Apparatus Rodent respirator (room air, 60 strokes/min, approximately 1-1.2 ml/100 g body weight). Rectal temperature was maintained at 36-37 °C with a heating pad and a lamp. The pithing rod served as a

stimulating electrode, and another rod was inserted under the skin of the back to serve as an indifferent electrode for stimulation of the spinal cord. This pithing procedure destroys the entire central nervous system, but leaves the emerging nerve trunks intact. Since those parts of the pithing rod in the sacral and cervical regions of the spinal cord were coated with high resistance varnish, the stimulating current applied through the pithing rod was restricted to the thoracolumbar region where the sympathetic preganglionic fibers are located. After curare (3 mg/kg) was given via the dorsal vein of the penis to eliminate skeletal muscle contractions, the preganglionic thoraco-lumbar sympathetic nerves were stimulated at a constant rate (3 Hz, 10 V, and 0.5 msec) to maintain blood pressure at physiological levels. Some rats required small supplemental doses of curare.

The advantages of using pithing combined with stimulation include: 1) sympathetic outflow can be maintained at a steady level by applying a constant stimulation to the sympathetic preganglionic fibers via the pithing rod, 2) basal blood pressure can be maintained at physiological levels, and 3) there are no centrally-mediated compensations. By using this model, the CNS mediated fluctuation in catecholamine release from the nerve terminals and the adrenal medulla is eliminated, thus, allowing the evaluation of the peripheral mechanisms of catecholamine release.

## H. ADRENAL GLAND DENERVATION

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Under aseptic conditions, two flank incisions were made on both left and right sides of the back just

below the costal margin. With the aid of a microscope, the adrenal glands were isolated from the surrounding connective tissues except for the adrenal artery and vein. The splanchnic nerve fibers innervating the adrenal gland and any other fibers which might enter the adrenals without joining the splanchnic nerves were served. Extra care was taken to leave the vessels intact to avoid necrotic damage to the adrenal glands. Phenol (10%) was then applied on the surface of the glands and the vessels. Sham operations were performed by exposing the adrenal glands without dissecting the nerves. The incisions were closed in layers, and rats received 40,000 IU penicillin (i.m.). The denervation procedure required about 20 min to complete, and a two week recovery period was allowed before the experiment.

The effectiveness of this adrenal denervation technique was verified in a separate group by demonstrating the failure of plasma epinephrine to become elevated in response to hemorrhage. Chronic adrenal denervated rats were subject to carotid artery cannulation and on the next day, the carotid cannula was extended to a 5 ml glass syringe via a PE-200 tubing, both pre-rinsed with heparin. The syringe served as a blood reservoir and blood was allowed to flow in or out of the reservoir depending on the height of the syringe above the rat. The blood pressure was monitored on the Grass oscillograph using a stopcock. The rats were bled to 70 mmHg (total volume 4.5-5 ml) in about 10 min by adjusting the syringe position. Blood samples were taken at predetermined times (15, 60 min) for catecholamine assay. Results showed that in sham denervated rats (n=3), hemorrhage induced significant elevations in plasma epinephrine ( $251 \pm 60$  pg/ml at 0 min,  $2286 \pm 465$  pg/ml at 60 min) and norepinephrine ( $377 \pm 44$  pg/ml at 0 min,  $787 \pm 204$



at 60 min). In contrast, plasma epinephrine elevations were abolished in adrenal denervated rats ( $n=3$ ,  $45\pm 16$  pg/ml at 0 min,  $48\pm 7$  pg/ml at 60 min) while plasma norepinephrine was still increased ( $394\pm 102$  pg/ml at 0 min,  $764\pm 75$  pg/ml at 60 min) in response to hemorrhage.

## I. BLOOD SAMPLE COLLECTION

Arterial blood samples ( $500\ \mu\text{l}$ ) were collected at predetermined times and placed in 1.5 microcentrifuge tubes (Sarstedt) containing  $10\ \mu\text{l}$  solution consisting of 90 mg/ml ethyleneglycol-bis-(B-amino-ethyl ether) $N,N'$ -tetraacetic acid (EGTA) and 60 mg/ml glutathione (pH 6-7). The plasma was separated following centrifugation ( $10,000\ \text{g} \times 2-3$  min, Eppendorf Model 5414 microfuge) and stored at  $-40\ ^\circ\text{C}$  (Revco freezer) until analysis for catecholamines. A  $500\ \mu\text{l}$  volume of donor blood from normal rats or equal volume of saline-resuspended blood cells was infused into the venous line following each sample withdrawal.

## J. PLASMA CATECHOLAMINE ASSAY

Plasma levels of norepinephrine and epinephrine were assayed according to the radio-enzymatic thin-layer chromatographic procedure described previously (189,193). Materials were supplied in kit form (Amersham, Arlington Heights, IL). This single isotope radioenzymatic method can measure epinephrine and norepinephrine simultaneously with a sensitivity of 2-5 pg per  $50\ \mu\text{l}$  sample (40-100 pg/ml plasma) and interassay variation of approximately 10%. The analysis is based on the use of the

isolated enzyme catecholamine-o-methyltransferase (COMT) to transfer a radioactive methyl group from S-adenosyl-L-methionine ( $^3\text{H}$ -SAM) to an endogenous catecholamine acceptor molecule to form a O-methylcatecholamine derivative. In the presence of  $^3\text{H}$ -SAM, epinephrine is converted to  $^3\text{H}$ -metanephrine and norepinephrine is converted to  $^3\text{H}$ -normetanephrine.

Duplicate 50  $\mu\text{l}$  aliquots of each plasma sample were added to incubates containing (in final concentration) 100 mM Tris, 30 mM  $\text{MgCl}_2$ , 10 mM EGTA, 10  $\mu\text{l}$  of COMT solution, 1 mM reduced glutathione, 5  $\mu\text{Ci}$  SAM ( $^3\text{H}$ -methyl, 4.3-5.5 mM). Total volume was 100  $\mu\text{l}$  and pH was approximately 8.1-8.3. For every 5 samples, one sample was added to four tubes rather than duplicate tubes and 100 pg of epinephrine and norepinephrine were added to two of the four tubes as internal standards. Reagent blank was constituted by substituting distilled  $\text{H}_2\text{O}$  for the plasma.

The samples were incubated in a shaking water bath at 37  $^\circ\text{C}$  for 60 min. The reaction was stopped by addition of 50  $\mu\text{l}$  of a solution consisting of 800 mM boric acid, 80 mM ethylenediamine tetraacetic acid-disodium salt ( $\text{EDTA-Na}_2$ ) and 4 mM each of metanephrine and normetanephrine in 1 NaOH. The resulting solution (pH 10.0) was mixed vigorously for 15 sec with 2 ml of toluene/isoamyl alcohol (3:2, v:v). The  $^3\text{H}$ -methoxycatecholamine derivatives are much more soluble in the organic phase than in aqueous phase. The aqueous and organic phases were separated by centrifugation (800 g x 2 min, Model UV centrifuge, International Equipment Company). The aqueous phase was rapidly frozen in an ethanol/dry ice bath, and the organic phase was decanted into a tube containing 100  $\mu\text{l}$  of 0.1 M acetic acid. The  $^3\text{H}$ -methylcatecholamine derivatives

were partitioned into the aqueous phase by mixing for 15 sec. The decrease in pH effected by the addition of acetic acid greatly reduced the solubility of the derivatives in the organic phase. After the tubes were centrifuged at 800 g for 2 min, the aqueous phase was quick-frozen, and the organic phase was discarded. One ml of the toluene-isoamyl alcohol was added to the acetic acid mixture. After mixing, centrifugation and freezing the aqueous phase, the organic layers were aspirated and discarded. One hundred  $\mu\text{l}$  of ethanol was then added to the final extract and the solution was applied to a silica gel thin glass plate (Uniplate, Analtech) by use of 250  $\mu\text{l}$  Hamilton syringes and a thin layer chromatograph (TLC) Multispotter (Analytical Instrumentation Specialties) while drying with a hot air blow dryer (60 °C). The plates were developed for 20-30 min in a paper-lined tank containing a solution of chloroform/t-amyl alcohol/methylamine (8/2/1, v/v/v). Two zones were visualized under 254 nm UV light. The upper zone contained metanephrine, and the lower zone contained normetanephrine. Each zone was then scraped from the plate into a correspondingly numbered scintillation vial.

The further steps for epinephrine and norepinephrine assay were identical. One ml of 0.05 M ammonium hydroxide was added to each vial to elute the amine from the silica gel. The  $^3\text{H}$ -catecholamine derivative was then oxidized to  $^3\text{H}$ -vanillin by addition of 50  $\mu\text{l}$  of sodium periodate for 5 min followed by the addition of 500  $\mu\text{l}$  of 10% glycerol. The solution was acidified by the addition of 1 ml of 0.1 M acetic acid and vigorously mixing. Ten ml of toluene/liquiflor (1000:50) was added to each vial and  $^3\text{H}$ -vanillin was partitioned into the organic phase with 30 sec shaking. Liquiflor is a 2.5-Diphenyloxazole--p-bis-[2-(5-phenyloxazolyl)]-benzene (PPO-POPPO) toluene

concentrate. Radioactivity in these samples was determined by a liquid scintillation counter (LKB Instruments Inc., MD). The sample plasma content for each catecholamine was calculated by computer using the following equation:

Catecholamine Concentration (pg/ml) =

$$\frac{\text{CPM sample-CPM blank}}{\text{CPM (sample+standard)-CPM sample}} \times \frac{\text{pg standard}}{\text{ml sample volume}}$$

Above counts per min (CPM) value was the average from each set of duplicates.

## K. AGENTS

1. Endotoxin (ETX): Lyophilized endotoxin derived from Salmonella enteritidis (lipopolysaccharide Boivin, control No. 723198) was purchased from Difco Laboratories (Detroit, MI). For use in intravenous administration, the lipopolysaccharide was prepared freshly in 0.9% sodium chloride (3 mg/ml for the dose of 5 mg/kg, and 1 mg/ml for the dose of 1.5 mg/kg).

2. Interleukin-1 $\beta$  (IL-1 $\beta$ ): Human recombinant interleukin-1 $\beta$  was purchased in a lyophilized form from Biosource International Inc. (Westlake Village, CA). The IL-1 was lot 215-110C with specific activity 1 x10<sup>7</sup> unit/mg, as assessed by a mitogenic assay stimulating <sup>3</sup>H-thymidine uptake into C3H/HEJ thymocytes. Endotoxin contamination was determined by Biosource to be less than 0.001 endotoxin units/ $\mu$ g (Limulus Amoebocyte Lystate Assay; 1 endotoxin unit = 0.1 ng of ETX).

3. Interleukin-6 (IL-6): Human recombinant interleukin-6 was obtained in a lyophilized form from Biosource International Inc. (Westlake Village, CA). The lot No. was 216-

002D, and the specific activity was  $6 \times 10^6$  unit/mg as determined by stimulation of  $^3\text{H}$ -thymidine uptake by B9 cells. Endotoxin contamination was same as in IL-1.

4. Tumor necrosis factor  $\alpha$  ( $\text{TNF}\alpha$ ): Human recombinant tumor necrosis factor  $\alpha$  was also purchased in a lyophilized form from Biosource International Inc. (Westlake Village, CA). The lot No. was 311-010D, and the specific activity was  $1 \times 10^8$  unit/mg as determined by the cytolysis of murine L929 cells in the presence of Actinomycin-D. Endotoxin contamination was also less than 0.001 endotoxin units/ $\mu\text{g}$ .

#### L. DATA ANALYSIS

Data are expressed as mean  $\pm$  SEM. A p value less than 0.05 was considered to be statistically significant. Cardiovascular responses, percent change of sympathetic nerve activity and plasma catecholamines following test treatments were compared using analysis of variance with repeated measurements. Student-Newman-Keul's test was used for individual group comparisons. Blood pressure and associated heart rate as well as RSNA responses to phenylephrine or nitroglycerine infusion to assess baroreceptor denervation were compared between the SAD and sham groups with unpaired Student t tests.

## CHAPTER IV

### INVOLVEMENT OF CENTRAL VS. PERIPHERAL MECHANISMS IN MEDIATING SYMPATHOADRENAL ACTIVATION IN ENDOTOXIC RATS

#### INTRODUCTION

Gram-negative sepsis with ensuing septic shock is a persistent health problem accounting for the major cause of mortality in hospitalized patients (1,32). Numerous studies have linked gram-negative bacterial endotoxins to the pathogenesis of sepsis (103,104,167,171), which supports the use of the endotoxic animal model for studying the basic pathophysiologic process of septic shock.

Studies of sepsis or endotoxemia in either clinical or experimental situations have demonstrated a marked sympathoadrenal activation. Supportive evidence includes elevations of plasma catecholamines (17,94,113,114,134), an enhanced norepinephrine turnover (188), augmented sympathetic nerve discharges (92,159,185) as well as depletion of the tissue catecholamine content in the end stage of septic shock (96,187). This sympathoadrenal activation may support the cardiovascular and metabolic adjustments to septic challenge, but overwhelming and sustained activation may contribute to the irreversibility of the shock process (31,88,125,148).

Mechanisms responsible for mediating the sympathoadrenal activation during endotoxemia are largely unknown. Norepinephrine release from the sympathetic nerve terminals and epinephrine release from the adrenal medulla are most likely neurally-controlled via post ganglionic sympathetic nerves and preganglionic adrenal sympathetic nerves, respectively. The critical role of centrally-mediated mechanisms and neural dependence of the sympathetic activation during developing endotoxic shock have been demonstrated in previous studies (71,150,176,253). However, recent experimental findings suggest that peripheral modulation of catecholamine secretion from the adrenal medulla during endotoxic shock may also be present (112,211). Non-neurogenic stimulation of adrenal epinephrine secretion is suggested by evidence that ganglionic blockade could not prevent catecholamine release from the adrenal medulla (211), and *in vitro* incubation of adrenal chromaffin cells with endotoxin or other endotoxin-elaborated agents enhance catecholamine secretion (130,141,177,225).

Thus, the purposes of the present study were: 1) to clarify whether both central and peripheral mechanisms are involved in mediating plasma catecholamine responses during endotoxemia, 2) to assess the relative contributions of each of the mechanisms to elevations in plasma norepinephrine and epinephrine following endotoxic challenge, and 3) to examine the neurogenic or non-neurogenic characteristics of peripheral modulations of adrenomedullary release following endotoxin, if the modulation indeed exists. Specifically, plasma catecholamine responses to endotoxin challenge were compared between rats with and without central regulatory mechanisms, i.e. conscious and pithed rats, to assess the contribution of central mechanisms. It was hypothesized that the major

elevation of plasma catecholamines depends on centrally-mediated mechanisms such that destruction of the central nervous system (by pithing) would significantly diminish the endotoxin-induced elevation in plasma catecholamines. Meanwhile, pithed rats with constant stimulations of the sympathetic chain do not have centrally-mediated fluctuations in sympathetic outflow and, thus, permit the evaluation of peripheral modulation of catecholamine release. It was hypothesized that peripheral modulations of the adrenal medulla are present, which would result in significant elevation of plasma epinephrine in pithed rats following administration of endotoxin. The possibility of non-neurogenic catecholamine secretion from the adrenal gland was further studied in pithed animals as well as conscious rats by means of adrenal denervation.



## MATERIALS AND METHODS

### Experimental Protocols

Two sets of experiments were conducted in male rats weighing  $407 \pm 10$  grams. In the first experiment sympathoadrenal responses were assessed in pithed, endotoxic rats with intact or denervated adrenal glands. Under pentobarbital anesthesia, bilateral adrenal glands were denervated or sham-operated and penicillin (40,000 IU) was given i.m. following the surgery. After two weeks of recovery, experiments were performed. Rats were pithed under ether anesthesia and immediately ventilated (60 strokes/min, 1-1.2 ml/100 g body weight). After administration of curare, the thoraco-lumbar preganglionic sympathetic nerve fibers were stimulated at a constant rate (3 Hz, 10 V, and 0.5 msec). Carotid artery and jugular vein were then cannulated. Following a 30 min equilibration period, endotoxin (1.5 mg/kg) was given intravenously to both adrenal sham-operated rats ( $n=8$ ) and adrenal denervated rats ( $n=7$ ). Saline treated sham ( $n=5$ ) and adrenal denervated ( $n=4$ ) rats served as controls. The hemodynamic response was assessed at 0, 30, 60 and 90 min, and plasma catecholamines were determined at 0, 60 and 90 min post endotoxin. Rectal temperature was maintained at 36-37 °C using a heating pad and a lamp throughout the protocol.

In the second experiment sympathoadrenal responses were evaluated in conscious rats with intact or denervated adrenal glands. Under halothane anesthesia chronically adrenal sham-operated or denervated rats underwent implantation of arterial and venous cannulae, and experiments were conducted the following day. Endotoxin was given

intravenously (1.5 mg/ml) to both sham operated (n=5) and denervated rats (n=5). Saline treated sham (n=5) and denervated (n=4) rats served as controls. Hemodynamic responses and plasma catecholamines were also assessed at 0, 30, 60, and 90 min post endotoxin.

### Pithing Procedures

Pithed rats were prepared according to the method described in chapter III. Briefly, after cannulation of the trachea under ether anesthesia, rats were pithed by inserting an aluminum rod through the orbit and foramen magnum, down the spinal cord to the sacral vertebrae. The pithing rod served as a stimulating electrode, and another rod inserted under the back skin served as an indifferent electrode for stimulation of the sympathetic chain. After minimizing skeletal muscle contraction with curare (3 mg/kg, i.v.), the preganglionic thoraco-lumbar sympathetic nerves were stimulated at a constant rate (3 Hz, 10 V, and 0.5 msec) to maintain blood pressure at physiological levels and to eliminate the fluctuations of catecholamine secretions.

### Adrenal Gland Denervation

As described in general methods, bilateral adrenal glands were denervated by severing all the surrounding connective tissues which contain the nerve fibers innervating the adrenals. Extra care was taken to leave the blood vessels intact to avoid necrotic damage to the adrenal glands. Phenol (10%) was then applied on the surface of the glands and the vessels. Sham operations were performed by exposing the adrenal glands

without dissecting the nerves. Two week recovery period was allowed before experiments. The effectiveness of this adrenal denervation technique was verified, in a separate group, by demonstrating the failure of the plasma epinephrine elevation in response to hemorrhage-induced hypotension (see Chapter III for details).

### Blood Sample Collection and Catecholamine Assay

Arterial blood samples (500  $\mu$ l) were collected at pre-determined times and treated as described in chapter III. A 500  $\mu$ l volume of donor blood from normal rats was infused into the venous line following each sample withdrawal. Plasma levels of norepinephrine and epinephrine were assayed in duplicate 50  $\mu$ l samples using the radio-enzymatic thin-layer chromatographic procedure described in general methods.

### Materials

Endotoxin (derived from *S. enteritidis*) was prepared freshly in 0.9% saline (1 mg/ml). Materials for catecholamine assay were obtained in kit form from Amersham (Arlington Heights, IL).

### Data Analysis

Data are expressed as mean  $\pm$  SEM (n). A p value less than 0.05 was considered to be statistically significant. Cardiovascular responses and plasma catecholamines were compared using analysis of variance with repeated measurements and Student-Newman-Keul's test for individual comparisons.

## RESULTS

### Hemodynamic and Sympathetic Responses in Pithed Rats Following Endotoxin

Thirty minutes after pithing, arterial blood pressure and heart rate in all animals with constant stimulation reached steady-state levels. As presented in figure 4-1, baseline mean blood pressure were not different between the groups. The mean blood pressure was within physiological range (100-120 mmHg), indicating that this level of electrical stimulation to the sympathetic chain was adequate to maintain the blood pressure in pithed rats comparable to levels in conscious rats. There was a gradual decline of the blood pressure with time in saline treated animals, as indicated in figure 4-1 where MBP was decreased from baseline value of  $110 \pm 10$  to  $88 \pm 6$  mmHg at 90 min in sham rats and  $99 \pm 3$  to  $87 \pm 10$  mmHg in denervated rats. By contrast, in both adrenal sham operated and denervated rats, intravenous endotoxin treatment induced much greater drop in MBP than the time-related fall. The decrease in MBP was similar in sham and denervated groups given endotoxin, from pretreatment values of  $107 \pm 4$  and  $117 \pm 5$  mmHg to  $42 \pm 3$  and  $41 \pm 4$  mmHg at 90 min, respectively. There were no differences in baseline heart rate between the groups. Heart rate was significantly accelerated to comparable levels at 60 and 90 min following endotoxin injection in sham and denervated rats. Saline treatment did not affect the heart rate during the protocol.

In both adrenal sham-operated and denervated animals, plasma norepinephrine was not significantly elevated by endotoxin administration during the 90 min protocol

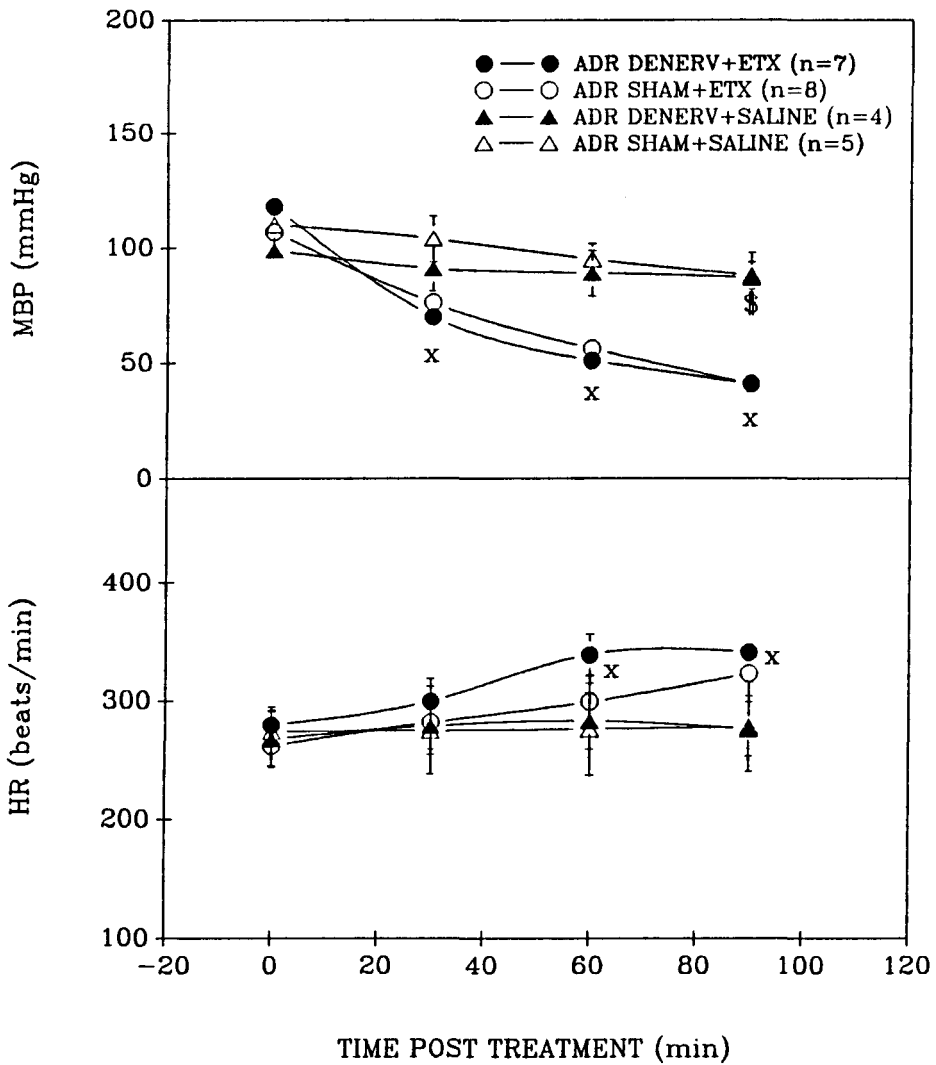


Figure 4-1. Mean blood pressure (MBP) and heart rate (HR) responses in pithed rats with adrenal denervation (ADR DENERV) or sham operation (ADR SHAM) following intravenous endotoxin (ETX, 1.5 mg/kg) or saline. x:  $p < 0.05$  for both ADR DENERV and ADR SHAM post endotoxin vs. 0 min; \$:  $p < 0.05$  for both ADR DENERV and ADR SHAM post saline vs. 0 min.

(figure 4-2). In contrast, intravenous endotoxin induced mild and similar increase in plasma epinephrine in both sham and denervated groups. Ninety min after endotoxin injection, plasma epinephrine was increased from pretreatment value of  $153 \pm 19$  and  $125 \pm 38$  to  $376 \pm 87$  and  $463 \pm 137$  pg/ml in sham and denervated groups, respectively. Saline treatment in sham and denervated animals altered neither plasma norepinephrine nor epinephrine.

#### Hemodynamic and Sympathetic Responses in Conscious Rats Following Endotoxin

In conscious sham-operated animals, intravenous endotoxin induced a significant drop in blood pressure at 60 min post treatment, which recovered at 90 min to a level not different from the baseline value (figure 4-3). In adrenal denervated conscious rats, however, hypotension appeared at 30 min following endotoxin injection, which was significantly lower compared to MBP in the sham group. Furthermore, the denervated animals remained in a hypotensive state thereafter with slight recovery at 90 min. In both sham and denervated groups, endotoxin treatment evoked a significant tachycardia as indicated in figure 4-3 at 30, 60 and 90 min post injection. The heart rate was significantly higher in denervated rats at 60 min in comparison to sham animals. Saline controls of both sham and denervated rats showed no alterations in MBP and heart rate.

Plasma catecholamine response to endotoxin challenge in conscious rats is presented in figure 4-4. In both adrenal sham-operated and denervated animals, plasma norepinephrine was similarly and significantly enhanced from baseline values

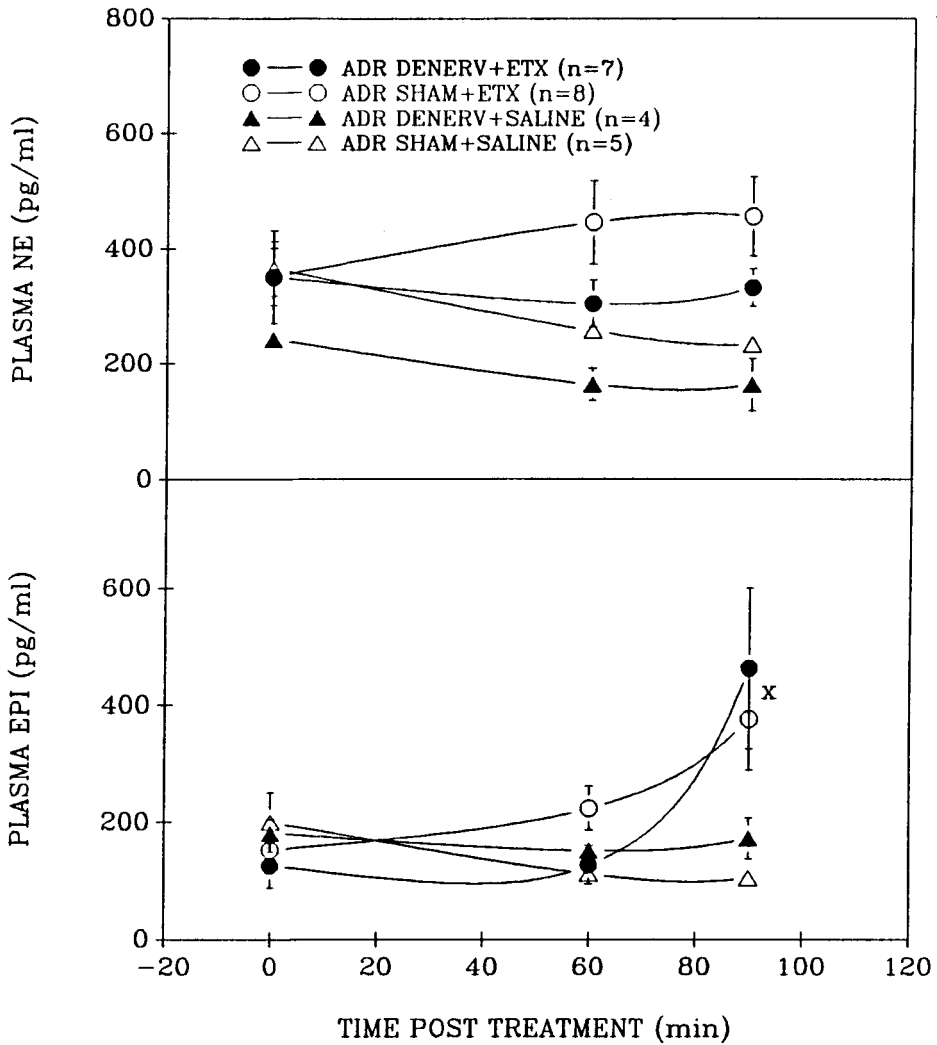


Figure 4-2. Plasma norepinephrine (NE) and epinephrine (EPI) responses in pithed rats with adrenal denervation (ADR DENERV) or sham operation (ADR SHAM) following intravenous endotoxin (ETX, 1.5 mg/kg) or saline. x:  $p < 0.05$  both ADR DENERV and ADR SHAM post endotoxin vs. 0 min.

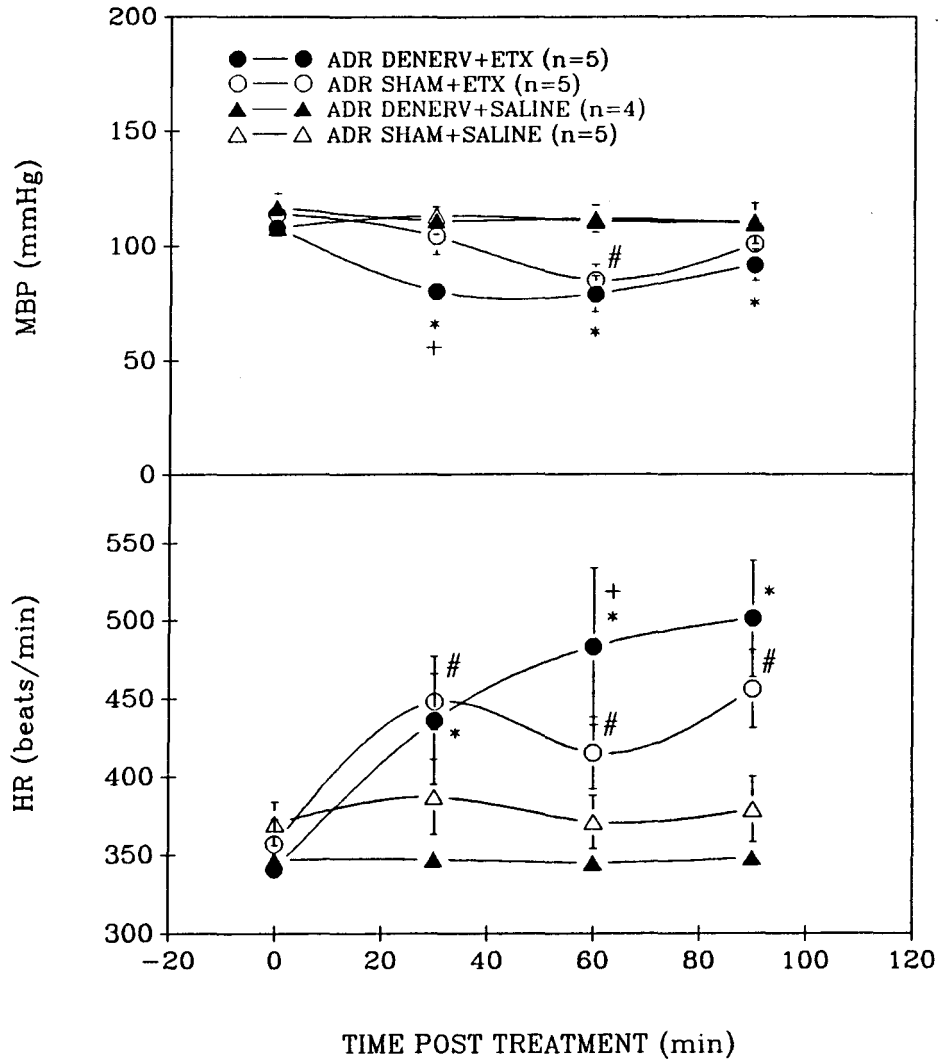


Figure 4-3. Mean blood pressure (MBP) and heart rate (HR) responses in conscious rats with adrenal denervation (ADR DENERV) or sham operation (ADR SHAM) following intravenous endotoxin (ETX, 1.5 mg/kg) or saline. \*:  $p < 0.05$  for ADR DENERV post endotoxin vs. 0 min; #:  $p < 0.05$  for ADR SHAM post endotoxin vs. 0 min; +:  $p < 0.05$  for endotoxin-treated ADR DENERV vs. ADR SHAM.



following endotoxin throughout the 90 min protocol. Although the norepinephrine level at 90 min in denervated group appeared to be higher than that in the sham group, the multivariate ANOVA with repeated measures did not indicate a significant difference, most likely due to the high variance in the denervated group. The response pattern of plasma epinephrine was dramatically different between the sham group and the denervated group. As shown in figure 4-4, in sham animals, plasma epinephrine was markedly elevated at 30 min following endotoxin and remain elevated thereafter. The maximum increment, approximately 12-13 fold above control, was achieved 30-60 min post endotoxin after which the increase diminished, but remained 4 fold higher than baseline value at 90 min. In contrast, in adrenal denervated rats, the same dose of endotoxin did not induce significant enhancement of plasma epinephrine until 90 min post injection where the increment was not different from that in sham rats. Saline treatment to both sham and denervated animals did not have any significant effects on plasma catecholamines during the protocol.

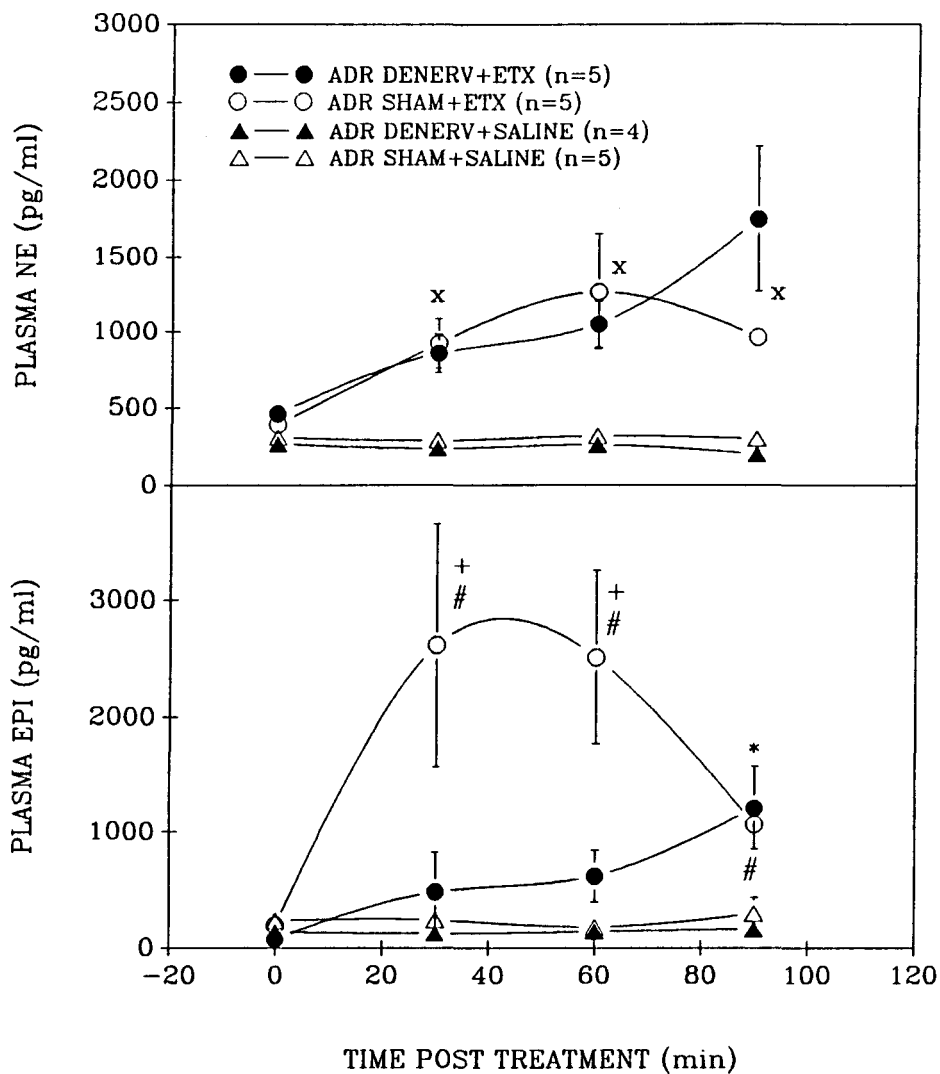


Figure 4-4. Plasma norepinephrine (NE) and epinephrine (EPI) responses in conscious rats with adrenal denervation (ADR DENERV) or sham operation (ADR SHAM) following intravenous endotoxin (ETX, 1.5 mg/kg) or saline. x:  $p < 0.05$  for both ADR DENERV and ADR SHAM post endotoxin vs. 0 min; \*:  $p < 0.05$  for ADR DENERV post endotoxin vs. 0 min; #:  $p < 0.05$  for ADR SHAM post endotoxin vs. 0 min; +:  $p < 0.05$  for endotoxin-treated ADR DENERV vs. ADR SHAM.

## DISCUSSION

The data presented in this study demonstrated that in pithed rats with no central regulatory mechanisms, intravenous endotoxin did not increase plasma norepinephrine during the 90 min protocol. However, endotoxin administration did induce a 2 to 3 fold elevation in plasma epinephrine at 90 min post injection. In contrast, data from the present and previous studies (114) showed that in intact conscious rats, both plasma norepinephrine and epinephrine were markedly elevated 30 min following endotoxin (figure 4-4). Thereafter, plasma norepinephrine remained elevated, and epinephrine declined gradually but was still significantly above pre-treatment controls. These results suggest that increases in plasma norepinephrine during endotoxemia, largely elaborated from sympathetic nerve terminals, depend on central mechanisms. Increases in plasma epinephrine with endotoxin, primarily released from the adrenal medulla, were shown to involve both central and peripheral regulations with central mechanism being dominant. This is demonstrated by the much lower and delayed elevation of plasma epinephrine in pithed rats (figure 4-2) compared to conscious rats following endotoxin (figure 4-4). Since endotoxin induced a similar elevation in plasma epinephrine in pithed rats both with and without adrenal innervation, it appears that peripheral mechanisms involve non-neurogenic modulation of release. This non-neurogenic adrenal epinephrine release was further substantiated in conscious, adrenal denervated rats, which accounted for 1/3 of the maximum epinephrine response as observed in intact rats (figure 4-4). Consistent with the finding in pithed rats, the epinephrine elevation in adrenal denervated

conscious rats also showed a lower magnitude and a delayed time course compared to intact animals. These results imply that central mechanisms are primarily responsible for the early epinephrine increments during endotoxicosis whereas the peripheral modulation contributes to the more prolonged plasma epinephrine elevation.

The present results are in agreement with previous reports where the crucial involvement of the central nervous system in the sympathoadrenal response during endotoxicosis was demonstrated. For example, acute lesion of the cerebellar fastigial nuclei in the dog prevents the recovery and maintenance of blood pressure following endotoxin-induced hypotension (149), suggesting that the sympathetic response to endotoxin challenge requires intact central regulatory mechanisms. Intact spinal cord also seems critical to endotoxin-evoked sympathetic response. Spinal transection at the C-7 level in dogs abolished the catecholamine release during endotoxicosis (71), implying that endotoxin-elicited sympathoadrenal response was presumably dependent on descending spinal pathways. Section of the splanchnic nerves in the dog eliminated the elevation of plasma epinephrine (176), and acute denervation of hemi-spleen protected against the norepinephrine depletion (253). These results suggest that increased catecholamine release during septic shock depends on nerve activation.

Unlike the present study, these authors did not report the non-neurogenic modulation of epinephrine release from the adrenal glands. This may be attributable to the fact that animals species, doses of endotoxin and sampling time course used in the present study and previous studies are different. Most importantly, it is noteworthy that the animal numbers in the previous studies were small ( $n=2-3$ ) and the results were variable.

Furthermore, the catecholamine assay used in their studies (biological or floremetric assay) was less sensitive.

The peripheral modulation of epinephrine secretion has been previously reported in pithed, endotoxic rats (112), but it was not known whether this modulation was neurally or non-neurally mediated. In both previous and present studies using pithed rats, sympathetic outflow to the adrenal glands was maintained at steady state levels by applying constant electrical stimulation to the paravertebral sympathetic chain. In those animals endotoxin-induced adrenal modulation could be due to an enhanced epinephrine release per nerve impulse or local modulation within the glands independent of nerve activity. The data from adrenal denervated rats in the present study suggest that such peripheral modulation is most likely non-neurogenic. Although incomplete adrenal denervation could account for epinephrine elevation with endotoxin, this is unlikely to be the case in the present study. First, the adrenal denervation completely abolished the increases in plasma epinephrine in response to hemorrhage-induced hypotension. Secondly, 90 min following endotoxin, adrenal denervated animals showed similar, rather than attenuated, epinephrine response compared to adrenal intact animals. The non-neurogenic stimulation of the adrenal epinephrine secretion has also been reported in a previous study in that ganglionic blockade could not prevent catecholamine release from the adrenal glands during endotoxemia in newborn rats (211).

One possible mechanism for the endotoxin-mediated non-neurogenic release may involve decreased oxygen supply to the adrenal medulla as a consequence of circulatory hypoxia and diminished blood flow. Hypoxia has been shown to be a potent stimulus for

adrenal catecholamine secretion in newborn rats (212), but in adult rats hypoxia effects are not prominent on catecholamine release from the adrenal gland (212). Alternatively, certain endogenous substances released into the circulation during endotoxicosis have also been demonstrated to act directly on the adrenal gland or cultured adrenal chromaffin cells to stimulate catecholamine secretion. Those substances include histamine, bradykinin, angiotensin, prostaglandins, and vasoactive intestinal polypeptide (130,141,153,177). Prolonged incubation of adrenal chromaffin cells with endotoxin *in vitro* also enhanced catecholamine release (225). Recent studies have shown that protein kinase C might be involved in endotoxin effects and activation of protein kinase C is associated with enhanced catecholamine release from cultured chromaffin cells (151,229).

The nature of the involvement of the central nervous system in mediating the sympathoadrenal activation during endotoxicosis is unknown. Although endotoxin-induced hypotension may unload the arterial baroreceptors and result in the sympathoadrenal activation via baroreflex deactivation, numerous studies have shown that the sympathoadrenal system can still be markedly activated during endotoxicosis in the absence of hypotension or remain activated after restoration of blood pressure (115,185). Acidosis with resultant alteration in pH may also increase the sympathetic outflow, possibly via stimulating the peripheral or central chemoreceptors (50). The activation of the hypothalamic-pituitary-adrenal axis, which frequently occurs in sepsis, has been linked to the activation of the sympathoadrenal system (38). Recent studies have suggested that most endotoxin effects are mediated by releasing cytokines (79). Some of

the cytokine effects involve the central nervous system and may be initiated in the brain (118,194). Elevation of plasma catecholamines following systemic or intracerebroventricular administration of tumor necrosis factor or interleukin-1 has been demonstrated (21,202,213,236).

Hemodynamic responses to endotoxin challenge in the present study appeared to follow the circulating catecholamine levels. Intravenous administration of endotoxin to pithed rats reduced the arterial blood pressure in a more rapid and severe manner and without a transient recovery compared with conscious or anesthetized rats receiving similar dose of endotoxin (114). Such results may be accounted for by the elimination of centrally-mediated compensatory mechanisms such as the major elevation of plasma catecholamines as a consequence of pithing. Thus, pithed rats are much susceptible to endotoxin insult. The late tachycardia in pithed rats was presumably due to the late increased circulating epinephrine from the adrenal gland. The hypotension and tachycardia responses following endotoxin in conscious animals were consistent with many previous results (113,114). The earlier and more profound endotoxin-induced decreases in MBP in denervated animals compared with sham rats are apparently attributable to the abolition of neurally mediated early epinephrine response. The explanation for greater tachycardia response at 60 min post endotoxin in denervated rats in comparison to that in sham rats is not clear.

Results from the present experiments suggest that plasma norepinephrine response to endotoxic challenge is solely mediated by central mechanisms whereas epinephrine response involves both central and peripheral regulations. Centrally-mediated mechanisms

are dominant and responsible for the major and early elevation of circulating epinephrine. Peripheral modulation of adrenal epinephrine secretion, most likely non-neurogenic, contributes to a lesser and prolonged epinephrine response during endotoxemia.



## CHAPTER V

### PLASMA CATECHOLAMINE RESPONSE IN SINOARTIC DENERVATED RATS FOLLOWING ENDOTOXIN

#### INTRODUCTION

Hyperactivation of the sympathoadrenal system is generally considered to be one of the important pathogenic components during the development of septic shock (157,176,251). The signals responsible for mediating the sympathoadrenal activation during septic challenge are not clear and are basically unexplored. Since hypotension typically occurs along with increases in heart rate, sympathetic discharge and plasma catecholamines during endotoxemia (93,113,185), it is possible that endotoxin-induced hypotension may be the primary cause of this activation by deactivating arterial baroreflexes. It is also important to recognize that in addition to hypotension, decreased pulsatile pressure without significant changes in mean blood pressure reduces the baroreceptor activity as well (46,47,70). A study reported by Chapleau et al. (48) showed that an endogenous factor derived from activated endothelial cells also diminished the baroreceptor afferent discharge. These findings may have implications in the sympathetic activation during sepsis, such that baroreflex deactivation, as a result of decreased

baroreceptor activity, may contribute to the enhanced sympathetic outflow.

A variety of experimental findings have challenged the concept that hypotension-induced baroreflex deactivation is a dominant factor in mediating sympathoadrenal response during septic shock. In rats treated with *E. coli* bacteria, plasma catecholamines are markedly elevated while blood pressure is not significantly altered (115). In experiments involving bolus administration of endotoxin to conscious rats, plasma catecholamines were markedly elevated before hypotension occurred (114). Direct nerve recording in endotoxic animals indicated that increased sympathetic discharges took place prior to the onset of hypotension and additional increases in nerve activity occurred following the fall in blood pressure (185). This activity persisted after blood pressure was restored to basal levels by volume infusion. Collectively, such evidence suggests that hypotension does not necessarily initiate or maintain the increased plasma catecholamines which occur with septic insult.

The aim of the present study was to examine the role of arterial baroreceptors in mediating sympathoadrenal activation during endotoxicosis by using acutely, as well as chronically, sinoaortic-baroreceptor-denervated rats. It was hypothesized that baroreflex deactivation may not be the major factor in mediating the sympathoadrenal activation during developing endotoxic shock and, thus, the denervation of the arterial baroreceptors would not eliminate the marked elevation of plasma catecholamines that occurs following administration of endotoxin. Since endotoxin will initiate dramatic increases in plasma catecholamines in rats, experiments were designed to treat all animals with endotoxin and to focus on potential differences between sinoaortic-baroreceptor-denervated and sham-

treated groups.

## MATERIALS AND METHODS

### Experimental Protocols

Three groups of experiments were conducted in male rats weighing  $386 \pm 12$  grams. The first group of experiments were performed in acutely sinoaortic baroreceptor denervated rats to obtain a preliminary assessment of the baroreceptor role in mediating the sympathoadrenal activation during endotoxemia. Bilateral sinoaortic denervation (SAD,  $n=6$ ) or sham operation (SHAM,  $n=6$ ) was performed under xylazine (10 mg/kg, i.p.) and  $\alpha$ -chloralose (70 mg/kg, i.p.) anesthesia as described below. Femoral arterial and venous cannulae were implanted, and baroreceptor reflexes were tested to confirm the completeness of SAD. After approximately 1.5 hours equilibration, endotoxin (5 mg/kg) was injected intravenously. MBP and HR were recorded continuously, and arterial blood samples were collected at 0, 30, 60, and 90 minutes after endotoxin administration.

The second group of experiments were performed in chronically sinoaortic baroreceptor denervated rats. This design was employed to prevent possible misleading results as a consequence of anesthesia and acute surgery. SAD and sham operation were performed as in acute preparations, and both groups of animals received 40,000 U penicillin (i.m.) following the surgery. After a 2-4 week recovery period, all rats underwent surgical implantation of femoral arterial and venous cannulae under halothane anesthesia (2.0% in oxygen). On the following day, baroreflexes were tested to confirm the SAD as described before. Endotoxin (5 mg/kg) was then given intravenously to both

SAD (n=4) and sham (n=5) rats. MBP and HR were recorded continuously, and blood samples were taken at 0, 30, 60, and 90 minutes after endotoxin administration. Separate groups of SAD (n=4) and sham animals (n=5) were given saline in place of endotoxin and were followed during the course of above protocol with subsequent sample collection and analysis.

The third group of experiments were designed to assess the singular contribution of hypotension-induced baroreceptor reflexes to the sympathetic activation in the absence of endotoxin treatment. The cardiovascular and sympathoadrenal responses of rats with either SAD (n=5) or sham operation (n=5) to hypotension induced by hydralazine, a vasodilator, were determined. The animals were prepared as in the second group of experiments, and on the day of the experiment, the baroreceptor reflex testing confirmed the completeness of the denervation. Hydralazine (1 mg/kg) was given intravenously as a bolus injection, and MBP and HR were monitored continuously and blood samples were taken at 0, 15, and 30 minutes.

#### Sinoaortic Baroreceptor Denervation

Sinoaortic baroreceptor denervation (SAD) was performed as described in chapter III. For aortic baroreceptor denervation, the superior laryngeal nerves, the aortic depressor nerves, and the superior cervical ganglia including a small segment of sympathetic chain were sectioned. For carotid sinus denervation, all connective tissue and nerve fibers were stripped from the thyroid, occipital, internal, external and common carotid arteries. Sham operation was performed by exposing the carotid sinuses bilaterally

without dissection of the region. Completeness of baroreceptor denervation was verified by determining the reflex changes in heart rate following pharmacological alterations in blood pressure. See general methods for details.

#### Blood Sample Collection and Catecholamine Assay

Arterial blood samples (500  $\mu$ l) were collected at pre-determined times and treated as described in chapter III. Plasma levels of norepinephrine (NE) and epinephrine (EPI) were assayed in duplicate 50  $\mu$ l samples using the radio-enzymatic thin-layer chromatographic procedure described in general methods.

#### Materials

Endotoxin (derived from S. enteritidis) was prepared fresh daily in 0.9% saline (3 mg/ml). Hydralazine was obtained in 20 mg/ml vial and diluted with saline to 1 mg/ml for i.v. injection. Materials for catecholamine assays were obtained in kit form from Amersham (Arlington Heights, IL).

#### Data analysis

Data are expressed as Mean $\pm$ SEM (n). A p value less than 0.05 was considered to be statistically significant. Cardiovascular responses and plasma catecholamines following endotoxin or hydralazine were compared using analysis of variance with repeated measurements and student-Newman-Keul's test for individual comparisons. Blood pressure and associated heart rate response to phenylephrine or nitroglycerine infusion

to assess baroreceptor denervation were compared between the SAD and sham groups with unpaired student t tests.

## RESULTS

### Effects of SAD on Baroreflex Control of Heart Rate

Effectiveness of the baroreceptor reflex was tested in all denervated and sham animals. Pharmacologic-induced changes in blood pressure were very transient lasting only a few minutes. As presented in figure 5-1, the increase in MBP induced by phenylephrine was not different between sham and SAD rats in either acute or chronic preparations although the chronic animals appeared to have a greater increase than the acute preparations. Nitroglycerine also caused a similar fall in MBP in acutely prepared SAD and sham animals, whereas it induced a slightly greater drop of MBP in chronic SAD compared to sham rats. Both acute and chronic SAD eliminated the bradycardia caused by the phenylephrine-induced hypertension and the tachycardia associated with the nitroglycerine-induced hypotension. In contrast, phenylephrine and nitroglycerine infusion evoked significant bradycardia and tachycardia, respectively, in sham-treated animals. Low control values of plasma catecholamines prior to the administration of endotoxin to either SAD or sham group suggest that there were no prolonged effects of the transient manipulation of blood pressure to test baroreceptor reflexes.

### Effects of Acute SAD on Hemodynamic and Sympathoadrenal Activation during Endotoxemia

The repeated measures ANOVA showed that the pattern of blood pressure responses at various times following endotoxin was the same in SAD and sham animals. Within



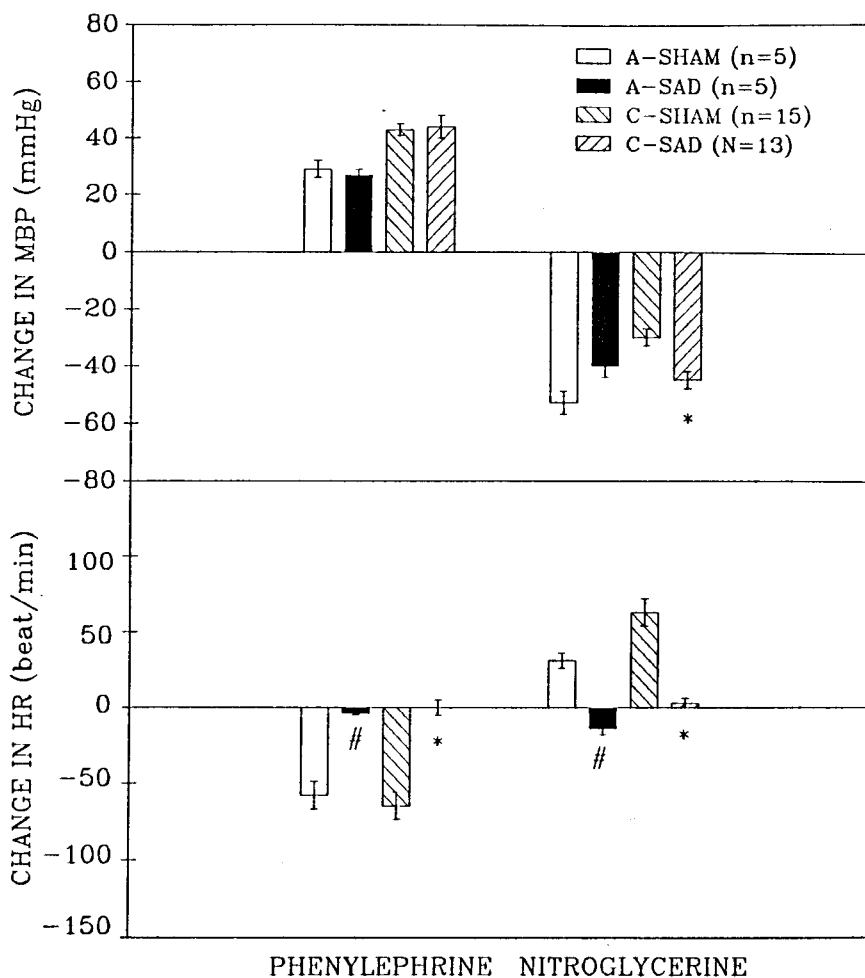


Figure 5-1. Changes of mean blood pressure (MBP) and heart rate (HR) in response to intravenous phenylephrine and nitroglycerine infusion in acute sham operation (A-SHAM), acute sinoaortic denervation (A-SAD), chronic sham operation (C-SHAM) and chronic sinoaortic denervation (C-SAD) groups. #:  $p < 0.05$  for A-SAD vs. A-SHAM; \*:  $p < 0.05$  for C-SAD vs. C-SHAM. Unpaired-student t test.

each group (i.e. SAD or sham) there were no differences in MBP at 0, 30, 60 and 90 minutes post endotoxin (figure 5-2). However, there was a significant overall difference (regardless of time) in blood pressures between the SAD and the sham groups following endotoxin administration. This difference can be seen in figure 5-2, where MBP was always lower in the sham group compared to the SAD group. HR was significantly increased (above baseline values) in SAD animals at 30, 60 and 90 minutes post endotoxin, whereas in the sham group, HR was significantly greater at 60 and 90 minutes compared to both 0 and 30 minutes.

As shown in figure 5-3, the pattern of plasma catecholamine response following endotoxin exposure was similar in SAD versus sham animals. In both groups, plasma NE and EPI become significantly elevated at 30, 60 and 90 min following endotoxin (figure 5-3).

#### Effects of Chronic SAD on Hemodynamic and Sympathoadrenal Activation during Endotoxemia

The pattern of blood pressure responses to endotoxin treatment was not altered by chronic SAD compared to sham group (figure 5-4). Endotoxin treatment did not induce significant hypotension in either group. Pre-endotoxin HR was higher in SAD rats compared with sham rats. There was also a gradual increase in HR with time following endotoxin administration in the sham group but not in the SAD group (figure 5-4).

As indicated in figure 5-5, the SAD and sham groups had a similar pattern of plasma NE elevation in response to endotoxin. Although it appeared that SAD animals

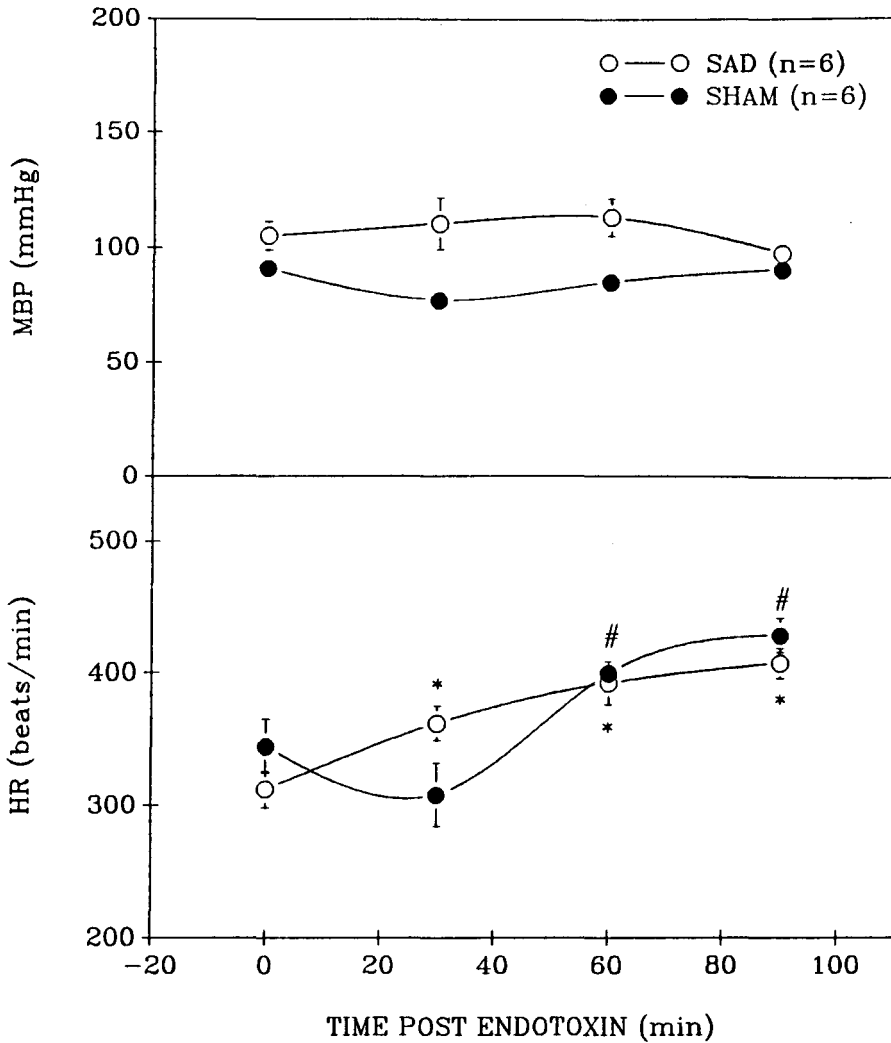


Figure 5-2. Mean blood pressure (MBP) and heart rate (HR) responses in anesthetized rats with acute sinoaortic denervation (SAD) or acute sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. \*:  $p < 0.05$  for SAD post endotoxin vs. 0 min; #:  $p < 0.05$  for SHAM post endotoxin vs. 0 min.

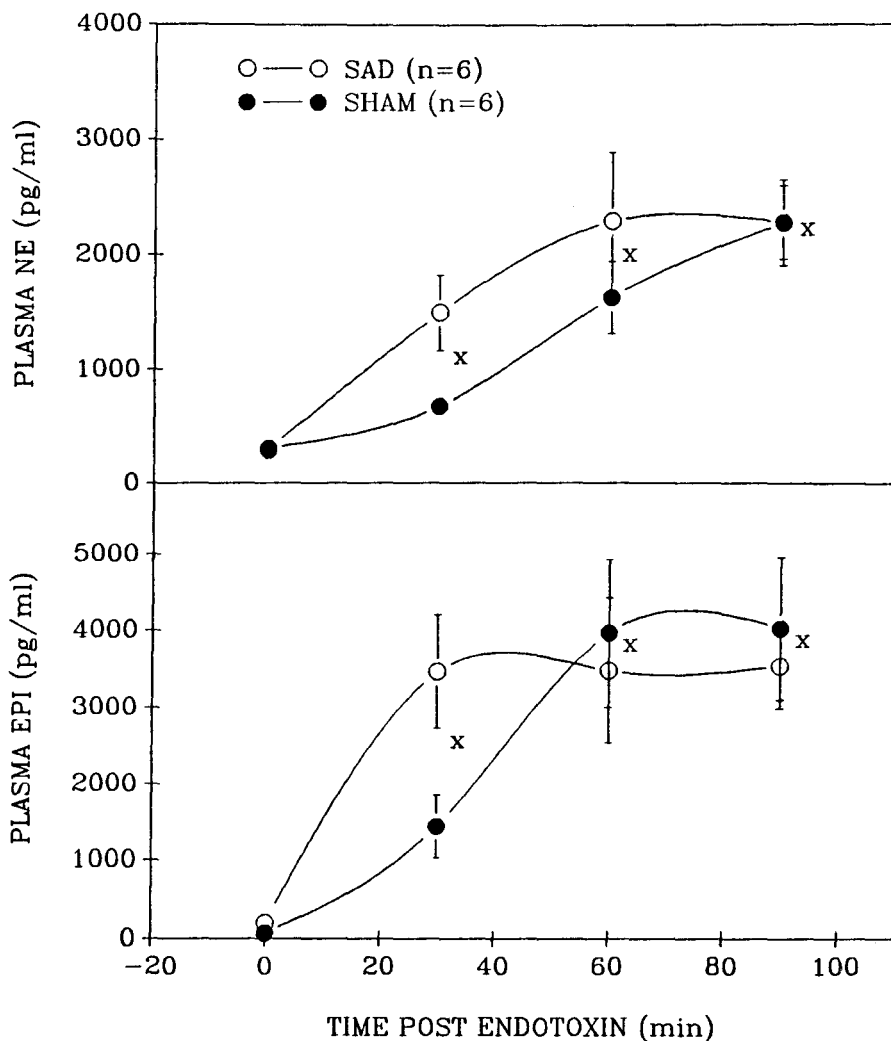


Figure 5-3. Plasma norepinephrine (NE) and epinephrine (EPI) concentrations in anesthetized rats with acute sinoaortic denervation (SAD) or acute sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. x:  $p < 0.05$  for both SAD and SHAM post endotoxin vs. 0 min.

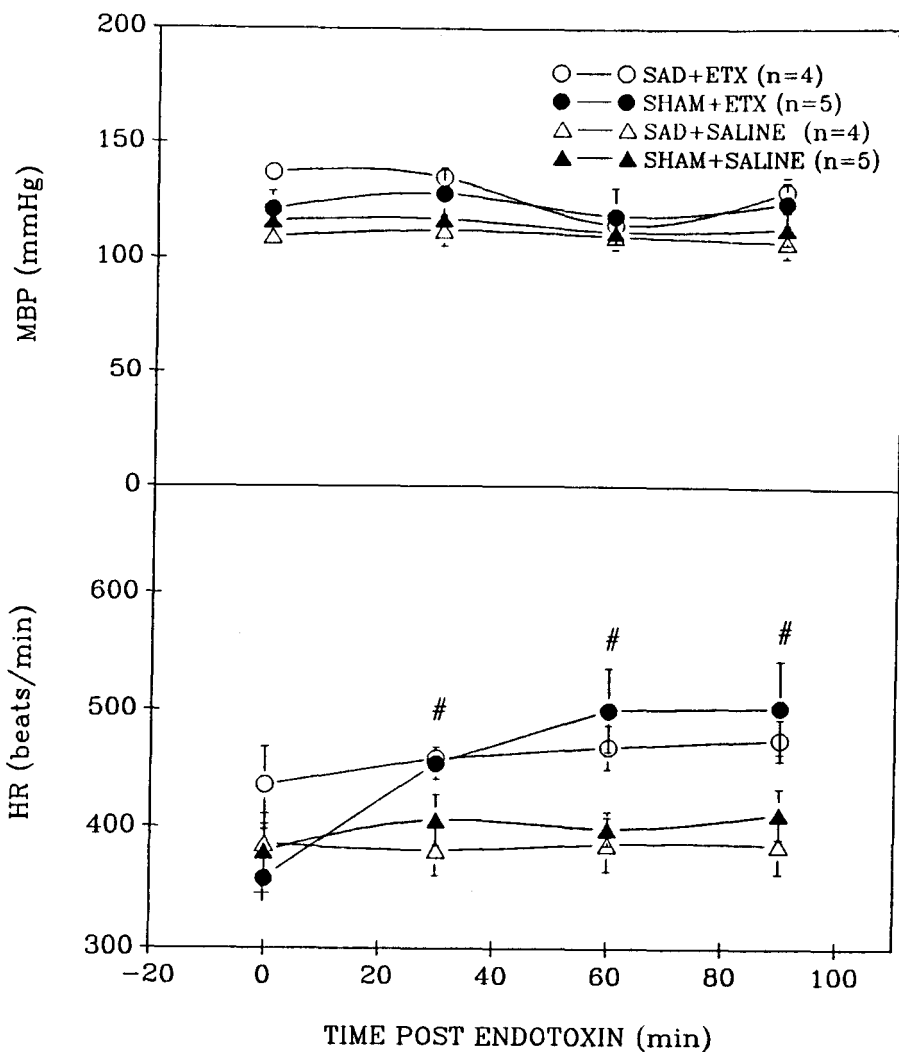


Figure 5-4. Mean blood pressure (MBP) and heart rate (HR) responses in conscious rats with chronic sinoaortic denervation (SAD) or chronic sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. #:  $p < 0.05$  for SHAM post endotoxin vs. 0 min. +:  $p < 0.05$  for endotoxin-treated SAD vs. SHAM.

had higher plasma EPI than sham animals at 30 min, lack of statistical difference in the between the two groups. Following endotoxin treatment, there were marked elevation plasma catecholamines in both SAD and sham groups over time, i.e. plasma NE and EPI at 30, 60 and 90 minutes post endotoxin were significantly increased compared to baseline values (figure 5-5). Saline-treated SAD and sham rats (as controls) showed no alterations in blood pressure, heart rate or plasma catecholamines during the experimental protocol.

#### Effects of SAD on Hemodynamic and Sympathoadrenal Response to Hydralazine

To test the degree of hypotension-induced sympathoadrenal activation with and without baroreceptor denervation in the absence of endotoxin, measurements of hemodynamic and plasma catecholamines were made following intravenous administration of hydralazine (1 mg/kg). This treatment caused a rapid and persistent reduction in mean blood pressure of both SAD and sham groups over 30 minutes (figure 5-6). However, the decrease in blood pressure was greater in the SAD compared to the sham group at 5, 10, 15 and 20 minutes post injection, with a peak fall to  $63 \pm 4$  mmHg in the SAD and  $78 \pm 3$  mmHg in the sham animals. This more profound hypotension in the SAD group is most likely due to the absence of baroreflex compensation for the blood pressure fall. The decreases in blood pressure were associated with increases in heart rate in both groups over time (figure 5-6). Although the sham group exhibited sustained tachycardia while the SAD animals showed variable changes in heart rate post hydralazine, the ANOVA statistic showed no significant difference between their patterns, likely due to

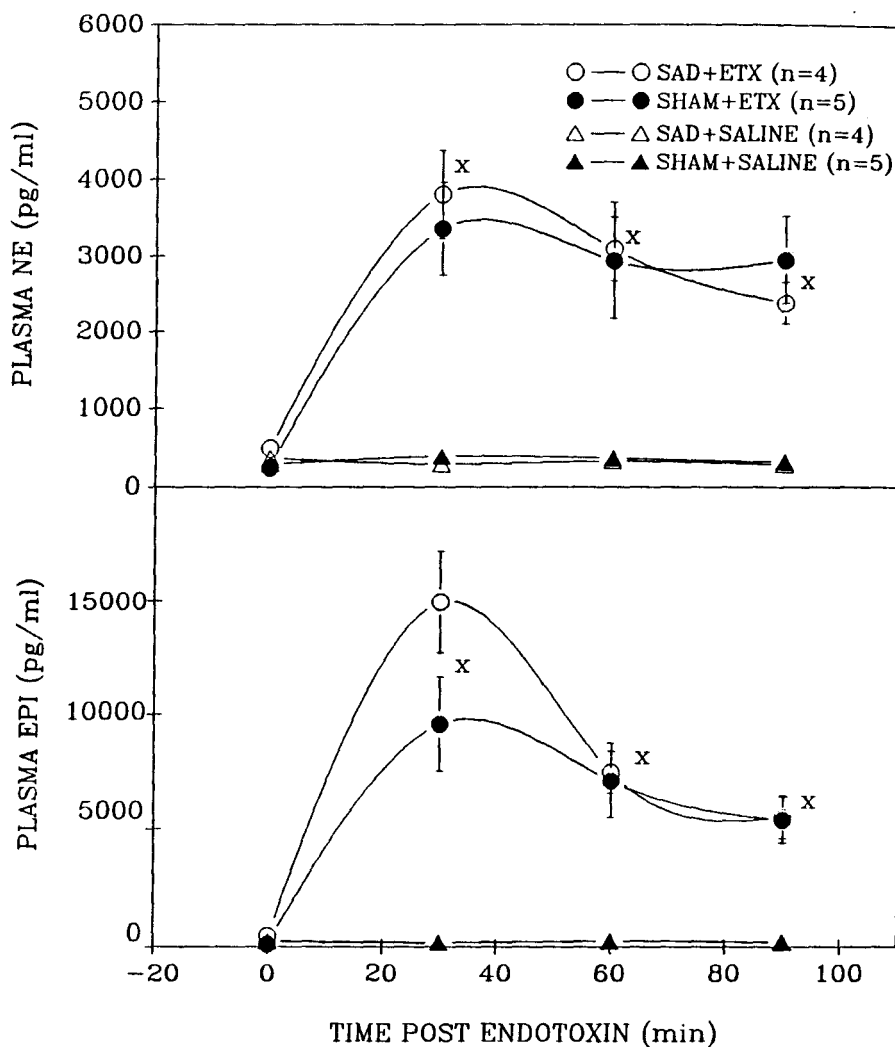


Figure 5-5. Plasma norepinephrine (NE) and epinephrine (EPI) concentrations in conscious rats with chronic sinoaortic denervation (SAD) or chronic sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. x:  $p < 0.05$  for both SAD and SHAM post endotoxin vs. 0 min.

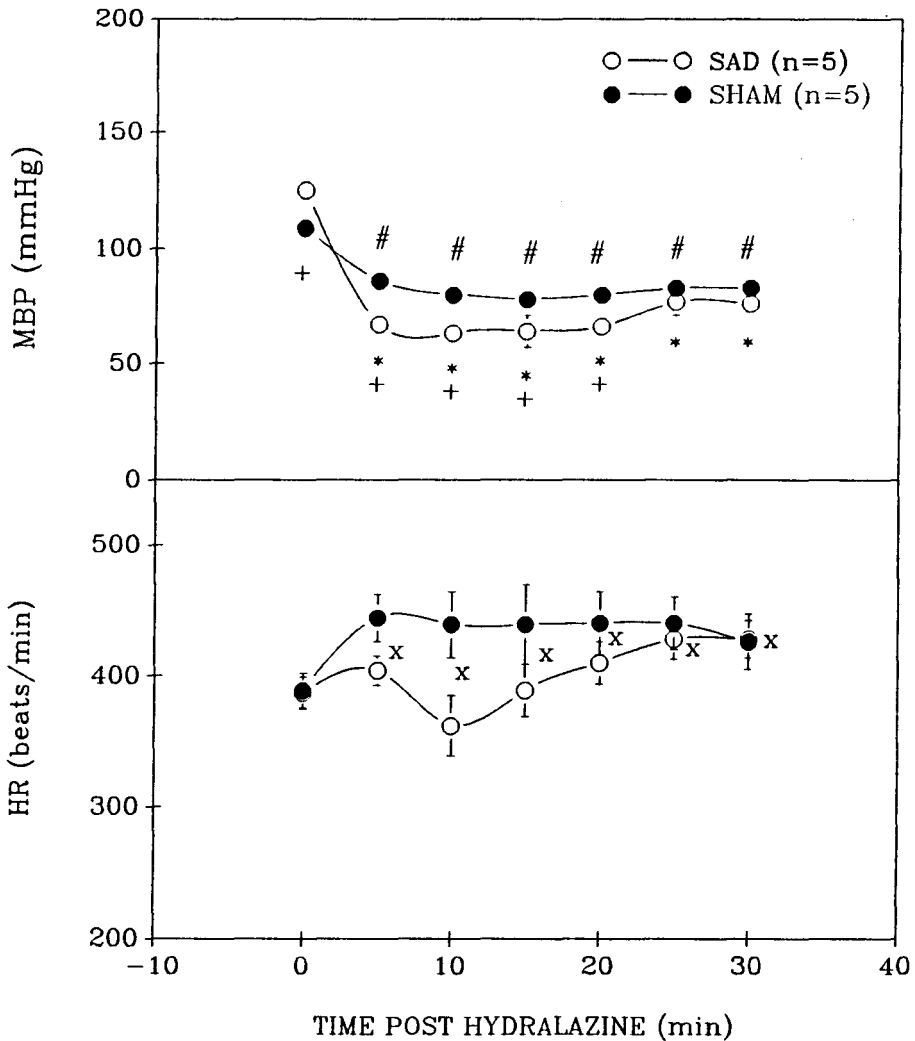


Figure 5-6. Mean blood pressure (MBP) and heart rate (HR) responses in conscious rats with chronic sinoaortic denervation (SAD) or sham operation (SHAM) following hydralazine infusion (1 mg/kg, i.v.). \*:  $p < 0.05$  for SAD post hydralazine vs. 0 min; #:  $p < 0.05$  for SHAM post hydralazine vs. 0 min; +:  $p < 0.05$  for SAD vs. SHAM; x:  $p < 0.05$  for both SAD and SHAM post hydralazine vs. 0 min.



the relatively high total variance. SAD treatment did alter the response pattern of plasma catecholamines to hydralazine compared to the sham.

As shown in figure 5-7, plasma NE and EPI of the sham group were markedly elevated at 15 and 30 minutes following hydralazine-induced hypotension, whereas in the SAD group, plasma NE and EPI were not significantly elevated in response to a greater drop in blood pressure. This indicates that SAD treatment greatly reduced the hypotension-induced baroreflex-mediated sympathoadrenal activation. Furthermore, the increases in plasma catecholamines of the SAD rats were significantly less than that of the sham group at 15 and 30 minutes post injection (figure 5-7).

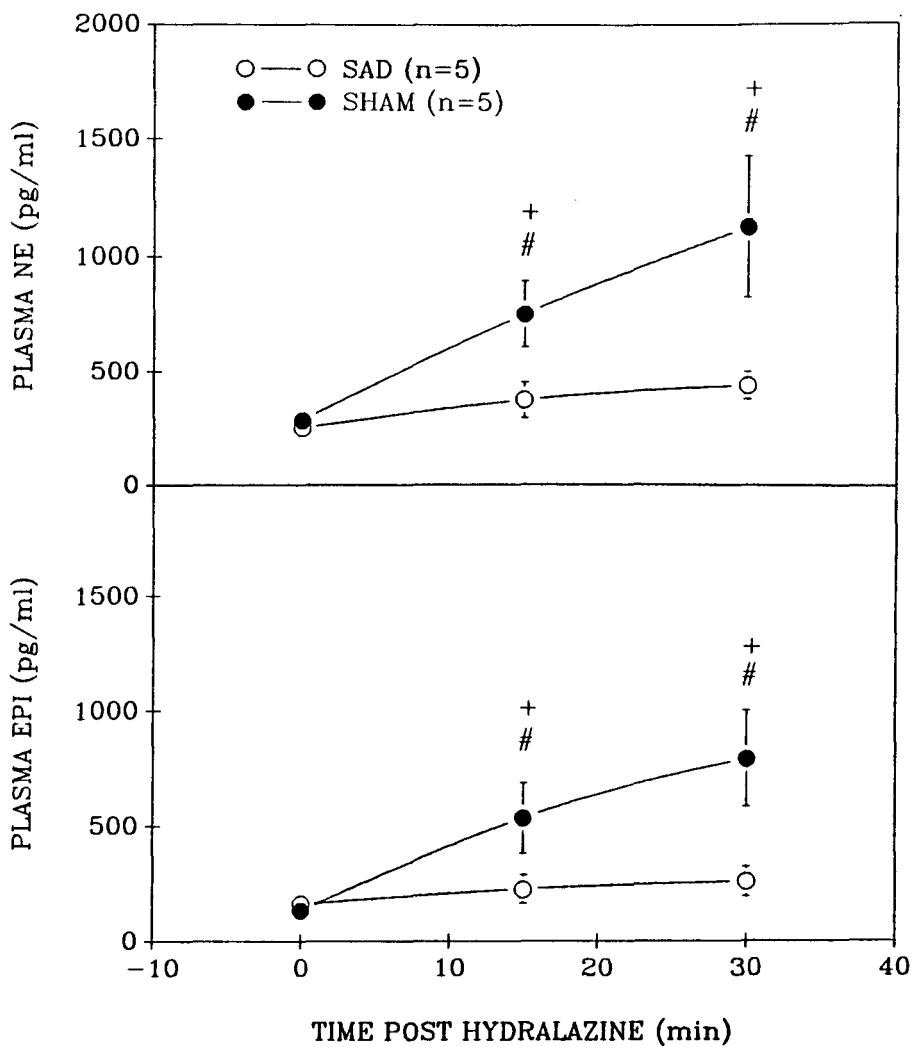


Figure 5-7. Plasma norepinephrine (NE) and epinephrine (EPI) concentrations in conscious rats with chronic sinoaortic denervation (SAD) or sham operation (SHAM) following hydralazine infusion (1 mg/kg, i.v.). #:  $p < 0.05$  for SHAM post hydralazine vs. 0 min. +:  $p < 0.05$  for SAD vs. SHAM.

## DISCUSSION

The mechanisms responsible for mediating the sympathoadrenal activation during endotoxemia are not clear. It is expected that a decrease in systemic blood pressure induced by endotoxin would unload the arterial baroreceptors and reflexly enhance sympathetic outflow. Previous studies by Halinen et al. (92,93) demonstrated that endotoxin administration induced a drop of blood pressure in anesthetized dogs and cats, which was associated with cessation of the aortic arch baroreceptor afferent impulses and increased cardiac sympathetic efferent nerve discharges during their 15 minute protocol. The authors concluded that the sympathetic pathways were primarily activated through cardiovascular receptor reflexes to maintain blood pressure. However, they did not report what happened later than 15 minutes. Furthermore, their findings did not necessarily demonstrate baroreflex mechanism as an exclusive mechanism for sympathoadrenal activation during endotoxemia or rule out the possibility of other parallel mechanisms for initiating the increased central sympathetic outflow.

The experimental data presented here show that SAD treatment did not significantly alter the response pattern of plasma catecholamines to endotoxin when compared to sham-operated controls. Although in both figure 5-3 and 5-5 it would appear that endotoxin induced plasma epinephrine elevations were greater at early stage in denervated rats compared to sham rats, the change was in a direction opposite from that anticipated if baroreceptors primarily mediate the sympathetic response. It is also important to recognize that the hemodynamic and sympathetic alterations observed in the present study

are due to the effects of endotoxin and not to the pressure-monitoring or blood sampling procedures since saline treatment in SAD and sham rats failed to induce changes in these variables. Collectively, these results suggest that arterial baroreceptor disinhibition is not the major factor stimulating the sympathoadrenal response to endotoxemia and suggests that non-baroreflex mechanisms may be involved. The profound sympathetic activation occurring in the absence of hypotension further supports that hypotension-induced baroreflex disinhibition is not essential for sympathoadrenal responses during endotoxemia.

Although previous studies in conscious rats with intact baroreceptors reported that endotoxin-induced sympathetic activation occurred in the presence of hypotension (113), sympathetic changes were observed in the absence of significant hypotension in the present study. This discrepancy may be attributable to the fact that the animal preparations were apparently different between the two studies. The chronic recovery (2-4 weeks) from a surgery in the present study may somehow altered the blood pressure response as compared to acute preparations. The lack of hypotension, however, does not limit the examination of the proposed hypothesis since the pattern and magnitude of plasma catecholamine response to endotoxin in sham rats were comparable to those observed in previous study where hypotension existed (196). Furthermore, decreases in baroreceptor afferent nerve activity and associated baroreflex deactivation can be induced by factors other than hypotension, such as decreases in pulsatile pressure or endothelial factors (46,47,70). Although a significant hypotension in the present protocol may presumably lead to an even greater elevation in plasma catecholamines, it is also possible

that severe hypotension may result in diminished central nervous system function and impaired sympathetic outflow.

Administration of bacterial endotoxin to anesthetized dogs subjected to SAD have also been reported by Koyoma et al. (129). Their results showed that endotoxin administration induced a fall in both blood pressure and cardiac output with a resultant increase in total peripheral resistance in both SAD and sham-operated animals. Such increases in total peripheral resistance reflect increased peripheral sympathetic activity to peripheral resistance vessels along with decreased cardiac output. In this sense, the present results are consistent with their findings. Studies by Mills (159) demonstrated that increased sympathetic discharge occurred before endotoxin-induced hypotension in rats, indicating that the initiation of sympathetic activation is independent of the baroreflex. Following the fall of blood pressure, sympathetic nerve activity increased further and was maintained even after blood pressure was restored to basal levels, suggesting that maintenance of sympathoadrenal activation is also independent of the baroreflex. Palsson et al. (185) observed somewhat similar findings with bolus injection of endotoxin in conscious rats in which endotoxin induced a markedly increased renal sympathetic nerve activity, that was not diminished after correcting hypotension.

Additional findings of the present study demonstrate the effectiveness of SAD treatment to alter the response pattern of plasma catecholamines to hydralazine-induced hypotension. Plasma catecholamines were significantly increased following hydralazine in the sham controls but not in the SAD rats during the 30 minute protocol. Thus, SAD appears to be an appropriate model to eliminate or significantly blunt the baroreceptor-

induced sympathoadrenal activation and to test whether such activation occurs during endotoxemia. The increments of plasma catecholamines induced by hydralazine-evoked hypotension were far less than that induced by endotoxin administration. Such results further suggest that mechanisms other than hypotension-induced baroreflex activation are involved in the sympathoadrenal response to endotoxin.

In both acute and chronic preparations, SAD animals had higher baseline blood pressure compared to sham animals. Such relative hypertension is in agreement with most of the previous reports (169,239) and may be attributable to the elimination of afferent input to the central nervous system, which normally inhibits sympathetic pathways. Thus, sinoaortic denervation results in higher basal central sympathetic outflow. In acute preparations blood pressures in the sham group were always lower compared to that in the SAD group post endotoxin. In an isolated carotid sinus model, Trank and Visscher (238) found that the relationship of baroreceptor discharge frequency versus intrasinus pressure was shifted to the left during endotoxemia. Baroreceptor-nerve activity was always higher for a given pressure stimulus, which led to a reflex inhibition of sympathetic outflow. By denervating arterial baroreceptors, this baroreceptor resetting might be eliminated, which could account for the sympathetic outflow being higher in SAD rats than in baroreflex intact rats. However, this explanation is unlikely to account for the difference in blood pressure following endotoxin between the SAD and sham groups, since this difference was not seen in chronic preparations. The most plausible factors responsible for the difference between acute and chronic preparations may be  $\alpha$ -chloralose anesthesia and the acute effect of SAD. Alpha-chloralose anesthesia has been

reported to possibly augment baroreceptor reflexes to maintain normal blood pressure (39). Acute SAD eliminates the inhibitory baroreflex and results in relative hypertension.

In the literature conflicting results concerning the HR changes following endotoxin have been reported (115,128,137). Both tachycardiac and bradycardic reactions have been observed. These different results might be due to differences in animal species, anesthesia, basal HR, and reactive states of the baroreceptor reflex. In the present study HR changes induced by endotoxin are not uniform from one preparation to another. In acute experiments HR of sham animals was not significantly elevated until 60 and 90 minutes post endotoxin, whereas the HR of SAD animals continuously increased with time following endotoxin. In chronic experiments, HR was increased with time post endotoxin in both sham and SAD animals, but only reached statistical significance in the sham group. Since endotoxin-treated SAD rats had higher basal HR compared to sham rats, the presence of tachycardia in SAD rats may make a further acceleration undramatic. Hydralazine-induced hypotension evoked a sustained tachycardia in sham animals, whereas SAD rats exhibited variable changes in HR.

In conclusion, the data from the present study suggest that the afferent input from the arterial baroreceptors is not the major factor, or not even essential, in mediating sympathoadrenal activation during endotoxemia. Non-baroreflexogenic mechanisms may be involved in stimulating such activation.

## CHAPTER VI

### RENAL SYMPATHETIC RESPONSE IN SINOARTIC DENERVATED, CONSCIOUS RATS FOLLOWING ENDOTOXIN

#### INTRODUCTION

Endotoxin-induced hypotension and associated baroreceptor reflexes have been suggested to be the primary cause of the sympathoadrenal activation during endotoxemia (92,93,148). However, accumulating experimental findings suggest that the sympathoadrenal system can be markedly activated by endotoxin or bacteria in the absence of significant hypotension (89,115) and remains activated after restoration of blood pressure with volume infusion (159). Results from previous chapter further demonstrated that in sinoartic baroreceptor denervated rats bacterial endotoxin administration elevated plasma catecholamines to a similar, if not higher, level compared to sham-operated animals (255). These results suggest that the afferent input from the arterial baroreceptors is not essential in elevating plasma catecholamines with endotoxemia. Although no hypotension was observed in those animals following endotoxin, decreased baroreceptor activity and resultant baroreflex deactivation can be evoked by factors other than hypotension (see Chapter V for details).



Plasma catecholamines, released from the peripheral sympathetic nerve terminals and the adrenal medulla, have been widely used as an index of sympathoadrenal activity. However, circulating catecholamine levels can be influenced not only by their release but also by neuronal uptake and peripheral catabolism (84), such that increases in plasma catecholamines may occur without alterations in catecholamine release (152). Therefore, plasma catecholamines may not adequately quantitate sympathetic activity as compared to direct nerve recording. Despite the frequent demonstration of an elevation of plasma catecholamines during clinical or experimental sepsis, contradictory results have been reported with direct measurements of sympathetic nerve activity, i.e. direct nerve recordings (127,128,159,185). In addition, differential sympathetic responses have been observed during shock states (232,242), such that the sympathetic outflow is increased to one organ while decreased to another. Thus, although plasma catecholamines and sympathetic discharge both represent the functional state of the sympathoadrenal system, they may not always change in a parallel manner.

Considering these factors as well as the fact that plasma catecholamines and sympathetic discharge have never been simultaneously determined in septic animals, the present study was conducted. Both plasma catecholamine measurements and renal sympathetic recording techniques were employed to assess the contributions of afferent neural input from arterial baroreceptors to sympathoadrenal responses in conscious, unstrained endotoxic rats. Endotoxin was used as a septic agent based on the large body of literature showing that endotoxin administration to experimental animals duplicates many pathophysiological changes observed in patients with sepsis or septic shock

(104,122,168,171). To accomplish our objective, conscious rats with either sinoaortic denervation (SAD) or intact baroreceptors were subjected to intravenous endotoxin treatment. Renal sympathetic nerve activity (RSNA) and circulating catecholamines were monitored both before and following the injection of endotoxin. It was hypothesized that removal of baroreceptor afferent input would not eliminate the endotoxin-evoked peripheral sympathoadrenal activation, as reflected in both the renal sympathetic nerve activity and plasma catecholamines.

## MATERIALS AND METHODS

### Experimental Protocols

Holzman Rats, weighing  $396 \pm 12$  grams, were anesthetized with sodium pentobarbital (60 mg/Kg, i.p.). Sinoaortic baroreceptor denervation and sham operation were performed as described in chapter III. Each animal received 40,000 U penicillin (i.m.) following the surgery. After a 2-4 week recovery period, all rats underwent surgical implantation of arterial and venous cannulae as well as electrode placement for recording renal sympathetic nerve activity under pentobarbital anesthesia (60 mg/kg, i.p.). After a 24 hr recovery, cannulae and electrodes were extended to instruments to record blood pressure and renal sympathetic nerve activity (RSNA). Following a 0.5 hr equilibration period, baroreflexes were tested to confirm the SAD. Endotoxin (5 mg/Kg) was then given intravenously to both SAD (n=6) and sham animals (n=7). Separate groups of SAD (n=4) and sham (n=5) rats also received equivalent amount of saline for time-matched control. Mean blood pressure (MBP), heart rate (HR), and RSNA were determined at 0, 5, 15, 30, 60, and 120 min after infusions. Blood samples were taken at 0, 15, 90, and 180 min post treatments.

### Sinoaortic Baroreceptor Denervation

Sinoaortic baroreceptor denervation (SAD) was performed by a method as described in chapter III. Briefly, for aortic baroreceptor denervation, the superior laryngeal nerves, the superior cervical ganglia including a small segment of sympathetic chain and the

aortic depressor nerves were sectioned. For carotid sinus denervation, all connective tissue and nerve fibers were stripped from the thyroid, occipital, internal, external and common carotid arteries. Sham operation was performed by exposing the carotid sinuses bilaterally without dissection of the region. Completeness of baroreceptor denervation was tested as described before.

### Sympathetic Nerve Recording

Renal sympathetic nerve activity (RSNA) was recorded from a renal nerve branch of the left greater splanchnic nerve. Briefly, the left kidney was exposed retroperitoneally, and the renal nerve branch was found in the aortic-renal artery angle. After the renal nerve branch was dissected free the nerve was carefully placed on a thin, bipolar gold electrode. When an optimal nerve signal was obtained, the electrode and the nerve was fixed with a rubber gel mixture. The electrode wires were exteriorized on the back of the neck. On the next day, the electrode wires were extended to the amplifier. The nerve signal was amplified 1,000-10,000 fold and filtered (low-frequency cutoff at 35 Hz and high-frequency cutoff at 2,000 Hz). Then the amplified signal was full-wave rectified and integrated by two separate models of integrator for simultaneous estimation of frequencies of RSNA (measured as spikes/min) and overall RSNA (measured as slopes). The rectified signal was continuously recorded on a Grass oscillograph. The background noise level for the nerve recording was determined 30 min after each animal was killed with pentobarbital overdose. All RSNA were expressed as percent of baseline control.

## Blood Sample Collection and Catecholamine Assay

Arterial blood samples (500  $\mu$ L) were collected at different time points as described above and assayed for plasma catecholamines using the radio-enzymatic thin-layer chromatographic procedure described in general methods.

## Materials

Endotoxin (derived from *S. enteritidis*) was prepared freshly in 0.9% saline (3 mg/ml). Materials for catecholamine assay were obtained in kit form from Amersham (Arlington Heights, IL). Phenylephrine, nitroglycerine and penicillin were purchased from Sigma, St. Louis, MO, Baxter, McGaw Park, IL and Pfizer, New York, NY, respectively.

## Data Analysis

Data are expressed as mean  $\pm$  SEM (n). A p value less than 0.05 was considered to be statistically significant. Cardiovascular responses, RSNA, and plasma catecholamines were compared using analysis of variance with repeated measurements and Student-Newman-Keul's test for individual comparison. Blood pressure and associated heart rate, RSNA responses to phenylephrine or nitroglycerine infusion were compared between the SAD and Sham groups with an unpaired Student t test.

## RESULTS

### Effects of SAD on Baroreflex Control of HR and RSNA:

Effectiveness of the baroreceptor reflex was tested in all animals. The pharmacologic-evoked changes in blood pressure were very transient, lasting only a few minutes. Phenylephrine administration induced an identical rise and nitroglycerine caused a similar fall of MBP in SAD rats compared to the sham group. The phenylephrine-induced hypertension was associated with profound bradycardia and inhibition of RSNA, and nitroglycerine induced-hypotension evoked significant tachycardia and excitation of RSNA in sham-treated animals (figure 6-1 and 6-3). In contrast, SAD eliminated or significantly attenuated reflex changes of HR and RSNA in response to those pharmacological alterations in blood pressure (figure 6-2 and 6-3).

### Hemodynamic Responses to Endotoxin Infusion:

The baseline MBP was not different among all animals. SAD and sham rats had similar patterns of blood pressure response after endotoxin administration with no significant alterations in MBP from control values (figure 6-4). There were also no significant differences in baseline HR between SAD and sham animals. Endotoxin induced a modest, but not significant, increase in HR in the sham group during the experiment. In contrast, SAD rats showed significant tachycardia at 5 min after endotoxin

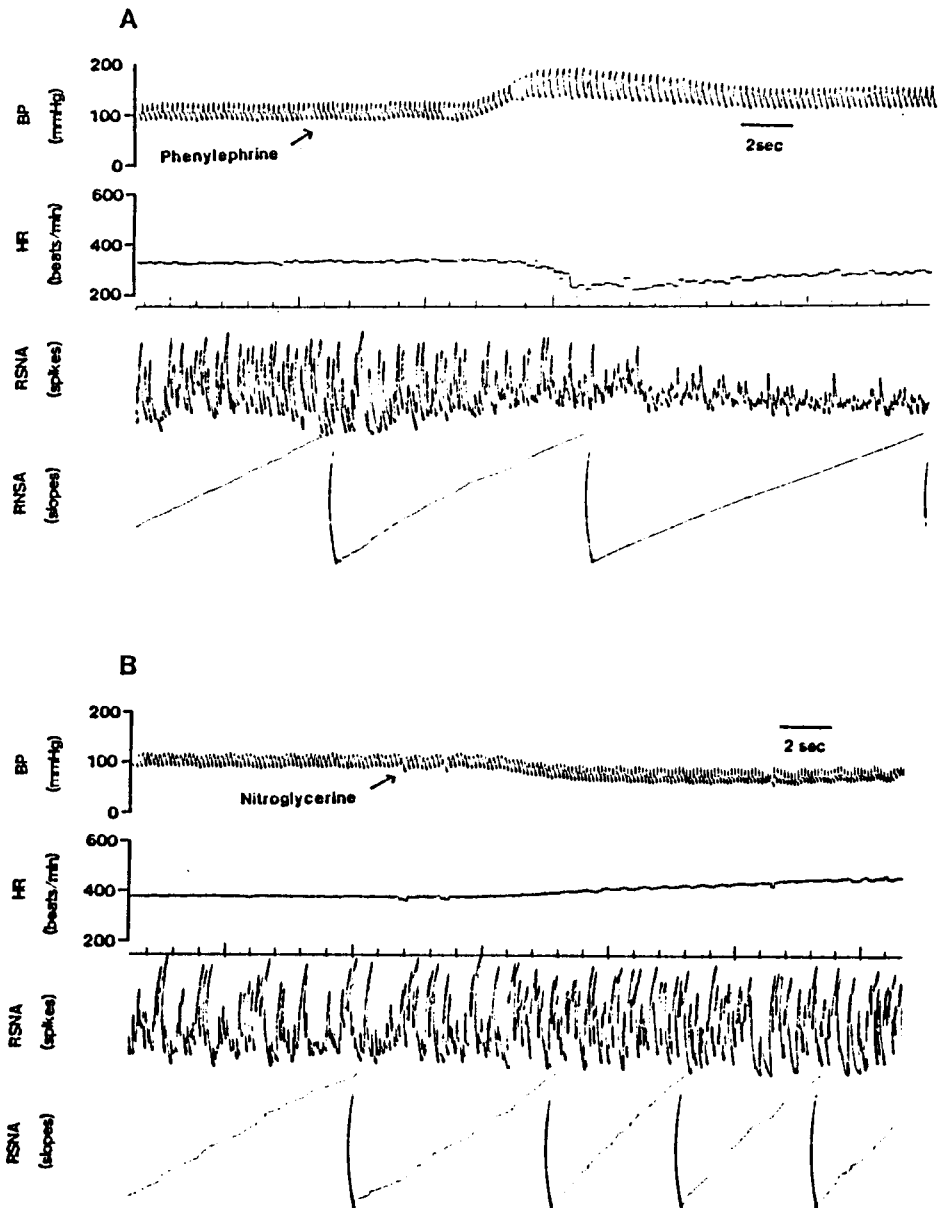


Figure 6-1. Baroreflex test from a conscious sham-operated rat showing blood pressure (BP), heart rate (HR), renal sympathetic nerve activity (RSNA) spike and slope responses to intravenous phenylephrine (A) and nitroglycerine (B).

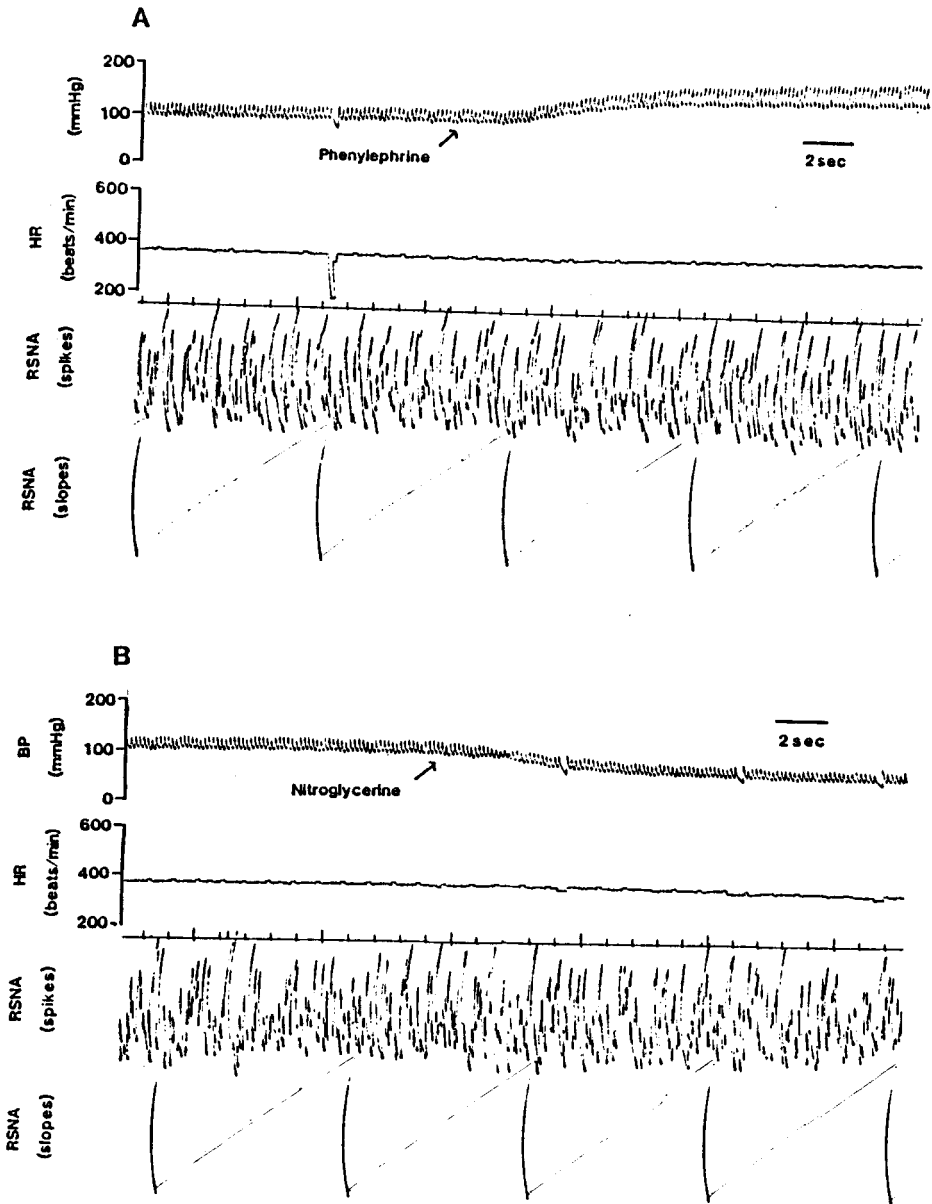


Figure 6-2. Baroreflex test from a conscious sinoaortic-denervated rat showing blood pressure (BP), heart rate (HR), renal sympathetic nerve activity (RSNA) spike and slope responses to intravenous phenylephrine (A) and nitroglycerine (B).



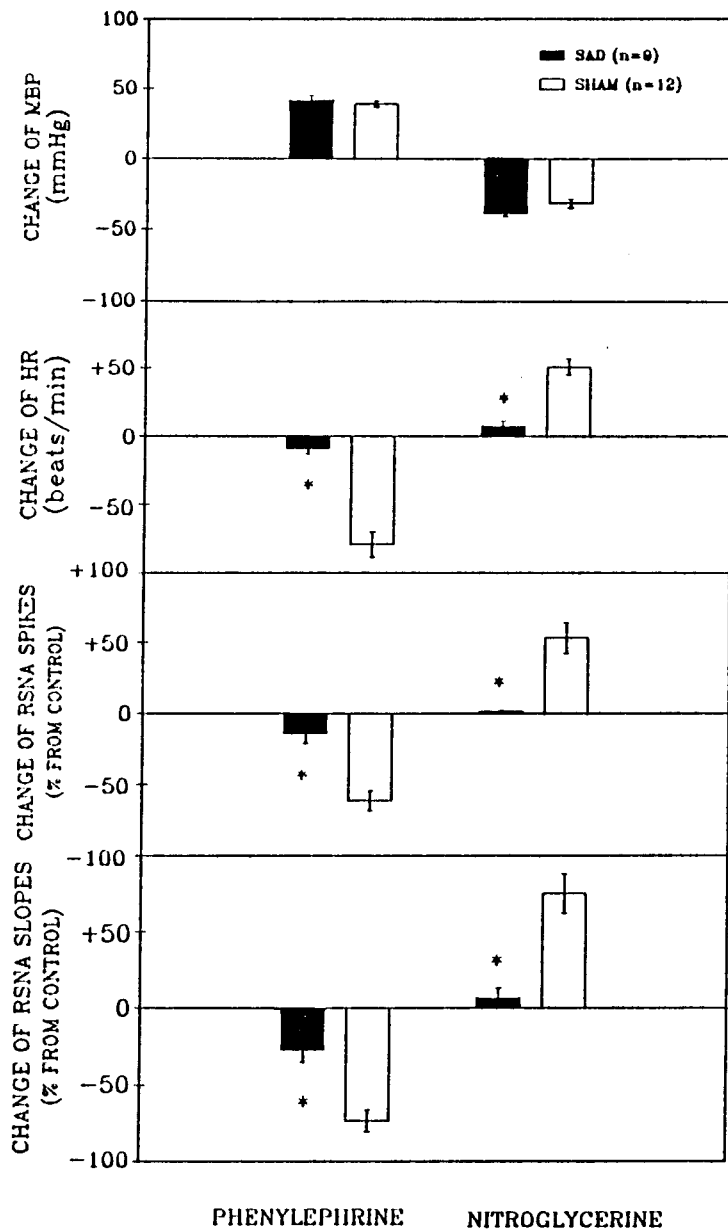


Figure 6-3. Changes of mean blood pressure (MBP), heart rate (HR), renal sympathetic nerve activity (RSNA) spikes and slopes in response to intravenous phenylephrine and nitroglycerine in conscious rats with sinoaortic denervation (SAD) or sham operation (SHAM). \*:  $p < 0.05$  for SAD vs. SHAM. Unpaired-student t test.

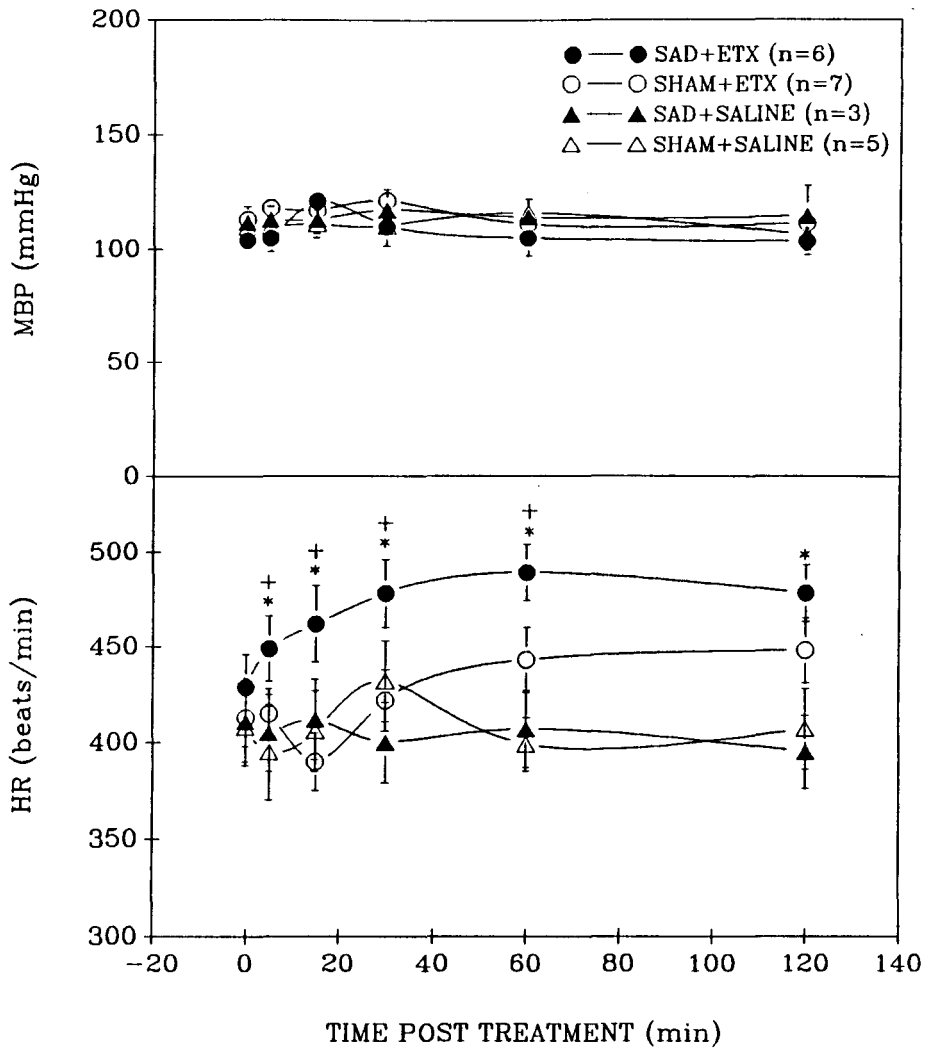


Figure 6-4. Mean blood pressure (MBP) and heart rate (HR) responses in conscious rats with sinoaortic denervation (SAD) or sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. \*:  $p < 0.05$  for SAD post endotoxin vs. 0 min; +:  $p < 0.05$  for endotoxin-treated SAD vs. SHAM.

infusion which was maintained for the duration of the protocol. In saline-treated SAD and sham rats, there was no alterations in MBP or HR over the course of the experiment.

#### Sympathetic Responses to Endotoxin Infusion:

Endotoxin administration induced very rapid and sustained increases in both RSNA and plasma catecholamines. For both SAD and sham groups, RSNA was markedly increased as early as 5 min after endotoxin infusion with the peak increase occurring between 15-30 min post endotoxin which remained elevated during the protocol (figure 6-5 and 6-6). RSNA spikes and slopes basically changed in a parallel manner, although the magnitudes are not identical. Interestingly, RSNA response at 5 and 15 min post endotoxin was 2 fold higher in SAD rats compared to sham rats. Rapid and significant elevations in plasma norepinephrine (NE) and epinephrine (EPI) were also apparent at 15, 90, and 180 min after endotoxin administration in both SAD and sham groups compared to baseline values (figure 6-7). Similar to the RSNA response, plasma NE and EPI levels at 15 min post endotoxin were also significantly higher in SAD group compared to the sham group. Saline-treated SAD and sham rats showed no alterations in RSNA and plasma catecholamines during the experimental protocol.

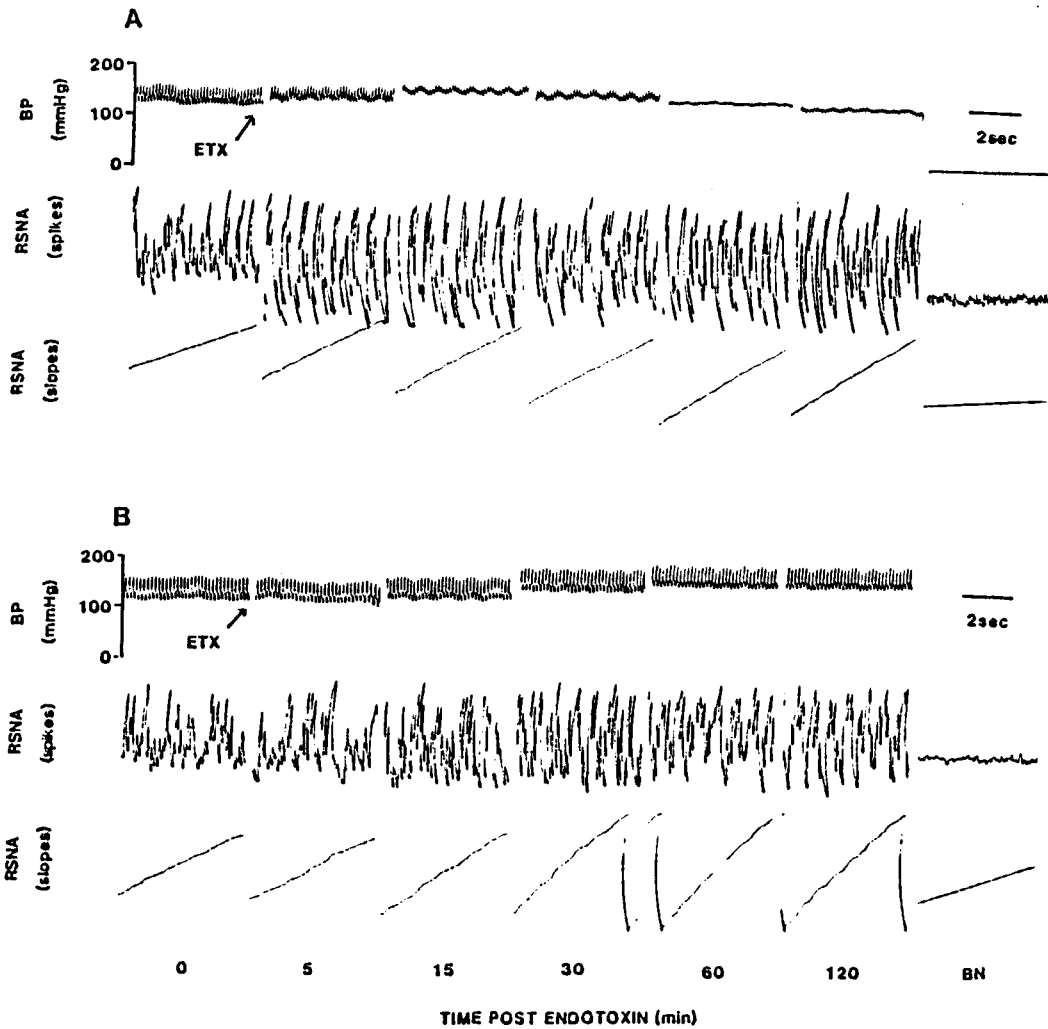


Figure 6-5. Blood pressure (BP), Renal sympathetic nerve activity (RSNA) spike and slope responses to intravenous endotoxin (ETX, 5 mg/kg) infusion from rats with sinoaortic denervation (SAD, A) or sham operation (SHAM, B). BN represents background noise level.

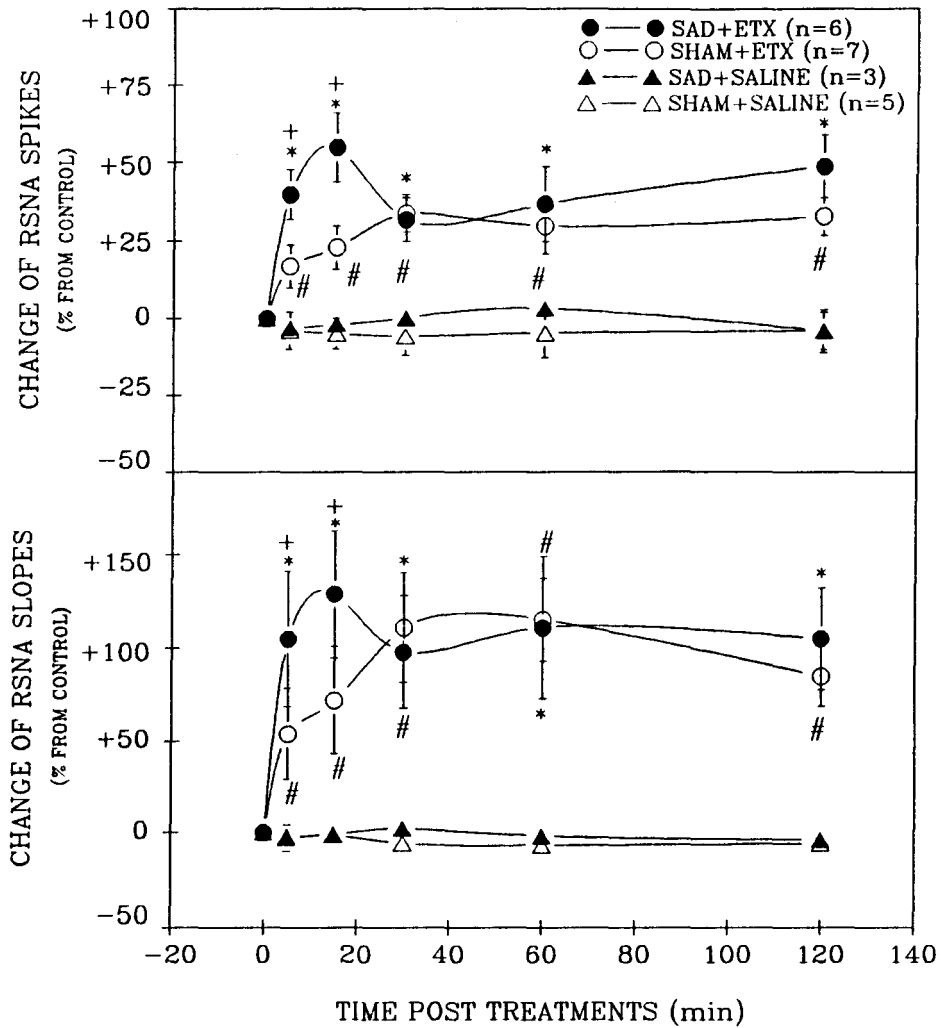


Figure 6-6. Percent changes of renal sympathetic nerve activity (RSNA) spikes and slopes in conscious rats with sinoaortic denervation (SAD) or sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. \*:  $p < 0.05$  for SAD post endotoxin vs. 0 min; #:  $p < 0.05$  for SHAM post endotoxin vs. 0 min; +:  $p < 0.05$  for endotoxin-treated SAD vs. SHAM.

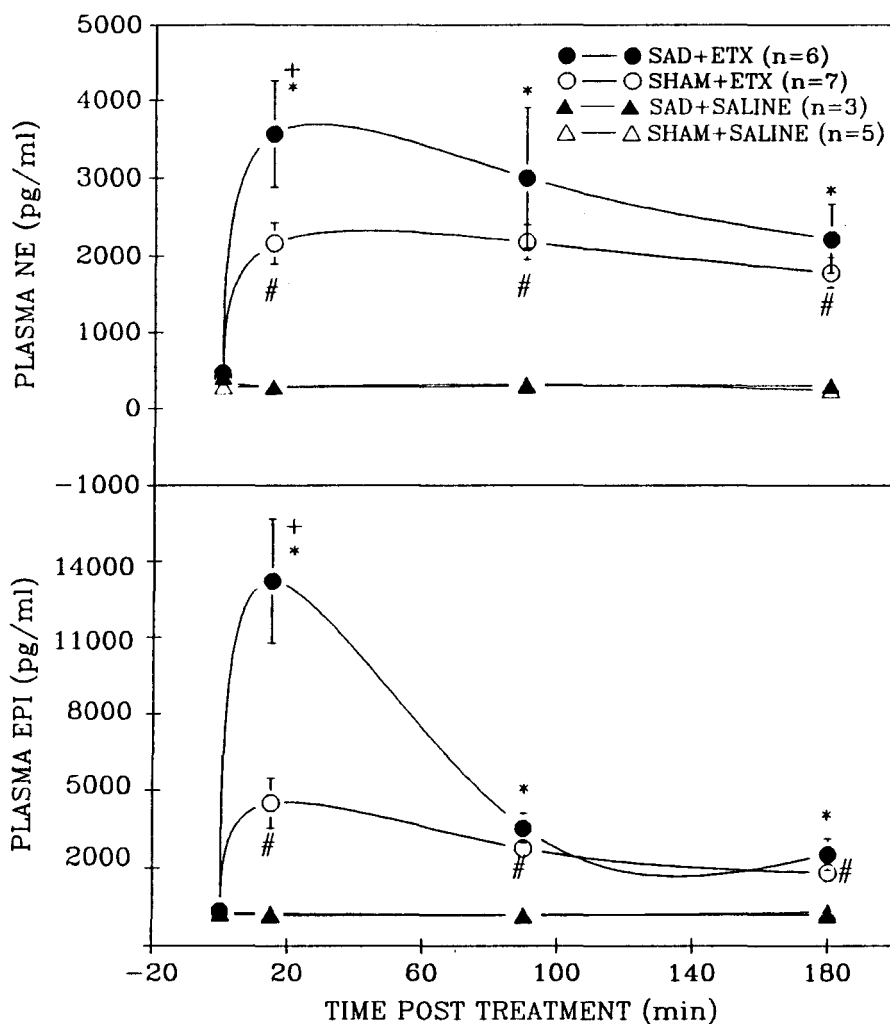


Figure 6-7. Plasma norepinephrine (NE) and epinephrine (EPI) concentrations in rats with sinoaortic denervation (SAD) or sham operation (SHAM) following intravenous endotoxin (ETX, 5 mg/kg) or saline. \*:  $p < 0.05$  for SAD post endotoxin vs. 0 min; #:  $p < 0.05$  for SHAM post endotoxin vs. 0 min; +:  $p < 0.05$  for endotoxin-treated SAD vs. SHAM.

## DISCUSSION

The principal findings of the present study demonstrate a marked elevation in sympathoadrenal activity following endotoxin in conscious rats, as indicated by parallel, but not identical, changes of direct renal sympathetic nerve recording and plasma catecholamine measurements. Such rapid onset and apparently simultaneous increases have not been previously reported, and the elevations are of a profound magnitude lasting for several hours. Furthermore, since these increments occurred in both sham and SAD rats, it suggests that the arterial baroreflex is not essential in mediating such activation.

The baroreflex control is intact in sham operated rats, as determined by reflex bradycardia and sympathoinhibition as well as reflex tachycardia and sympathoexcitation in response to pharmacologically induced alterations in blood pressure. With intact arterial baroreceptor control of the circulation, intravenous endotoxin administration induced rapid and significant increases in both renal sympathetic activity and plasma catecholamines. These results further corroborate and also extend previous findings which demonstrated significant elevation of plasma catecholamines in different septic models of various animal species and septic patients (17,75,113,115,218). The markedly increased RSNA in the present study also agrees with the reports from the literature showing augmented sympathetic discharge following septic insult in different species (93,159,185). Although Koyama et al. reported that efferent sympathetic nerve activity in rabbits and cats was decreased following sepsis (127,128), the differences in species and the use of anesthesia may account for these observations.

In SAD rats, both major arterial baroreceptors, i.e. carotid sinus and aortic arch baroreceptors are deafferented. The effectiveness of this treatment is indicated by the failure of reflex changes of HR in response to arterial pressure change induced by pharmacological agents. Such observations are routinely used as the criteria of the completeness of the SAD (8,13,15,169). Direct recording of reflex changes of renal sympathetic activity also suggests that baroreflex control of the circulation is greatly blunted in SAD compared to intact animals. The significant attenuation in reflex RSNA changes in SAD group corresponds to the same directional change of HR. However, the degree of attenuation of RSNA changes with vasoconstrictor and vasodilator agents is less than that of correspondent HR changes. This observation raises the possibility that the cardiomotor component of arterial baroreflex is completely abolished by SAD while the vasomotor component of the reflex is not (90). Several lines of evidence suggest that this is unlikely. First, we have shown that the same SAD preparation in our laboratory significantly abolished reflex elevation of plasma catecholamines in response to hydralazine-induced hypotension compared to intact control rats (255), suggesting that overall baroreflex-mediated sympathoadrenal activation is disrupted by SAD. Second, we and other investigators have observed that SAD markedly potentiates the fall in arterial pressure with hydralazine (224,255), indicating that the vasomotor component of baroreflexes is also significantly reduced. Third, SAD has been shown to be able to eliminate both the cardiomotor and sympathetically-mediated vasomotor components of the baroreflex in a parallel manner (90). Taken together, SAD is capable of eliminating or significantly blunting the baroreflex-mediated sympathoadrenal activation. The



remaining changes of RSNA with arterial pressure alterations may be explained by the observation that there is still a selective renal sympathetic activation to pressure changes after the baroreflex arc is destroyed. Hubbard et al. (108) reported that plasma renin and plasma NE were elevated by hydralazine-induced severe hypotension in rats with nucleus tractus solitarii lesion (NTS). These elevations were completely abolished by bilateral renal denervation, indicating a selective renal sympathetic activation which is controlled by an area lying outside of the NTS, independent of baroreflex arc. Another possibility is that cardiopulmonary receptor reflex may mediate the RSNA changes (232). These receptors are primarily innervated by vagal nerves and not disrupted by SAD. Furthermore, these receptors can differentially regulate sympathetic responses, e.g. RSNA versus adrenal sympathetic nerve activity (232).

After disruption of arterial baroreflex control with SAD, RSNA as well as plasma catecholamines were still rapidly and markedly elevated by endotoxin. This result implies that the baroreflex does not play a major role in turning on as well as maintaining sympathoadrenal activation with endotoxin administration. The significant sympathoadrenal activation in association with the absence of hypotension following endotoxin in both sham and SAD animals in the present study further supports this concept. The explanation for the absence of hypotension following endotoxin in the present study may again involve chronic recovery (2-4 weeks) from a major surgery, which somehow altered the blood pressure response as compared to acute preparations.

Interestingly and surprisingly, the sympathetic responses to endotoxin were more profound in the early phase in SAD animals compared to sham-operated controls. This

heightened response was also accompanied by a greater increase in HR in the SAD group versus sham rats. Results from Barron and Heesch (15) may provide a possible explanation for the present findings. They demonstrated that baroreceptor denervation potentiates the cardiovascular effects and sympathetic outflow elicited by the central nervous system activation such as posterior hypothalamic stimulation and intracerebroventricular injection of angiotensin II. They proposed that arterial baroreceptor reflexes exert an effective buffering of pressor stimuli initiated from the central nervous system and that elimination of this buffering mechanism with SAD would augment the centrally-mediated cardiovascular and sympathetic responses. It is important to recognize that the profound sympathoadrenal responses to endotoxin involve intact central nervous system (112) and such responses tend to elevate BP. Analogous to Barron and Heesch's finding, this endotoxin-induced, centrally-mediated pressor stimuli also can not be buffered in SAD animals, since this modulating mechanism is interrupted with SAD. As a consequence, higher RSNA and plasma catecholamine following endotoxin are seen in the SAD group.

The mechanisms responsible for mediating sympathoadrenal activation during endotoxemia can not be determined in the present experiment. Such rapid and profound activation may suggest a direct action of circulating mediators on the central nervous system during endotoxemia. Alternatively, rapid peripherally-elicited, centrally-mediated reflex mechanisms might be involved. Although local modulation of catecholamine release from the adrenal gland has been suggested in endotoxic animals (112,211), more studies are needed to determine its importance. It is concluded from the present study,

that the sympathoadrenal response to endotoxin infusion is a very rapidly-occurring and long-lasting event and that afferent neural input from arterial baroreceptors is not essential in mediating such activation during developing septic shock. The elimination of feedback buffering mechanisms with SAD may account for the augmented sympathetic response seen in SAD animals. More studies need to be done to elucidate the mechanisms responsible for this activation.

## CHAPTER VII

# SYMPATHOADRENAL RESPONSES TO CENTRAL ADMINISTRATION OF ENDOTOXIN, INTERLEUKIN-1 $\beta$ , INTERLEUKIN-6, AND TUMOR NECROSIS FACTOR $\alpha$ IN CONSCIOUS RAT

## INTRODUCTION

Systemic administration of bacterial endotoxin, as a model of septic insult, results in the profound sympathoadrenal activation, as indicated by the direct sympathetic nerve recording and plasma catecholamine measurements (93,113,185). Endotoxin effects are recognized to be mediated primarily by the release of cytokines, a family of protein molecules produced by macrophages and other cells. Interleukin-1 (IL-1), tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ), and interleukin-6 (IL-6) have been extensively studied and shown in particular to be linked to the pathogenesis of the septic syndrome (37,79). Among the multifunctional actions of these cytokines are actions on the central nervous system, some of which may be initiated on the brain side of the blood brain barrier. The central effects include induction of fever, activation of the hypothalamic-pituitary-adrenal axis, suppression of food intake, slow wave sleep, and acute phase response (66,118,194,214). The brain site of cytokine actions has been proposed in the hypothalamus (69,194). Brain

tissues have receptors for, and can respond directly to certain cytokines (107,117,194,250). Furthermore, IL-1 immunoreactive innervation has been shown to exist in various brain areas including the hypothalamus (34,136), and also some brain cells can produce IL-1, TNF and IL-6 in response to endotoxin or inflammatory challenge either *in vitro* or *in vivo* (82,95,135,138,200).

Thus, cytokines might be plausible factors in mediating the sympathoadrenal activation with systemic endotoxin via their central actions. To test this hypothesis, endotoxin, IL-1, TNF or IL-6 was administered into the lateral ventricle of conscious rats, and renal sympathetic nerve activity (RSNA) as well as plasma catecholamine responses were assessed. Among the potential brain sites of cytokine effects on the sympathetic nervous system, the paraventricular nucleus of the hypothalamus (PVN) is a likely candidate. First, the PVN has extensive projections to sympathetic neurons in the spinal cord (56) and electrical and chemical stimulations of the PVN increase sympathetic outflow and plasma catecholamines (226). Second, electrical activity of neurons in the PVN is altered during immune responses (23,205) and the PVN may be involved as an IL-1 action site for induction of corticotropin releasing factor (CRF) and pituitary-adrenal axis activation (203). Third, IL-1 immunoreactive innervation to the PVN has been demonstrated (34,136). Thus, to test whether the PVN is a site of cytokine actions in mediating sympathoadrenal response, each of the cytokines was administered to the PVN, and sympathetic responses were determined.

## MATERIALS AND METHODS

### Experimental Protocols

Holtzman rats, weighing  $396 \pm 12$  grams, were used in this study. Under pentobarbital anesthesia (60 mg/kg, i.p.), a carotid artery and a jugular vein were cannulated with polyethylene tubing. Bipolar, gold electrodes were then implanted on one renal branch of the left greater splanchnic nerve for recording renal sympathetic nerve activity (RSNA). The ends of cannulae and electrode wires were exteriorized on the back of the neck. A final procedure under the anesthesia was to insert a stainless steel cannula into the lateral ventricle through the skull or a bilateral cannula to both sides of PVN. After a 24 hr recovery, cannulae and electrodes were extended to instruments to record blood pressure and RSNA. Following a 0.5 hr equilibration period, endotoxin (ETX, 1000 ng, n=6), IL-1  $\beta$  (100 ng, n=5, 1000 ng, n=5), IL-6 (100 ng, n=5, 1000 ng, n=5), TNF  $\alpha$  (100 ng, n=5, 1000 ng, n=5), or saline vehicle (n=5), all in 10  $\mu$ l volume, was administered ICV using a Hamilton syringe. For PVN injection, IL-1  $\beta$  (1000 ng, n=7), IL-6 (1000 ng, n=5), TNF  $\alpha$  (1000 ng, n=6), or saline vehicle (n=5), all in 0.5  $\mu$ l volume, was delivered bilaterally to the PVN. Those doses were chosen mostly based on the doses used in previous studies reported in the literature (194,202,213). Hemodynamic responses, RSNA and plasma catecholamines were assessed at 0, 30, 60, 90, and 180 min post injection. The localizations of the tips of the ICV and PVN cannulae were histologically determined at the termination of the experiments.

In addition, a control group (n=4) was setup to demonstrate a positive sympathetic response with PVN injection of a synthetic opioid peptide [D-Ala<sup>2</sup>,NMe-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]enkephalin (DAGO). DAGO, when injected into the PVN, induces catecholamine secretion through activation of the central sympathetic outflow to the adrenal medulla and sympathetic nerve terminals. Bilateral PVN administration of DAGO (10  $\mu$ g in 500 nl volume) caused significant elevations of RSNA spikes ( $25\pm 8\%$  at 5 min,  $38\pm 11\%$  at 15 min) and slopes ( $45\pm 18\%$  at 5 min,  $75\pm 5\%$  at 15 min). Saline infusion to the same group of rats changed neither RSNA spikes ( $-1\pm 3\%$  at 5 min,  $-2\pm 1\%$  at 15 min) nor slopes ( $-2\pm 4\%$  at 5 min,  $-1\pm 4\%$  at 15 min).

### Sympathetic Nerve Recording

Renal sympathetic nerve activity (RSNA) was recorded from a renal nerve branch of the left greater splanchnic nerve, as described in chapter III. After exposing the left kidney retroperitoneally, the renal nerve branch was separated from fat and connective tissues. It was carefully placed on a thin, bipolar gold electrode. When an optimal nerve signal was obtained, the electrode and the nerve was fixed with a gel mixture. The electrode wires were exteriorized on the back of the neck. On the next day the nerve signal was amplified, rectified and integrated for estimation of frequencies of RSNA (measured as spikes/min) as well as overall RSNA (measured as slopes). The integrated signals were continuously recorded on a Grass oscillograph. The post mortem renal nerve activity was recorded in all animals as a measure of the background noise level. All RSNA was expressed as percent change from baseline control.

### Implantation of Intracerebroventricular (ICV) Cannulae

Under anesthesia, a 30 gauge stainless steel cannula was implanted into the lateral ventricle with the tip stereotaxic coordinates: 0.5 mm caudal to bregma, 1.5 mm lateral to the midline, and 4.5 mm below the surface of the skull. The end of the cannula was pre-bent to a right angle and connected to a piece of PE-10 tubing (5 cm long), filled with saline (3-4  $\mu$ l) and heat sealed. On the day of experiment, the cannula was extended to a 10  $\mu$ l Hamilton microsyringe filled with test solution. Ten  $\mu$ l test solution (plus 3-4  $\mu$ l saline in the cannula) was infused over a period of 5 min. The ICV injection was verified by showing ventricular staining after a dye injection via the cannula (examples shown in figure 7-1).

### Implantation of PVN Cannulae

Using a stereotaxic apparatus, a 26 gauge bilateral guide cannula comprised of steel tubes with a 1.0 mm distance between the centers was implanted to the bilateral PVN. The coordinates for PVN were 1.7 mm caudal to bregma, 0.5 mm lateral to the midsagittal suture and 8.0 mm ventral to the skull surface. A dummy wire with two leads was inserted into the guide cannula to prevent clotting and to prevent dust from getting into the cannula. On the day of experiments, the dummy wire was removed and a double injector cannula (33 gauge) which was constructed 2 mm longer than the guide cannula was inserted into the guide cannula. The injector cannula was connected to a 10  $\mu$ l Hamilton microsyringe. A testing solution (500 nl) was injected bilaterally over a 5 min period. The exact injection site was histologically verified in each animal at the end of



experiments. Only those animals with the cannula terminating in the PVN without damage of PVN neurons were included in the study (examples shown in figure 7-2).

#### Blood Sample Collection and Catecholamine Assay

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A black and white photograph showing two coronal sections of a rat brain. The sections are positioned side-by-side, showing the internal structure of the brain. The lateral ventricles are clearly visible and appear to be filled with a dark, dense material, which is the dye used for staining. The surrounding brain tissue is lighter in color, providing a clear contrast with the stained ventricles.

IL-1 and TNF were prepared in 0.9 % sodium chloride at concentrations of 0.2 mg/ml

**Figure 7-1. Coronal brain sections showing staining of the lateral ventricles after dye injection via an intracerebroventricular (ICV) cannula.**

Peninsula, San Carlos, CA. Materials for catecholamine assay were obtained in a kit form from Amersham (Arlington Heights, IL).

#### Data Analysis

Data are expressed as mean  $\pm$  SEM (n). A p value less than 0.05 was considered

experiments. Only those animals with the cannula terminated in the dorsal border of the PVN without damage of PVN neurons were included in the statistical evaluation (example shown in figure 7-2).

### Blood Sample Collection and Catecholamine Assay

Arterial blood samples (500  $\mu$ l) were collected at different time points as described above. Plasma levels of norepinephrine and epinephrine were assayed in duplicate 50  $\mu$ l samples using the radio-enzymatic thin-layer chromatographic procedure described previously.

### Materials

For ICV infusion, endotoxin, IL-1, IL-6, and TNF were prepared in phosphate-buffered saline containing 0.2 % bovine serum albumin at a concentration of 10  $\mu$ g/ml for the dose of 100 ng/rat and 100  $\mu$ g/ml for the dose of 1000 ng/rat. For PVN infusion, IL-1 and TNF were prepared in 0.9 % sodium chloride at concentrations of 0.2 mg/ml for the dose of 100 ng/rat and 2 mg/ml for the dose of 1000 ng/rat. Saline vehicle contained equivalent amount of bovine serum albumin. DAGO was purchased from Peninsula, San Carlos, CA. Materials for catecholamine assay were obtained in a kit form from Amersham (Arlington Heights, IL).

### Data Analysis

Data are expressed as mean  $\pm$  SEM (n). A p value less than 0.05 was considered to



Figure 7-2. Coronal brain sections at the level of paraventricular nucleus of the hypothalamus (PVN). The tips of the cannula were shown to terminate at the dorsal border of the PVN.

be statistically significant. Cardiovascular responses, RSNA, and plasma catecholamines were compared using analysis of variance with repeated measurements and Student-Newman-Keul's test for individual comparisons.

## RESULTS

### Hemodynamic and Sympathoadrenal Responses to ICV Injection of Endotoxin

As shown in figure 7-3, ICV administration of endotoxin (1000 ng/rat), given over a 5 min period, did not cause significant changes in arterial mean blood pressure (MBP) or heart rate (HR) during the 120 min protocol. There were also no significant alterations in RSNA, both spikes and slopes, over times following endotoxin. Post treatment plasma norepinephrine (NE) and epinephrine (EPI) remained at the levels similar to the controls. ICV injection of saline failed to elicit any changes in MBP, HR, RSNA or plasma catecholamines.

### Hemodynamic and Sympathoadrenal Responses to ICV injection of IL-1 $\beta$

As indicated in figure 7-4, neither MBP nor HR was significantly altered by ICV administration of low and high doses (100 ng, 1000 ng) of IL-1  $\beta$ . These two doses of IL-1 induced basically similar patterns of increases in RSNA, as indicated by both spikes and slopes. The increments of RSNA were modest, approximately 20-30% of baseline values. Peak elevations were reached between 30 to 90 min post injection and remained elevated thereafter. In parallel, ICV IL-1 treatment evoked significant elevations of plasma catecholamines. Both low dose and high dose of IL-1 elevated plasma NE throughout the 180 min protocol. At 60 min, the NE elevation induced by high dose was higher than that induced by low dose whereas at 180 min the opposite was seen. Plasma EPI was elevated by low dose of IL-1 at 90 and 180 min post injection.

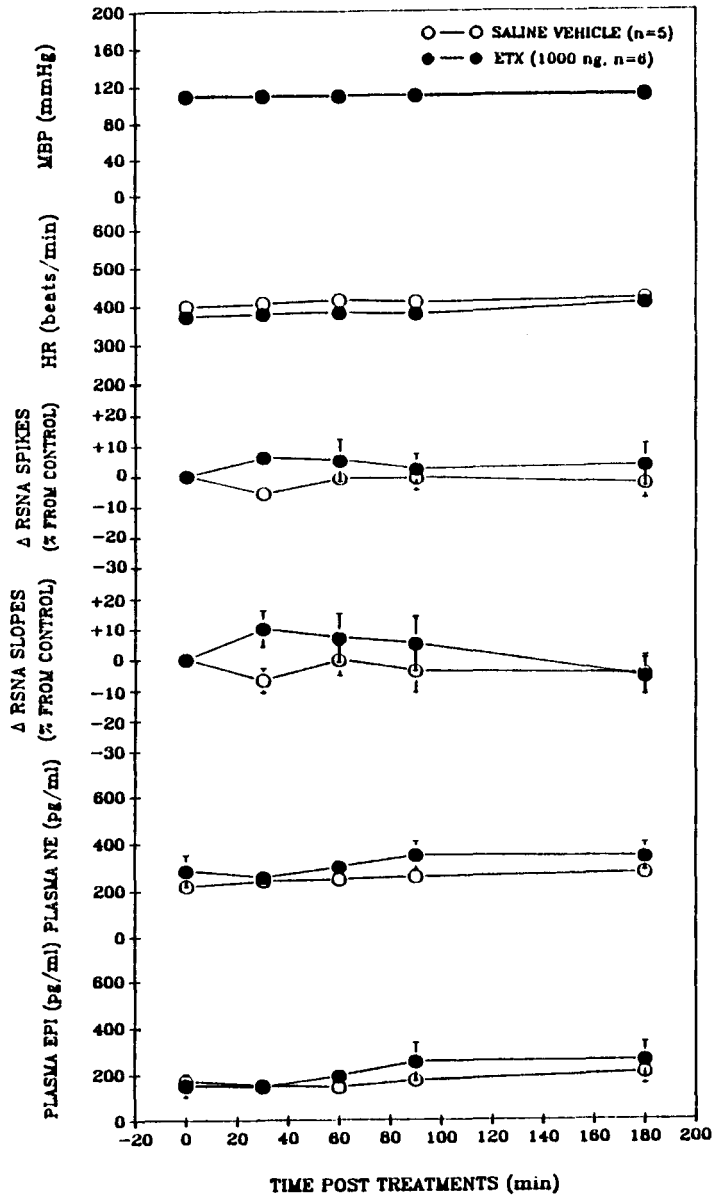


Figure 7-3. Hemodynamic, renal sympathetic nerve activity and plasma catecholamine responses to ICV injection of endotoxin (ETX) or saline in conscious rats. MBP: mean blood pressure; HR: heart rate; RSNA: renal sympathetic nerve activity; NE: norepinephrine; EPI: epinephrine.

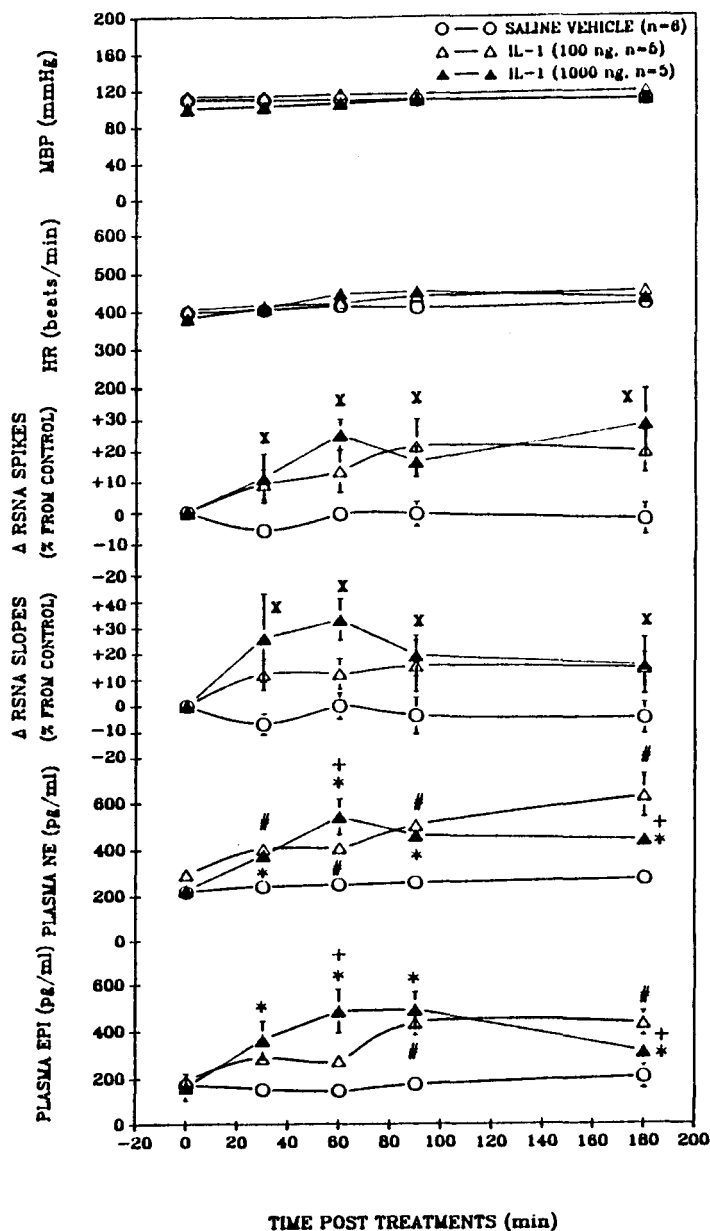


Figure 7-4. Hemodynamic, renal sympathetic nerve activity and plasma catecholamine responses to ICV injection of low and high doses of IL-1  $\beta$  or saline in conscious rats. MBP: mean blood pressure; HR: heart rate; RSNA: renal sympathetic nerve activity; NE: norepinephrine; EPI: epinephrine. x:  $p < 0.05$  for both low and high dose groups, compared with baseline values; #  $p < 0.05$  for low dose group, compared with baseline values; \*  $p < 0.05$  for high dose group, compared with baseline values; +  $p < 0.05$  low dose group vs. high dose group.

High dose of IL-1 evoked elevation of plasma EPI at all sampling times during 180 min. The increments in EPI evoked by low dose of IL-1 were higher than those induced by high dose.

#### Hemodynamic and Sympathoadrenal Responses to ICV injection of TNF $\alpha$

Neither MBP nor HR was altered within 180 min following ICV administration of TNF (figure 7-5). TNF induced significant decrease in RSNA, as indicated by both spikes and slopes (approximately 20%). The pattern of these depressions induced by the two doses of TNF were similar to each other. The depression in spikes reached significance at 30, 60, 90, and 180 min while the depression in slopes reached significance at 60, 90, and 180 min post treatment. Plasma NE or EPI did not change significantly following ICV injection of TNF.

#### Hemodynamic and Sympathoadrenal Responses to ICV injection of IL-6

As presented in figure 7-6, ICV injection of IL-6 at doses of 100 ng and 1000 ng did not alter MBP or HR significantly. The pattern of RSNA responses, both spikes or slopes, to low dose and high dose of IL-6 were quite different from each other. Low dose of IL-6 did not change RSNA spikes or slopes at any time post injection whereas high dose of IL-6 induced significant depression in both spikes and slopes during the 180 min sampling period. The peak depression was approximately 20% for RSNA spikes and was about 30% for RSNA slopes. Plasma NE or EPI did not change significantly following IL-6.



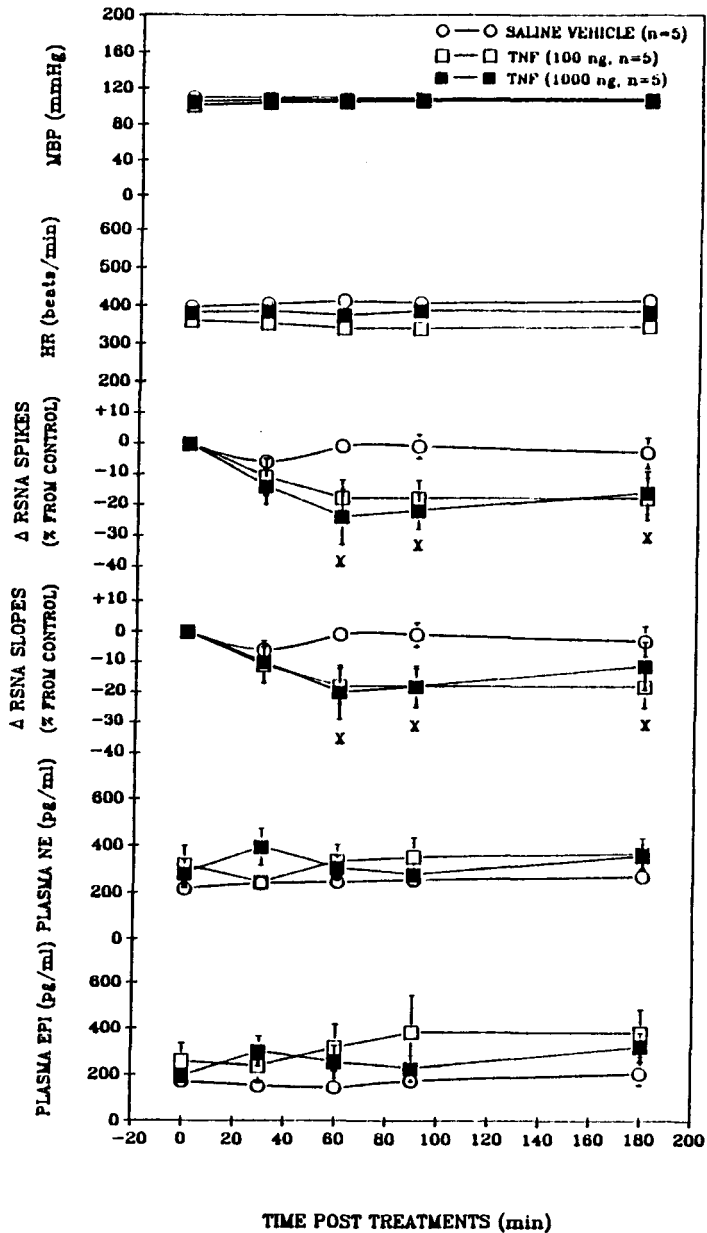


Figure 7-5. Hemodynamic, renal sympathetic nerve activity and plasma catecholamine responses to ICV injection of low or high doses of TNF  $\alpha$  or saline in conscious rats. MBP: mean blood pressure; HR: heart rate; RSNA: renal sympathetic nerve activity; NE: norepinephrine; EPI epinephrine. x:  $p < 0.05$  for both low and high dose groups, compared with baseline values.

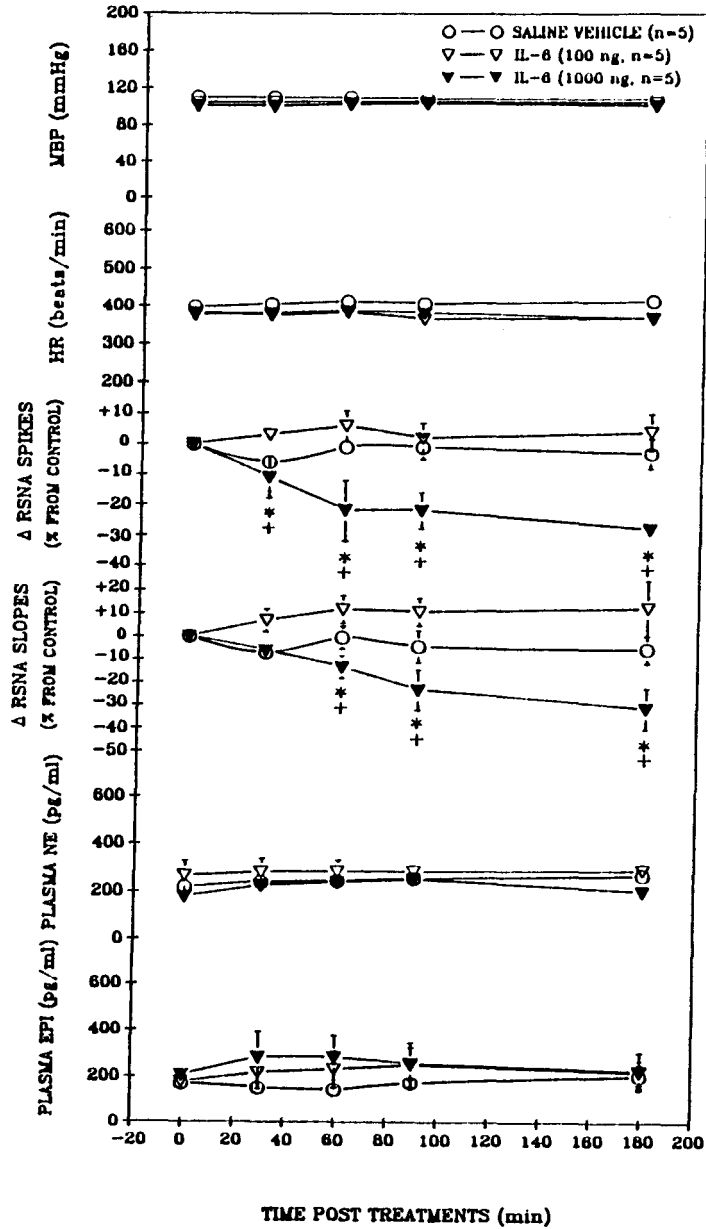


Figure 7-6. Hemodynamic, renal sympathetic nerve activity and plasma catecholamine responses to ICV injection of low and high doses of IL-6 or saline in conscious rats. MBP: mean blood pressure; HR: heart rate; RSNA: renal sympathetic nerve activity; NE: norepinephrine, EPI: epinephrine. \*:  $p < 0.05$  for high dose group, compared with baseline values; +:  $p < 0.05$  low dose group vs. high dose group.

### Hemodynamic and Sympathoadrenal Responses to PVN Injection of IL-1

As shown in table 7-1, PVN injection of IL-1 induced mild but significant elevation in mean arterial blood pressure at 60, 90, and 180 min post injection as compared to the baseline values. Simultaneously heart rate was slightly but not significantly increased. PVN injection of IL-1 had no marked effects on RSNA, both spikes and slopes (table 7-2). Plasma NE and EPI were slightly elevated at 90 and 180 min after IL-1 injection (table 7-3). Plasma NE increased from preinjection value of  $331 \pm 45$  pg/ml to  $520 \pm 61$  pg/ml at 90 min and  $567 \pm 103$  pg/ml at 120 min. Plasma EPI increased from baseline value of  $159 \pm 23$  pg/ml to  $349 \pm 64$  pg/ml at 90 min and  $378 \pm 59$  pg/ml at 120 min. PVN injection of saline did not evoke any significant changes in MBP, HR, RSNA, and plasma catecholamines.

### Hemodynamic and Sympathoadrenal Responses to PVN Injection of TNF

There were no significant alterations in blood pressure or heart rate following PVN injection of TNF (table 7-1). PVN TNF induced slight (< 10 %) but significant decreases in RSNA slopes at 60, 90, 120 min post injection without significant changes in RSNA spikes (table 7-2). PVN TNF had no effects on plasma catecholamines (table 7-3).

### Hemodynamic and Sympathoadrenal Responses to PVN injection of IL-6

As shown in table 7-1, IL-6 injection to PVN induced an increase in HR at 60 min without significant alteration in blood pressure. RSNA (both spikes and slopes) and

Table 7-1. Effects of saline vehicle, IL-1  $\beta$ , TNF  $\alpha$  and IL-6 injections into the paraventricular nucleus of the hypothalamus (PVN) on mean blood pressure (MBP) and heart rate (HR). \*:  $p < 0.05$  post treatment values vs. baseline value.

POST TREATMENT MBP (mmHg)					
Time (min)	0	30	60	90	180
SALINE	101 $\pm$ 1 (n=5)	102 $\pm$ 1	105 $\pm$ 2	105 $\pm$ 2	103 $\pm$ 2
IL-1 (1000 ng)	108 $\pm$ 3 (n=7)	112 $\pm$ 5	116 $\pm$ 4*	118 $\pm$ 4*	116 $\pm$ 4*
TNF (1000 ng)	109 $\pm$ 3 (n=6)	109 $\pm$ 2	110 $\pm$ 2	110 $\pm$ 2	112 $\pm$ 4
IL-6 (1000 ng)	111 $\pm$ 4 (n=5)	111 $\pm$ 4	115 $\pm$ 8	117 $\pm$ 8	115 $\pm$ 7
POST TREATMENT HR (beats/min)					
Time (min)	0	30	60	90	180
SALINE	398 $\pm$ 7 (n=5)	388 $\pm$ 22	393 $\pm$ 21	394 $\pm$ 20	401 $\pm$ 21
IL-1 (1000 ng)	404 $\pm$ 5 (n=7)	411 $\pm$ 8	431 $\pm$ 10	435 $\pm$ 11	436 $\pm$ 14
TNF (1000 ng)	398 $\pm$ 8 (n=6)	415 $\pm$ 13	400 $\pm$ 16	398 $\pm$ 19	400 $\pm$ 17
IL-6 (1000 ng)	420 $\pm$ 8 (n=5)	456 $\pm$ 21	476 $\pm$ 20*	444 $\pm$ 22	445 $\pm$ 22

Table 7-2. Effects of saline vehicle, IL-1  $\beta$ , TNF  $\alpha$  and IL-6 injections into the paraventricular nucleus of the hypothalamus (PVN) on renal sympathetic nerve activity (RSNA) spikes and slopes in conscious rats. \*:  $p < 0.05$  post treatment values vs. baseline value.

CHANGE OF RSNA SPIKES (% control)					
Time (min)	0	30	60	90	180
SALINE	100 $\pm$ 0 (n=5)	97 $\pm$ 5	97 $\pm$ 3	99 $\pm$ 3	99 $\pm$ 4
IL-1 (1000 ng)	100 $\pm$ 0 (n=7)	114 $\pm$ 9	99 $\pm$ 6	95 $\pm$ 7	92 $\pm$ 5
TNF (1000 ng)	100 $\pm$ 0 (n=6)	98 $\pm$ 5	96 $\pm$ 4	95 $\pm$ 3	90 $\pm$ 8
IL-6 (1000 ng)	100 $\pm$ 0 (n=5)	96 $\pm$ 3	93 $\pm$ 2	94 $\pm$ 3	100 $\pm$ 6
CHANGE OF RSNA SLOPES (% control)					
Time (min)	0	30	60	90	180
SALINE	100 $\pm$ 0 (n=5)	99 $\pm$ 3	96 $\pm$ 2	99 $\pm$ 1	99 $\pm$ 1
IL-1 (1000 ng)	100 $\pm$ 0 (n=7)	114 $\pm$ 9	98 $\pm$ 8	97 $\pm$ 9	94 $\pm$ 10
TNF (1000 ng)	100 $\pm$ 0 (n=6)	96 $\pm$ 2	93 $\pm$ 3*	91 $\pm$ 2*	92 $\pm$ 3*
IL-6 (1000 ng)	100 $\pm$ 0 (n=5)	98 $\pm$ 3	97 $\pm$ 2	99 $\pm$ 4	96 $\pm$ 5

Table 7-3. Effects of saline vehicle, IL-1  $\beta$ , TNF  $\alpha$  and IL-6 injections into the paraventricular nucleus of the hypothalamus (PVN) on plasma norepinephrine (NE) and epinephrine (EPI) in conscious rats. \*:  $p < 0.05$  post treatment values vs. baseline value.

PLASMA NOREPINEPHRINE (pg/ml)					
Time (min)	0	30	60	90	180
SALINE	406 $\pm$ 78 (n=5)	423 $\pm$ 21	462 $\pm$ 21	443 $\pm$ 38	456 $\pm$ 42
IL-1 (1000 ng)	331 $\pm$ 45 (n=7)	430 $\pm$ 62	441 $\pm$ 48	520 $\pm$ 61*	567 $\pm$ 103*
TNF (1000 ng)	257 $\pm$ 47 (n=6)	325 $\pm$ 17	319 $\pm$ 37	339 $\pm$ 27	335 $\pm$ 27
IL-6 (1000 ng)	354 $\pm$ 84 (n=5)	372 $\pm$ 71	395 $\pm$ 95	528 $\pm$ 164	360 $\pm$ 130
PLASMA EPINEPHRINE (pg/ml)					
Time (min)	0	30	60	90	180
SALINE	200 $\pm$ 41 (n=5)	256 $\pm$ 43	217 $\pm$ 41	212 $\pm$ 33	233 $\pm$ 36
IL-1 (1000 ng)	159 $\pm$ 23 (n=7)	207 $\pm$ 26	256 $\pm$ 48	349 $\pm$ 64*	378 $\pm$ 59*
TNF (1000 ng)	123 $\pm$ 22 (n=6)	165 $\pm$ 18	159 $\pm$ 34	162 $\pm$ 28	164 $\pm$ 31
IL-6 (1000 ng)	141 $\pm$ 17 (n=5)	197 $\pm$ 43	166 $\pm$ 44	176 $\pm$ 54	142 $\pm$ 41

plasma catecholamines were unaffected by PVN injection of IL-6 (table 7-2 and table 7-3).

## DISCUSSION

Systemic administration of TNF has been shown to induce modest to marked elevation of plasma catecholamines, depending on the doses and animal species used (11,53,236). Intravenous treatment of IL-1 has also been demonstrated to evoke slight activation of the sympathoadrenal system (21,202). Such activation induced by these two cytokines can occur in the absence of hypotension (21,53), which suggests that baroreflex mechanisms are not likely to be important in mediating cytokine effects. A variety of experimental findings have suggested that many systemic cytokine effects are mediated via their actions on the central nervous system (CNS), particularly the hypothalamus (194,201). However, the specific mechanisms by which the central cytokines mediate the observed increases in sympathetic outflow is not yet known.

In the present study we investigated the effects of centrally- administered endotoxin and cytokines on the sympathetic outflow in order to gain some insight as to the mechanism of sympathoadrenal activation during sepsis. The principle findings of this study demonstrated that ICV administration of endotoxin did not cause any change in sympathetic activity. ICV injection of IL-1 induced a modest activation of the sympathetic nervous system where RSNA and plasma catecholamines changed basically in a parallel manner. ICV injection of IL-6 or TNF, however, suppressed the RSNA without significant alterations in plasma catecholamines. PVN administrations of IL-1 induced a mild elevation of plasma catecholamines without significant effects on RSNA. PVN treatment of TNF slightly decreased RSNA slopes but did not affect plasma



catecholamines. IL-6 injection to PVN altered neither RSNA nor plasma catecholamines. These results suggest that IL-1 may be one of the mechanisms involved in mediating sympathoadrenal activation during septic shock via its central actions, partially in the PVN.

The enhanced catecholamine secretion following ventricular IL-1 in the present study is in agreement with the previous report by Rivier et al. (202). In their study similar doses of ICV IL-1 induced comparable elevations of plasma catecholamines. The magnitude of catecholamine increments with ICV IL-1 also resembled that induced by systemic IL-1 treatment where higher doses of IL-1 were often used (21,202). The augmented sympathetic outflow with IL-1 was further substantiated, in the present study, by demonstration of an increased renal sympathetic nerve activity. Although the magnitude of the sympathetic response, in this study and in previous study, is apparently less compared to the sympathetic activation following systemic endotoxin (93,113), the doses of IL-1 used may not reproduce the true local IL-1 concentration in the brain parenchyma during endotoxemia. The time course of ICV IL-1 induced sympathetic activation is, however, delayed (peak at 30-90 min) compared to systemic endotoxin evoked activation (peak at 15-30 min), even without considering the time needed for the synthesis of IL-1 following endotoxin challenge. These differences may suggest that either the doses of IL-1 given are not high enough to induce full stimulation of sympathetic nervous system or IL-1 is only one of several factors responsible for the initiation of the sympathetic activation. At least we can conclude that the sympathoadrenal activation during the development of septic shock may be partially

mediated by IL-1 via its central actions.

The mechanisms by which ICV IL-1 leads to the activation of the sympathoadrenal system can not be determined in the present study, but an apparent lack of hypotension following central IL-1 does not suggest the involvement of cardiovascular reflexes. Several other possibilities should also be considered. First, IL-1 may directly affect the CNS neurons which control the central sympathetic outflow. The hypothalamus would appear to be the most likely area since it has been shown to be involved in many IL-1 induced central effects such as fever, acute phase response, CRF secretion and suppression of food intake (66,118,194,214). Electrophysiological studies show that IL-1 can directly stimulate hypothalamic neurons to alter the firing rate (107). Furthermore, the hypothalamus, particularly the paraventricular nucleus (PVN), is involved in central regulation of the sympathetic nerve system (226). However, IL-1 delivery to the PVN at concentration higher than achieved with ICV route evoked a smaller and somewhat delayed sympathoadrenal response compared to that induced by ICV IL-1. This result suggests that other brain areas in addition to the PVN are involved in ICV IL-1 effects. Second, pyrogenic effects of IL-1 usually involve activation of the sympathetic nervous system in selective organs. However, this is unlikely to be the case in present study because IL-1 induced fever usually involves cutaneous sympathetic activation and simultaneous renal sympathetic inhibition (204). Although temperature was not evaluated in this study, renal sympathetic nerve activity was not decreased but increased. Third, centrally administered IL-1 can stimulate the hypothalamus to secrete corticotropin-releasing factor (CRF) (22,206). Central CRF is known to act within the brain to

stimulate sympathetic outflow and result in elevation of plasma catecholamines (38).

ICV administration of endotoxin has been demonstrated to induce fever production (165) and suppression of peripheral immune systems (227). Thus, endotoxin can somehow directly interact with the brain elements to cause functional changes. Some of the effects can be abolished or attenuated by cyclooxygenase inhibitor or  $\alpha$ -MSH (165,227). Since prostaglandins have been shown to be the secondary signal of IL-1 induced fever and  $\alpha$ -MSH has anti-IL-1 effects, endotoxin effects may indeed be mediated by brain-produced IL-1. Studies have shown that IL-1 activity is detected in cerebral spinal fluid following central endotoxin administration (54). The failure of ICV endotoxin injection to evoke sympathetic activation in the present study does not rule out the possibility that brain cells can produce cytokines *in vivo*. Likewise, the endotoxin-induced IL-1 may not be sufficient enough to stimulate central sympathetic outflow.

In contrast to IL-1, TNF (at both high and low doses) significantly suppressed the renal sympathetic nerve activity following ICV administration. Previous studies have shown that TNF  $\alpha$  administered peripherally to rabbits at the dose of 10  $\mu$ g/kg resulted in a decrease in ear temperature, indicative of cutaneous sympathetic activation, and simultaneous inhibition of renal sympathetic nerve activity at the initial phase (30 min) which returned to control levels around 60 min post treatment (204). The parallel changes of renal sympathetic activity following peripheral TNF in this previous study and the central TNF in the present study suggest a possible central site of TNF action. The much higher dose required for peripheral route as compared to the central route adds further support to the notion that TNF effects on renal sympathetic outflow are mediated

via its central actions. The longer durations of renal sympathetic suppression in the present study may be attributed to high central concentration achieved. A previous study using ICV TNF  $\alpha$  in rats also reported decreased sympathetic activity to the interscapular brown adipose tissue (106). The absence of plasma catecholamine changes concomitant with depression of renal sympathetic activity may be due to the net results of non-uniform alterations of sympathetic efferents to different organs, i.e. sympathetic impulse to some organs may be depressed while to others augmented. It should be noted, however, that systemic TNF usually induces modest to marked elevations of plasma catecholamines (11,53,236) whereas centrally administered TNF, in this study, does not affect plasma catecholamine levels. This discrepancy may suggest that either the local concentration of TNF in the effective site is not sufficiently high or systemic TNF effects on sympathetic nervous system are not exclusively mediated via its central actions. To expose brain parenchyma cells to a higher local TNF concentration, TNF was further administered directly to PVN, one of the important central regulatory areas for the sympathetic nervous system as well as the potential site of cytokine actions. TNF injection to PVN induced a slight decrease in RSNA slopes and spikes (the latter was not statistically significant), but did not affect plasma catecholamines. The smaller decreases in RSNA caused by TNF injection to PVN compared to that induced by TNF injection to ICV may indicate that PVN is not likely the only site where TNF acts to induce the sympathetic suppression.

One possible mechanism of central TNF action may be a direct effect on the electrophysiology of sympathetic regulatory neurons. TNF has been shown to decrease

the transmembrane potential of skeletal muscle (237) and influence the firing rate of the neurons in the CNS (180,194). For example, electrophoretically applied recombinant human-TNF (rh-TNF) suppressed the activity of glucose-sensitive neurons in the rat lateral hypothalamic area (194) and increased neuronal activity of glucoreceptor neurons in the rat ventromedial hypothalamic nucleus (180). The ionic mechanism of the inhibitory effect of TNF has been proposed to be the inactivation of resting sodium conductance (209). A decrease in potassium conductance may underline the excitatory effects of TNF (208). We speculate that ventricularly applied TNF may somehow directly or indirectly either stimulate the inhibitory neurons or suppress the excitatory neurons in the sympathetic control circuit and results in decreases of the renal sympathetic activity.

Similar to TNF, ICV injection of IL-6, at a high dose, also induced suppression of the renal sympathetic activity. The mechanisms of this finding are not known, but apparently ICV IL-6 effects on the sympathetic nervous system are not mediated in PVN since IL-6 injection to PVN affected neither RSNA nor plasma catecholamines. Previous studies have shown that IL-6 has various effects on the central nervous system such as fever induction and stimulation of hypothalamic-pituitary-adrenal axis (100,170). IL-6 induced ACTH release in rats could be abolished by pretreatment with anti-CRF antibodies, suggesting that IL-6 effects was mediated via CRF.

As discussed above, the alterations of sympathetic nerve activity associated with PVN injection of either IL-1 or TNF were somewhat delayed and less in magnitude compared to those induced by ICV injection of a similar dose of these cytokines. An

alternative explanation for this observation is that cytokines injected into the PVN may migrate with time to adjacent areas such as cerebral ventricles and thereby cause subsequent changes in sympathetic nerve activity.

Whether cytokines can cross the blood brain barrier and gain direct access to the brain parenchymal cells is controversial. Generally, it is believed that molecules as big as endotoxin or cytokines are unlikely to cross the blood brain barrier. Studies with intravenous administration of radiolabeled endotoxin and IL-1 have shown inconsistent results in terms of detection of radioactivity in the brain (12,20,67,109). Several authors have proposed that cytokines may gain access to the brain, particularly the hypothalamus, via circumventricular organs such as organum vasculosum laminae terminalis (OVLT), where there is no blood brain barrier (26,116,222). Furthermore, all three cytokines can be produced *in vivo* and *in vitro* by brain cells such as astrocytes, microglial cells or even neurons in response to endotoxin challenge or various other pathological conditions (82,95,135,182,200). Recently, Breder et al. reported that IL-1 immunoreactive fibers were found innervating the key endocrine and autonomic cell groups and were distributed in a manner as to suggest that it functions as a neuromodulator or neurotransmitter (34,136). Collectively, the substrates for potential interaction of brain cells with circulating, even locally produced cytokine, are possibly present.

It is not known how much the ventricularly applied cytokines might mimic the true exposure of the brain cells to circulating, or even locally produced, cytokines during septic states. Considering the normal volume of cerebral spinal fluid (300  $\mu$ l) and rate of secretion ( $2.2 \pm 0.3$   $\mu$ l/min) in the rat, the concentrations of the cytokines at 30, 60,

90 and 180 min after ICV injection would be 81, 66, 53, and 28% of the initial amount. It is also difficult to estimate how much the timely-diluted cytokines diffuse from CSF across the ependymal epithelia and pial surfaces into the brain parenchyma. From previous reports on radiolabeled peptides and chemicals, after ICV administration, their concentrations within the brain target region surrounding the ventricular cavities (e.g. the hypothalamus) and subarachnoid space may be less than 2% of the initial amount. This represents less than 20 ng after a dose of 1  $\mu\text{g}/\text{rat}$  of cytokines without taking enzymatic peptide degradation and turnover of CSF into account. Although injection of 1000 ng of cytokines into the brain parenchyma seems unphysiological, considering the potential local production of cytokines in the brain, an even greater amount of the cytokines than that measured in the circulation, i.e. approximately  $10^8$  to  $10^{10}$  g/ml, may be available to neuronal target sites.

In summary, the present study suggests that the sympathoadrenal activation during septic challenge may be partially mediated by IL-1 via its central actions. Furthermore, paraventricular nucleus of the hypothalamus appears to be one of the sites mediating cytokine induced sympathetic response.

## CHAPTER VIII

### SUMMARY AND CONCLUSIONS

Sympathoadrenal activation is one of the most dramatic pathophysiological changes which occur during septic/endotoxic shock. Such increased sympathetic nerve activity has been linked to both beneficial and detrimental effects on the development of the shock process. The present dissertation study examined potential mechanisms involved in mediating sympathoadrenal activation during experimental endotoxemia.

The first project evaluated the overall contributions of central versus peripheral mechanisms to the sympathoadrenal activation in endotoxic animals. Specifically, plasma catecholamine responses to endotoxin challenge were compared between rats with and without central regulatory mechanisms, i.e. conscious and pithed rats, respectively. Plasma EPI following endotoxin was further assessed in adrenal denervated conscious and pithed rats to illustrate the neurogenic or non-neurogenic characteristics of peripheral modulation of adrenal EPI release.

Summary of results from the first project:

1. Endotoxin induced a marked elevation in plasma NE in conscious rats, but elevations were not seen in pithed rats.
2. Endotoxin also induced a profound increase in plasma EPI in conscious rats, but



increases were much less and delayed in pithed rats.

3. Plasma EPI elevation following endotoxin was delayed in conscious adrenal denervated rats compared to adrenal sham-operated rats. This elevation was approximately one third of the maximum response observed in sham controls.

4. The delayed plasma EPI elevation was also seen in pithed adrenal denervated rats, which was not different from that in pithed adrenal intact rats.

Conclusions drawn from the first project:

1. The increased plasma NE in response to endotoxin challenge is centrally mediated.
2. The increased plasma EPI following endotoxin involves both central and peripheral mechanisms with the former being dominant.
3. Peripheral modulation of adrenal EPI release is most likely non-neurogenic, which contributes to the prolonged plasma EPI response during endotoxemia.

The second project examined the role of afferent neural input from arterial baroreceptors in mediating the sympathoadrenal activation during endotoxemia. Specifically, plasma catecholamines and/or RSNA were assessed in anesthetized rats with acute SAD and conscious rats with chronic SAD.

Summary of results from the second project:

1. Endotoxin induced a similar, if not higher, elevation in plasma catecholamines in anesthetized rats with acute SAD compared to baroreceptor intact sham rats.
2. Endotoxin induced rapid and sustained increases in both plasma catecholamines and RSNA in conscious rats with chronic SAD. The early increments in plasma

catecholamines and RSNA were more profound in SAD rats than sham animals.

3. Plasma catecholamines were significantly increased by hydralazine-induced hypotension in sham rats, but not in SAD rats. This elevation was far less than that induced by endotoxin.

Conclusions drawn from the second project:

1. Afferent neural input from arterial baroreceptors is not essential in mediating sympathoadrenal activation during endotoxemia, and non-baroreflexogenic mechanisms are involved in stimulating such activation.
2. The elimination of feedback buffering mechanisms with SAD may account for the augmented sympathetic response seen in SAD animals.

The third project tested the hypothesis that cytokines may be involved in mediating the sympathoadrenal activation during endotoxemia, via their actions on the central nervous system. Specifically, IL-1, TNF, or IL-6 in addition to endotoxin was administered into the lateral ventricle of conscious rats, and plasma catecholamines and renal sympathetic nerve activities were assessed. To examine whether the PVN is the anatomical site of cytokine actions, each of the cytokines was delivered into the region of the PVN, and plasma catecholamines and RSNA were determined following the PVN injection.

Summary of results from the third project:

1. ICV injection of endotoxin did not significantly change plasma catecholamines or RSNA.

2. ICV injection of IL-1 induced modest increases in both plasma catecholamines and RSNA. PVN injection of IL-1 also induced a mild elevation of plasma catecholamines without significant effects on RSNA.
3. ICV injection of TNF resulted in a slight attenuation of RSNA without significant effects on plasma catecholamines. RSNA was also slightly decreased following PVN injection of TNF without significant alteration in plasma catecholamines.
4. ICV injection of IL-6 caused decreases in RSNA and no changes in plasma catecholamines. PVN injection of IL-6 altered neither RSNA nor plasma catecholamines.

Conclusions drawn from the third project:

1. The sympathoadrenal activation during developing septic/endotoxic shock may be partially mediated by IL-1 via its central actions while the direct effects of cerebral ventricular TNF and IL-6 suppress renal sympathetic activity.
2. The alterations of sympathoadrenal activity associated with ventricular IL-1 and TNF may be mediated, in part, by their actions on PVN. The sympathetic suppression with ventricular IL-6 is not likely mediated by actions on PVN.

The sympathoadrenal activation in response to septic/endotoxic challenge involves very complicated processes in peripheral sensory structures, afferent nerves, central neuronal networks, efferent sympathetic nerves, and the adrenal medulla. Endotoxin, endotoxin-elaborated host factors and various pathophysiological alterations associated with septic shock may interact with those neuronal elements at different levels and result in enhanced activity of the sympathoadrenal system.

As can be integrated from the present study as well as previous works, a schematic representation of sympathoadrenal regulation during septic/endotoxic shock is presented in Fig. 8-1. Endotoxin, released from the invading gram-negative bacterial wall, stimulates macrophages as well as a variety of other cell populations to release cytokines and numerous other host factors such as eicosanoids, bradykinin and histamine. These factors, particularly cytokines, are important mediators of the hemodynamic, metabolic, and neuroendocrine alterations associated with septic/endotoxic shock.

In terms of neuronal reflex mechanisms, decreased blood pressure and venous return with ultimate volume depletion during septic shock may deactivate both arterial baroreceptors and cardiopulmonary volume receptors, and reflexly result in increment of central sympathetic outflow. Blood borne factors such as epithelial derived factors, PGI<sub>2</sub>, opiates, and neurotensin may also modulate baroreflexes at different levels of the baroreflex arc including baroreceptors and NTS (48,49,228,256), which may also lead to augmented sympathetic activity. However, marked sympathoadrenal activation could occur in septic patients and animals without hypotension or hypovolemia (89,115). Alternatively, such activation persists after recovery of hypotension with volume infusion (185). Furthermore, profound sympathetic activation is observed in baroreceptor denervated endotoxic animals in the present study (255). These results suggest that baro- and volume- receptor reflexes are not likely to be essential for turning on and maintaining the sympathoadrenal activation during septic shock, despite their participation in such activation. It is noteworthy that stimulations of mechano- or chemo-sensitive nerve endings in abdominal visceral organs also evoke cardiovascular reflexes, which are

frequently excitatory (144,145). Such reflexes can be stimulated by passive distension, local temperature changes, altering vessel pressure and intraarterial injection or topical application of certain chemicals such as bradykinin, histamine and capsaicin (144-146,181). Although this reflex mechanism has implications in the sympathetic response during septic shock, the importance has not been studied.

Metabolic alterations associated with septic shock may also be involved in sympathetic response. Changes in blood pH as a result of hyperlacticemia and acidosis may stimulate peripheral and central chemoreceptors and result in enhanced sympathetic outflow. A previous study showed that the plasma epinephrine response to hemorrhagic shock was blunted after prevention of acidosis by intravenous administration of sodium bicarbonate (58). Insulin-induced hypoglycemia also leads to elevations of plasma catecholamines presumably through both neurogenic and non-neurogenic mechanisms (120). The neurogenic mechanism likely involves stimulations of gluco-sensitive receptors in the hypothalamus and spinal cord (175). Since these metabolic alterations are usually delayed processes, they do not appear to account for the rapid onset of sympathoadrenal activation during developing septic shock.

Recently, increasing attention has been drawn to the cytokine-mediated modulation of neuroendocrine systems, which is a plausible mechanism for sympathoadrenal activation during septic shock. Although cytokines appear unable to cross the blood brain barrier in significant quantities, recent studies suggest that cytokines may enter the brain at sites where blood brain barrier is absent such as OVLT (26,116,222). A further possibility is that circulating cytokines can somehow interact with the brain and stimulate

its own cytokine productions (34,203). Systemically and centrally administered cytokines, particularly IL-1, have been shown to exert many actions in the brain including the stimulation of the sympathoadrenal system (53,202,203). This sympathetic activation can be evoked by IL-1 via its direct effects on sympathetic regulatory neurons or via some secondary neural or humoral factors. For example, IL-1 is a potent endogenous pyrogen which can induce fever, and the associated thermogenesis is usually accompanied by selective activation of efferent sympathetics and adrenergic mediated vasoconstriction (91). IL-1 also stimulates the hypothalamic-pituitary-adrenal axis primarily via releasing CRF from the hypothalamus, and CRF has long been recognized to enhance central sympathetic outflow (38). PVN seems to be one of IL-1 action sites in evoking sympathetic activation.

Non-neurogenic modulation of adrenal catecholamine release may involve direct actions of hypoxia, endotoxin-elaborated endogenous mediators or endotoxin itself on the adrenal medulla (166,225). Numerous endogenous substances such as histamine, bradykinin, angiotensin II, and vasoactive intestinal polypeptide have been shown to stimulate adrenal gland or chromaffin cells to secrete catecholamines (130,142,153,177).

Further studies are needed to determine, in a more systematic and comprehensive manner, the role of cytokine mediated modulation of the sympathoadrenal response during developing septic shock and the mechanisms underlying this modulation. Hypophysectomized animals could be used to determine whether the hypothalamus is the primary center of cytokine modulation. Since the OVLT has been proposed to be the entry or action site of circulating cytokines to the hypothalamus to mediate their

pyrogenic effects and hypothalamic-pituitary-adrenal stimulation, experiments with OVLT lesions or direct injection of cytokines to OVLT may help understanding the OVLT involvement in sympathetic response to endotoxin and cytokines. Electrophoretically sampling in a confined hypothalamic area may allow the evaluation of the quantity and time course of local cytokine production during endotoxemia as well as its correlation with sympathetic activation. Assessment of sympathetic activity in septic animals after application of specific cytokine antibodies to the brain areas suspected to be the cytokine production and action sites would provide additional valuable information on central cytokine involvements in sympathetic response during developing septic shock.

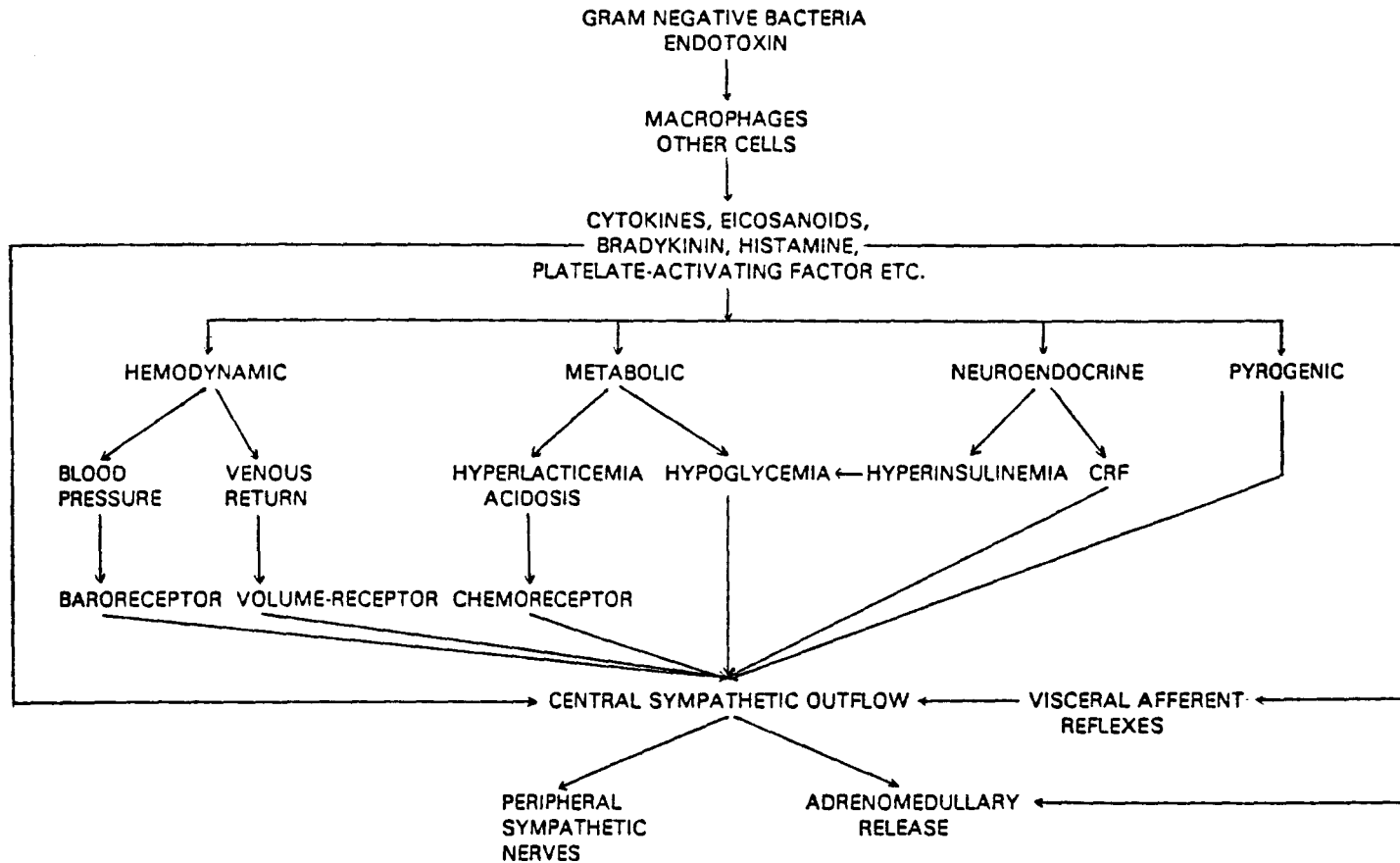


Figure 8-1. Schematic representation of possible regulation of the sympathoadrenal system during developing septic shock. CRF: corticotropin releasing factor.



## CHAPTER IX

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

2/27/92

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