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STAGE OF SEPSIS DETERMINES THE MECHANISM OF VASCULAR SMOOTH MUSCLE CONTRACTILE DYSFUNCTION

presented by

Samuel Howard Wurster

has been accepted towards fulfillment
of the requirements for

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Major professor
Irshad H. Chaudry, Ph.D.

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STAGE OF SEPSIS DETERMINES THE MECHANISM OF VASCULAR SMOOTH MUSCLE CONTRACTILE DYSFUNCTION

By

Samuel Howard Wurster

A THESIS

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ABSTRACT

STAGE OF SEPSIS DETERMINES THE MECHANISM
OF VASCULAR SMOOTH MUSCLE CONTRACTILE DYSFUNCTION

By

Samuel Howard Wurster

Although vascular smooth muscle (VSM) contractile function is impaired during late stages of sepsis, it is not known whether this impairment also occurs in the early stages of sepsis and, if so, whether different mechanisms are responsible for this dysfunction. To determine this, rats were subjected to sepsis by cecal ligation and puncture (CLP). Septic and sham rats were then sacrificed at 5, 10, 20, or 35 hours post-CLP and aortic rings were prepared for contraction studies using organ chamber technique. Dose-response contractions to norepinephrine (NE) and KCl were determined in rings with or without intact endothelium. Additionally, following peak contraction to NE, N^G-monomethyl-L-arginine (L-NMMA, an inhibitor of NO synthase) was added to the organ chambers of endothelium denuded aortic rings harvested at 10, 20 and 35 hours post-CLP. Endothelium removal at 10 and 20 hrs after CLP restored the contraction induced by NE and KCl toward sham levels. In contrast, the smooth muscle contractility at 35 hrs post-CLP remained impaired despite removal of septic endothelium.

For

Zachary,

Nathaniel

and

Caroline

ACKNOWLEDGMENTS

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Thanks to Carol Ayala, Zheng F. Ba, Mary Morrison and Margy Lynch for their technical expertise. Thanks also to my thesis committee: Drs. Alfred Ayala, Lana Kaiser and John Kisala.

And finally..., I would like to thank my wife, Ann, for her faith in me.

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KEY TO ABBREVIATIONS

ACh . . . . . Acetylcholine

ANOVA . . . . . Analysis of Variance

CLP . . . . . Cecal ligation and Puncture

cNOS . . . . . Constitutive Nitric Oxide Synthase

cGMP . . . . . Cyclic Guanosine Monophosphate

EDRF . . . . . Endothelium-Derived Relaxing Factor

EM . . . . . Electron Micrograph

iNOS . . . . . Inducible Nitric Oxide Synthase

KCl . . . . . Potassium Chloride

L-NMMA . . . . . N^G-monomethyl-L-arginine

NE . . . . . Norepinephrine

NO . . . . . Nitric Oxide

NOS . . . . . Nitric Oxide Synthase

O_2 . . . . . Oxygen

SE . . . . . Standard Error

TNF . . . . . Tumor Necrosis Factor

VSM . . . . . Vascular Smooth Muscle

INTRODUCTION

CLINICAL SIGNIFICANCE

Over the last decade laboratory investigations have greatly improved our understanding of the complex pathologic processes referred to as sepsis. Initially, the manifestations of sepsis were presumed to be mediated solely by infecting microbes. Presently, it is clear that microbial pathogens are simply the initiators of a complex cascade of endogenous mediators which comprise the final common pathway of sepsis or systemic inflammatory response syndrome. Despite this delineation of the mechanisms of sepsis, the efficacy of therapies to combat sepsis has not improved greatly since the plateau reached following the advent of antibiotics over fifty years ago.[1,2] Despite limited application of experimental treatments, such as endotoxin filtration and immune stimulation,[3] as well as, monoclonal antibodies directed against endotoxin[4] or inflammatory cytokines;[5] sepsis continues to be the most common cause of death in U.S. intensive care units. Current estimates of mortality from septic shock range as high as

100,000 deaths annually.[6,7] Sepsis which progresses to the point of multiple organ dysfunction remains almost universally fatal[8].

VASCULAR PATHOPHYSIOLOGY DURING SEPSIS

In addition to increased morbidity and mortality associated with septic complications, various investigators have postulated that increased susceptibility to sepsis may explain the occurrence of irreversible hemorrhagic shock, as well.[9,10,11] It has been well documented that the early response to sepsis is characterized by hyperdynamic circulation and hypermetabolism.[12,13] Both cardiac output[14,15,16] and microvascular perfusion in the liver, kidney, spleen and intestine are increased during the early stages of experimentally induced sepsis in rats (5-10 hrs post-CLP).[14,15] In contrast, tissue microvascular perfusion was found to be decreased during late stages of sepsis (>20 hrs post-CLP).[14,15]

The Role of Endothelium

Not long ago, the sole function ascribed to endothelial cells was as a protective and antithrombotic "cellophane" lining of the vascular tree.[17,18] In fact, it was only discovered as late as 1980 that acetylcholine induced vascular smooth muscle relaxation requires the presence of

intact endothelial cells.[19] Endothelial cells are situated at the vital interface between the blood and tissues where they can sense both mechanical and chemical changes in the environment. The endothelium can then process these signals and respond via the synthesis and release of a myriad of factors which have profound local and systemic effects.

It is known that patients in the end stages of sepsis often manifest a profound hypotension that is refractory to exogenous catecholamine administration.[20,21,22] In fact, human mortality from septic shock is correlated with decreased systemic vascular resistance.[23] Similarly, rats with experimentally induced endotoxemia exhibit diminished pressor responses *in vivo*.[24-28] Aortic vessel rings harvested from septic or endotoxemic rats demonstrate a marked diminution of norepinephrine (NE)-induced maximal contraction.[29,30] Partial or complete restoration of contractility following removal of endothelium from septic vascular tissue indicates that endothelium-derived factors play a major role in producing contractile depression of VSM.[29]

The Role of Nitric Oxide

In 1980, Furchgott and Zawadzki demonstrated that acetylcholine-induced relaxation of vascular smooth muscle is mediated by an "endothelium-derived relaxation factor" (EDRF).[19] The discovery that EDRF is nitric oxide (NO)

precipitated an explosion of research into its bioregulatory and cytotoxic properties.[30-34] EDRF/NO has a biological half-life of less than 30 seconds and resembles nitrovasodilators in that it activates VSM soluble guanylate cyclase, producing a rise in muscle cell cyclic guanosine monophosphate (cGMP).[35] Many studies have confirmed that NO is synthesized from the conversion of L-arginine to citrulline by at least two different isoforms of or nitric oxide synthases (NOS).[36-40]

One synthase isoform is constitutive, membrane associated, calcium/calmodulin dependent[41-46], and produces small quantities (picomoles)[47] of NO in response to stimulation/activation. This enzyme (cNOS) is an important transduction mechanism in the regulation of multiple physiologic processes such as the maintenance of normal vascular tone[48] and neurotransmission[49,50]. The other NO synthase is cytosolic, and calcium/calmodulin independent. This enzyme is induced in various cell types including endothelial cell[51] and vascular smooth muscle following activation by endotoxin, as well as, various cytokines[52] and is therefore referred to as inducible NO synthase (iNOS). Once activated the iNOS isoform produces larger quantities (nanomoles) of NO for prolonged periods, when compared to cNOS.[53] Both NOS isoforms require tetrahydrobiopterin, among other cofactors, in order to synthesize nitric oxide.[36-40] Thus far, the only confirmed role of NO produced following

iNOS activation is as a cytotoxic molecule[54] for invading microorganisms[55] and tumor cells.[56]

It has been postulated that activation of iNOS may result in host tissue damage via pathological vasodilation and/or direct cytotoxic effects.[57] Cytotoxicity of NO is due in large part to its reaction with superoxide anion to produce peroxynitrite[58,59] and subsequent hydroxyl radical formation.[60] Nitric oxide mediated increases in target cell cGMP results in protein phosphorylation by cGMP dependent kinases, which is the first step in many of the physiologic effects mediated by NO.

The recent discovery of a shear stress regulatory element[61,62] which regulates NOS activity in response to alterations in endothelial shear forces may explain the phenomenon of flow dependent vasodilation[63-66] at both the macro and microcirculatory levels. In addition to its short half-life, NO is rapidly inactivated by superoxide anions and hemoglobin[67] and is protected by superoxide dismutase. There is additional evidence that NO production exerts a feedback inhibition upon NOS.[68,69] Like prostacyclin, NO can relax smooth muscle as well as inhibit platelet aggregation.[70]

Exposure of vascular tissue to septic mediators such as endotoxin[71] and tumor necrosis factor (TNF)[72] has yielded important information as to their individual effects upon the contractile function of vascular smooth muscle.

However, true reproduction of clinically relevant sepsis seems to require the presence of all septic mediators, which are present only *in vivo*. This presumption is supported by findings of a greater suppression of agonist induced contractility in vascular tissue removed from rats previously injected with endotoxin, as compared to vascular tissue incubated with endotoxin in a serum free environment[24].

Several studies have indicated that the regulation of *in vivo* vascular tone is influenced by multiple factors including endothelin-1,[73,74] cytokine release,[75] and blood flow velocity.[76,77] Furthermore, other investigators have suggested that an alteration in alpha adrenoreceptor-coupled signal transduction is a primary mechanism of sepsis induced VSM contractile depression.[24,78]

Although it is known that VSM contractile function is depressed in the late stages of sepsis, it remains unclear whether vascular contractility is altered in the early stages of sepsis and, if so, whether different mechanisms are responsible for this dysfunction. Since it is difficult to accurately assess alterations in vascular reactivity *in vivo*, it was postulated that *ex vivo* testing of vascular responsiveness to both alpha-adrenoreceptor and non-receptor-dependent contractile agonists, in the presence and absence of intact endothelium, at selected timepoints during

experimentally induced sepsis, should provide insight into the dominant mechanisms involved.

Purpose

This study attempts to define more precisely the roles which septic endothelium and receptor-mediated phenomena play in producing VSM contractile dysfunction from early to late stages of sepsis. The contribution of vessel wall iNOS activation to VSM contractile derangement was investigated by blockade of NO production at precise intervals following CLP-induced sepsis. It was hypothesized that structural damage to the vascular smooth muscle itself would be evidenced by a diminished contractile response which is independent of contractile agonist, the presence of endothelium, or inhibition of nitric oxide synthesis.

MATERIALS AND METHODS

ANIMAL MODEL

The adult male Sprague-Dawley rat was chosen as the animal model for studying the effects of sepsis upon vascular smooth muscle contractility. Rats subjected to cecal ligation and puncture (CLP) progress through the early and late stages of sepsis in a predictable fashion similar to that observed in humans. Additionally, the methodology for determination of vascular smooth muscle contractility is standardized and highly reproducible. Rats are also small, easy to house, and relatively inexpensive.

GENERAL

Disease free male Sprague-Dawley rats (from Charles River Laboratory, Portage, MI) weighing 250-300 g were used throughout the studies. The animals were housed and acclimatized for at least 72 hours at the University Laboratory Animal Research (ULAR) building prior to experimentation. The rats were maintained on a consistent 12 hour light/dark cycle. Prior to experimentation, they

were fasted overnight (approximately 16 hours) but allowed water *ad libitum*. All protocols were carried out in accordance with the guidelines set forth in the Animal Welfare Act and as outlined in the Guide for Care and Use of Laboratory Animals by the National Institutes of Health Publications.

Sepsis Model

Intra-abdominal sepsis was produced in male Sprague-Dawley rats (250-300 g) by cecal ligation and puncture (CLP), according to the method of Wichterman *et al.*[16] The rats were lightly anesthetized with ether, and a 2-cm ventral midline incision was performed. The cecum was then exposed, ligated just distal to the ileocecal valve with 3-0 silk suture to avoid intestinal obstruction, punctured twice along the antimesenteric border with an 18 gauge needle, and returned to the abdomen. The incision was closed in two layers with 3-0 nylon continuous suture. The animals then received 3 ml/100 g body wt normal saline injected subcutaneously immediately following abdominal closure.

Sham-operated rats underwent the same surgical procedure with the exception that the cecum was neither ligated or punctured. Sham-operated animals also received 3 ml/100 g body wt normal saline by subcutaneous injection following abdominal closure. Both CLP and sham-operated animals were then returned to their cages for observation.

CLP and sham-operated animals were randomly divided into four groups which were sacrificed at 5, 10, 20 or 35 hours after CLP or sham operation. There were no deaths prior to sacrifice in rats observed from 5 to 20 hours following CLP. However, 2 out of 13 rats expired prior to the 35 hour time point following CLP induced sepsis.

PART I

Study of VSM Contractility
Utilizing Receptor and Non-receptor Mediated Agonists
From Early to Late Stages of Sepsis

The experimental animals were killed by cervical dislocation prior to immediate thoracotomy. The heart, lungs and thoracic aorta were then removed quickly and placed into ice-cold Krebs-Ringer HCO_3 solution (composition in mM: Nacl, 118.3; KCl, 4.7; $CaCL_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; Ca-EDTA, 0.026; glucose, 11.1), which was aerated with 95% O_2 : 5% CO_2 (pH=7.4; pO_2=580 mmHg). The thoracic aorta was sharply dissected from adjacent tissue with microinstruments to prevent damage to the vessel wall or endothelium. The isolated descending thoracic aorta was then sliced with a razor blade into two rings, each approximately 2.5 mm in length. The endothelium was then removed from one of the two aortic rings by inserting the shank of a small forceps into the lumen of the vessel ring,

and then gently rolling the vessel ring along the operators finger for four complete revolutions.[79,80]

The aortic rings were then suspended between two specimen holders and placed in jacketed glass organ chambers containing 20 ml of Krebs-Ringer HCO_3 solution aerated continuously with a 95% O2 and 5% CO_2 gas mixture maintained at a constant temperature of 37°C. The lower specimen holder was stationary and the upper specimen holder was connected to an isometric force-displacement transducer (Model FTO3, Grass Instruments) coupled to a polygraph (Model 7D, Grass Instruments). The vessels were then allowed to equilibrate for 60 minutes at a resting tension of 2000 mg, during which the organ chamber was rinsed at 15 minute intervals with fresh aerated Krebs-Ringer HCO_3 solution. When the basal tension was stable, the presence or absence of intact endothelium was determined by the response of the vessel ring to an endothelium-dependent vasodilator, acetylcholine (ACh).[19] From baseline, ~75% of maximal contraction of the vessel rings was achieved by adding 2 X 10^{-7} M norepinephrine (NE, Sigma, St. Louis, MO). Immediately following submaximal contraction, 1 X 10^{-6} M ACh (Sigma, St. Louis, MO.) was added to the organ chamber to test the functional integrity of the endothelium. Aortic rings demonstrating greater than 50% relaxation upon addition of ACh were considered to have intact endothelium.[81] In this experiment, the endothelium-denuded rings did not demonstrate any significant vascular relaxation following

addition of ACh. The vessels were then allowed to equilibrate for 3 rinse cycles (45 minutes). When a stable baseline tension was again established, cumulative dose-response curves for NE (concentration ranges from 10^{-9} to 10^{-5} M) were carried out in all aortic rings.

Following three washes with Krebs-Ringer HCO_3 solution, a second agonist, KCl (concentration range from 7.5 to 90 mM) was added to each organ chamber in cumulative fashion, and the change in vascular tension was recorded. Immediately following the final contraction curve, the aortic rings were removed from the organ chambers, blotted on tissue paper, and weighed.

PART II

Study of the Effects of Nitric Oxide Synthase Blockade Upon Peak VSM Contractility From Early to Late Stages of Sepsis

Following CLP-induced sepsis male rats (250-300 g) were sacrificed at 10, 20 and 35 hours, both the septic and sham aortic rings were then prepared for contractile studies in the previously described fashion with the exception that the endothelium was removed from all septic and sham aortic rings. Following attainment of stable baseline at a resting tension of 2000 mg, submaximal contraction of aortic ring VSM was achieved by addition of 2×10^{-7} M NE to the organ chambers. Immediately following submaximal contraction, 1

x 10^{-6} M ACh was added to the organ chambers to test for the presence of intact (functional) endothelium. Again, none of the endothelium denuded vessel rings exhibited any significant ACh-induced relaxation.

The aortic rings were then allowed to equilibrate for 45 minutes, during which time the organ chamber was rinsed at 15 minute intervals with Krebs-Ringer HCO_3 solution. When basal tension was stable, cumulative dose-response curves for NE (concentration ranges from 10^{-9} to 10^{-5} M) were carried out in all aortic rings.

At peak NE-induced contraction, 300μM of N^G-monomethyl-L-arginine (L-NMMA) (Calbiochem, La Jolla, CA) a competitive inhibitor of nitric oxide (NO) synthesis was added to each organ chamber and the NE-induced peak contraction was recorded both before and after addition of L-NMMA.

Following maximal response to L-NMMA administration, a total of 3mM of L-arginine hydrochloride (Sigma, St. Louis, MO) was added to the organ chambers (3 aliquots of 1mM L-arginine, 5 minutes apart) and the change in peak vascular contractile response was recorded. Immediately following the final contraction curve, the aortic rings were removed from the organ chambers, blotted on tissue paper, and weighed.

Electron Micrographic Study of Aortic Ring Ultrastructure at Different Stages of Sepsis

Two representative vessel rings were obtained from a rat at 35 hours after sham operation. The two vessel rings were then immediately immersed into capped test tubes containing 4% buffered gluteraldehyde solution prior to electron microscopic (EM) inspection. Two vessel rings were then harvested from rats at 10, 20 and 35 hours following CLP-induced sepsis and each were prepared for EM analysis in the said fashion. In total, (including sham vessel rings) aortic rings from four different rats were inspected.

STATISTICAL ANALYSIS

All vascular contraction values were determined as peak changes from control levels at each concentration of contractile agonist. The contraction induced by the agonist was expressed as mg tension per mg of tissue weight. Since NE-induced peak vascular contraction occurred at 1 X 10^{-6} M concentration, these values were used to represent NE-induced peak vascular contraction. The peak contraction induced by KCl occurred at a concentration of 90 mM. The statistical tests utilized were as follows:

1. Comparison of vascular contractile function between receptor (NE) and non-receptor mediated contractile agonists at 5 to 35 hours of sepsis was accomplished with a one-way analysis of variance (ANOVA) followed by Tukey's test. The differences were considered significant at $P < 0.05$. Results are presented as mean ± standard error of the mean (SEM). N = 6-7 per group.

2. The effect of L-NMMA upon NE-induced peak vascular contractile function in endothelium denuded vessel rings at 10 to 35 hours of sepsis was analyzed with a one-way analysis of variance (ANOVA followed by Tukey's test. The results are presented as mean ± SEM and differences were considered significant at $P < 0.05$. N = 4-5 per group.

RESULTS

PART I

CONTRACTILE RESPONSE TO RECEPTOR AND NON-RECEPTOR MEDIATED AGONISTS FROM EARLY TO LATE STAGES OF SEPSIS

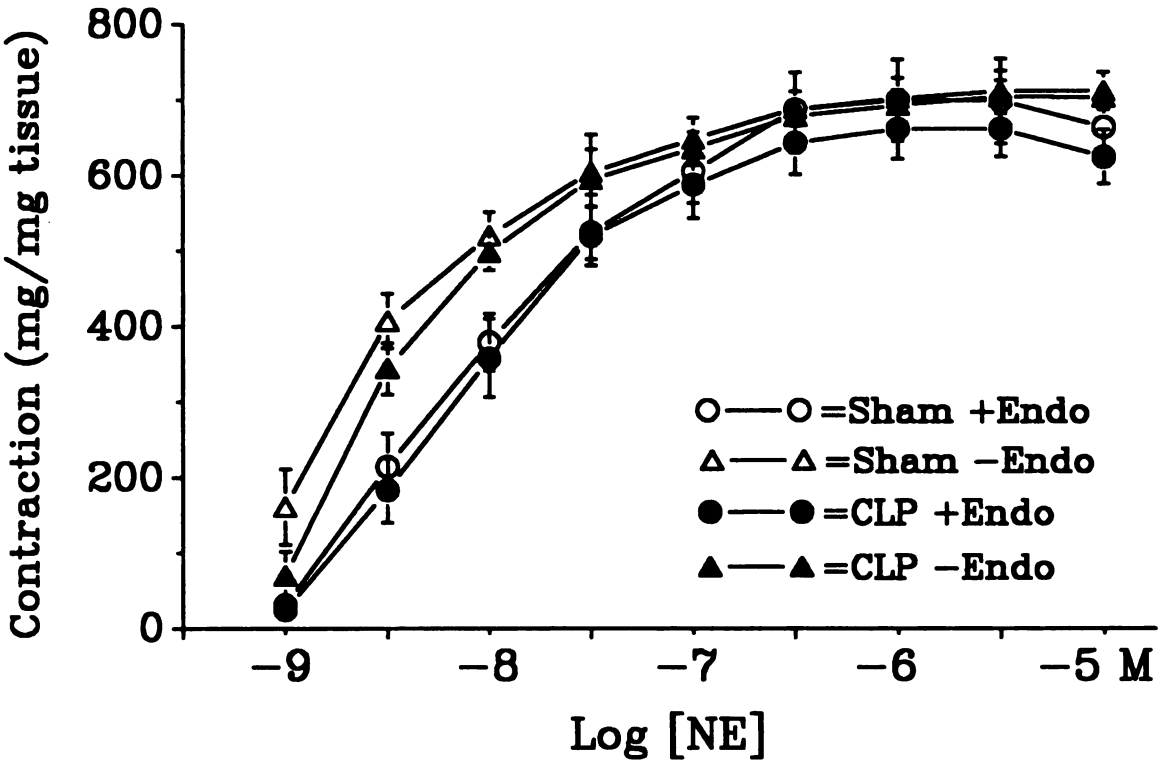
Norepinephrine-induced vascular contraction:

Aortic rings harvested from both septic and sham rats 5 hours after CLP demonstrated no significant differences in either cumulative dose response (Figure 1) or peak NE-induced vascular smooth muscle contractility (Figure 2). This finding was not altered by the presence or absence of intact endothelium in either sham or septic aortic rings.

At 10 hours following CLP-induced sepsis, there is a significant decrease in NE-induced peak vascular contraction in septic aortic rings with intact endothelium. Endothelium removal, however, completely restored NE-induced peak vascular contractility of septic aortic rings to sham levels (Figures 3 & 4).

At 20 hours following CLP, there is a marked decrease in NE-induced vascular contractility in septic aortic rings with intact endothelium. Removal of septic endothelium 20 hours post-CLP significantly improves peak vascular

Figure 1. Cumulative dose-response curve to various concentrations of norepinephrine (NE) in aortic rings at 5 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.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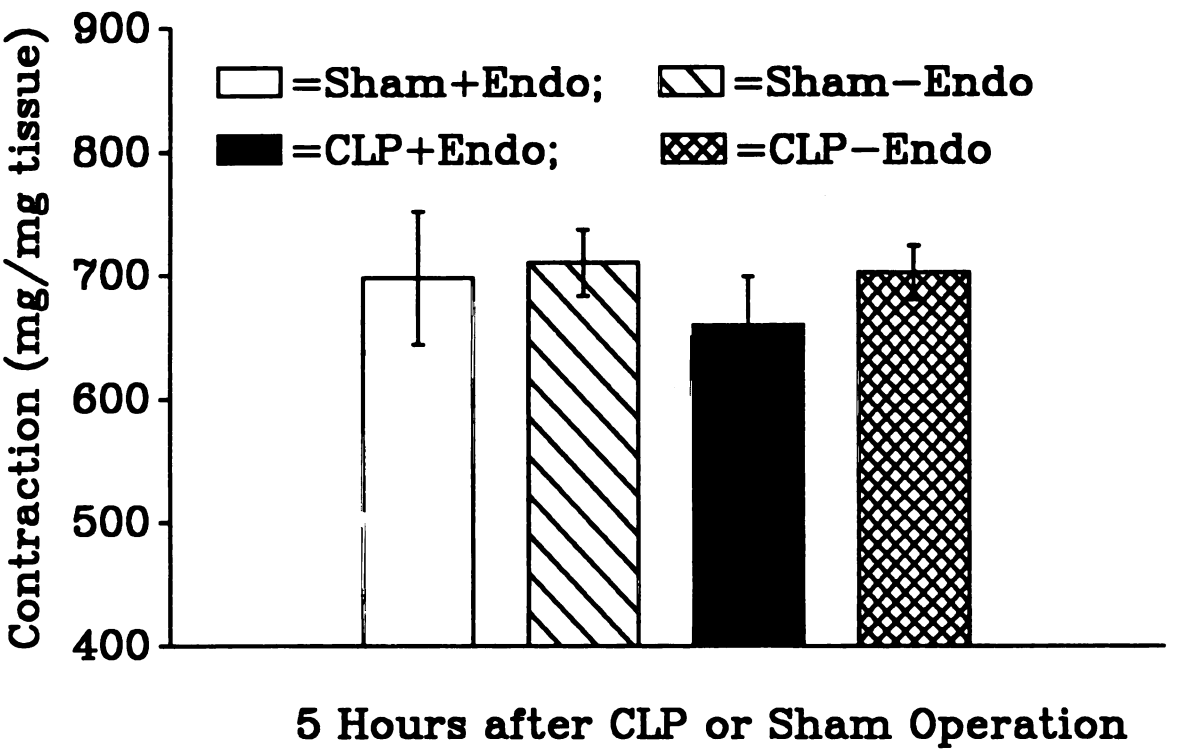
Log [NE]
O—O =Sham +Endo
Δ—Δ =Sham −Endo
●—● =CLP +Endo
▲—▲ =CLP −Endo

Figure 2. The peak vascular contraction induced by 1 X 10-6 M NE at 5 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.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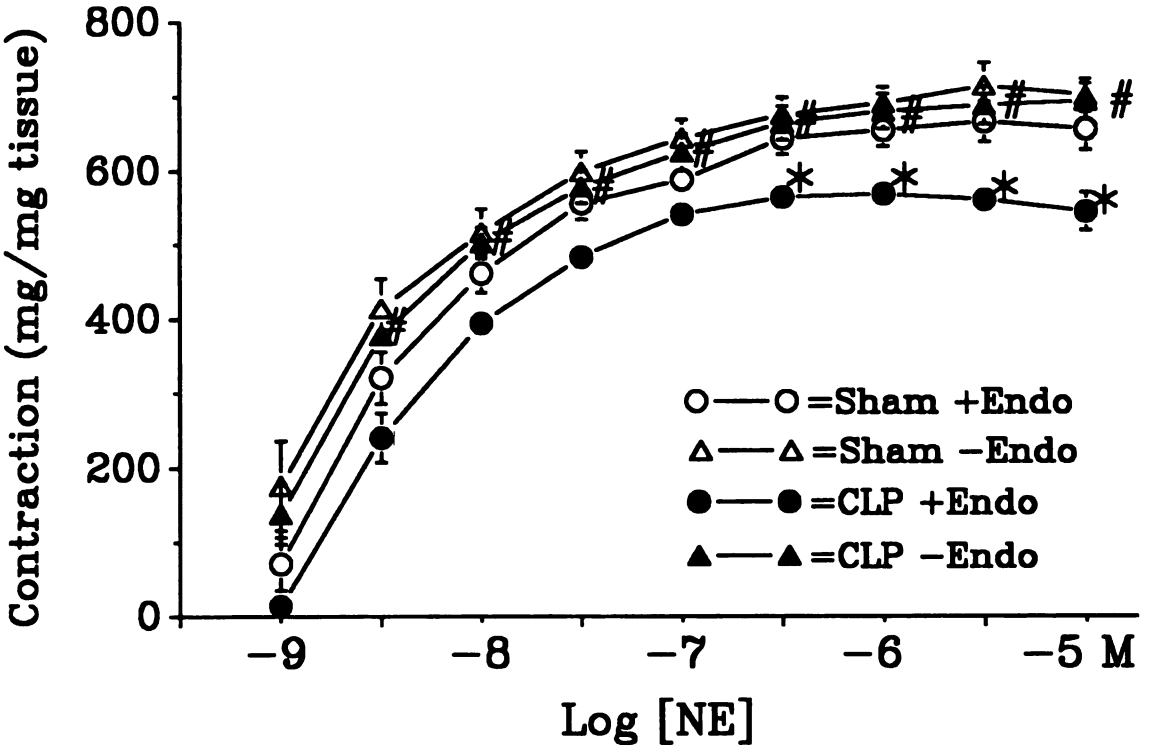
Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
5 Hours after CLP or Sham Operation

Figure 3. Cumulative dose-response curve to various concentrations of norepinephrine (NE) in aortic rings at 10 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.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●—●=CLP +Endo
▲—▲=CLP −Endo
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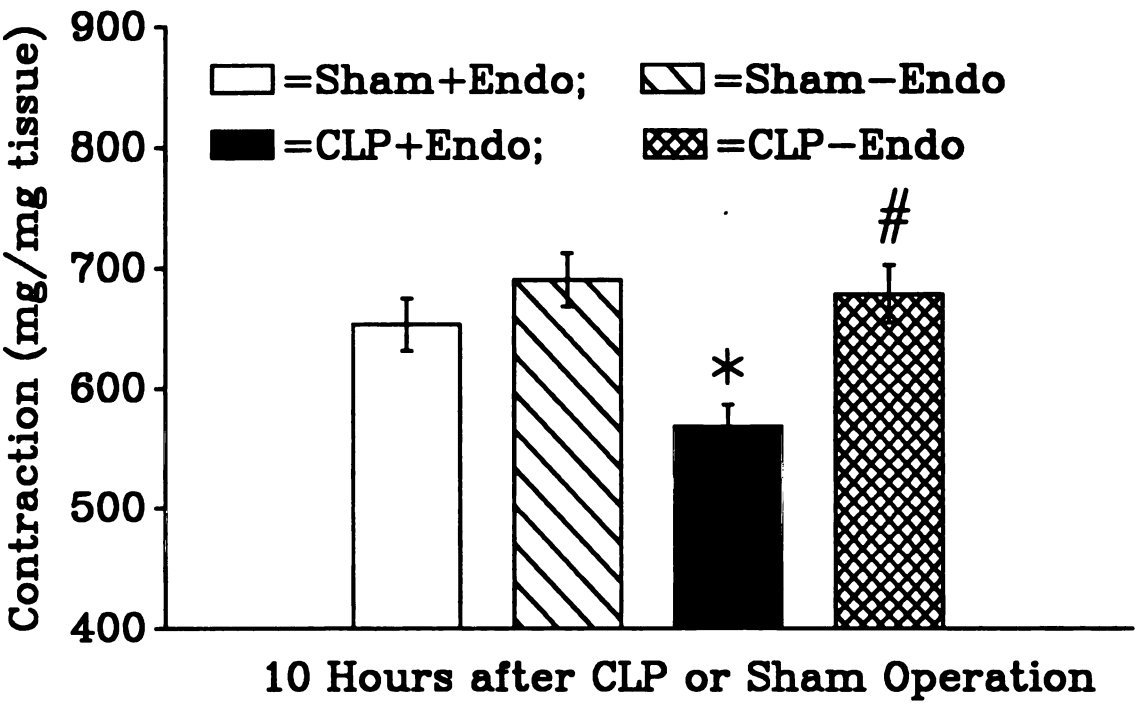
Figure 4. The peak vascular contraction induced by 1 X 10-6M NE at 10 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact rings. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.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Contraction (mg/mg tissue)
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#
10 Hours after CLP or Sham Operation

contractility, but does not completely restore maximal contractile function to sham levels (Figures 5 & 6).

At 35 hours following CLP, there is a severe depression of NE-induced contractility in septic vessels with intact endothelium. Contractile function remains markedly depressed despite removal of septic endothelium (Figures 7 & 8).

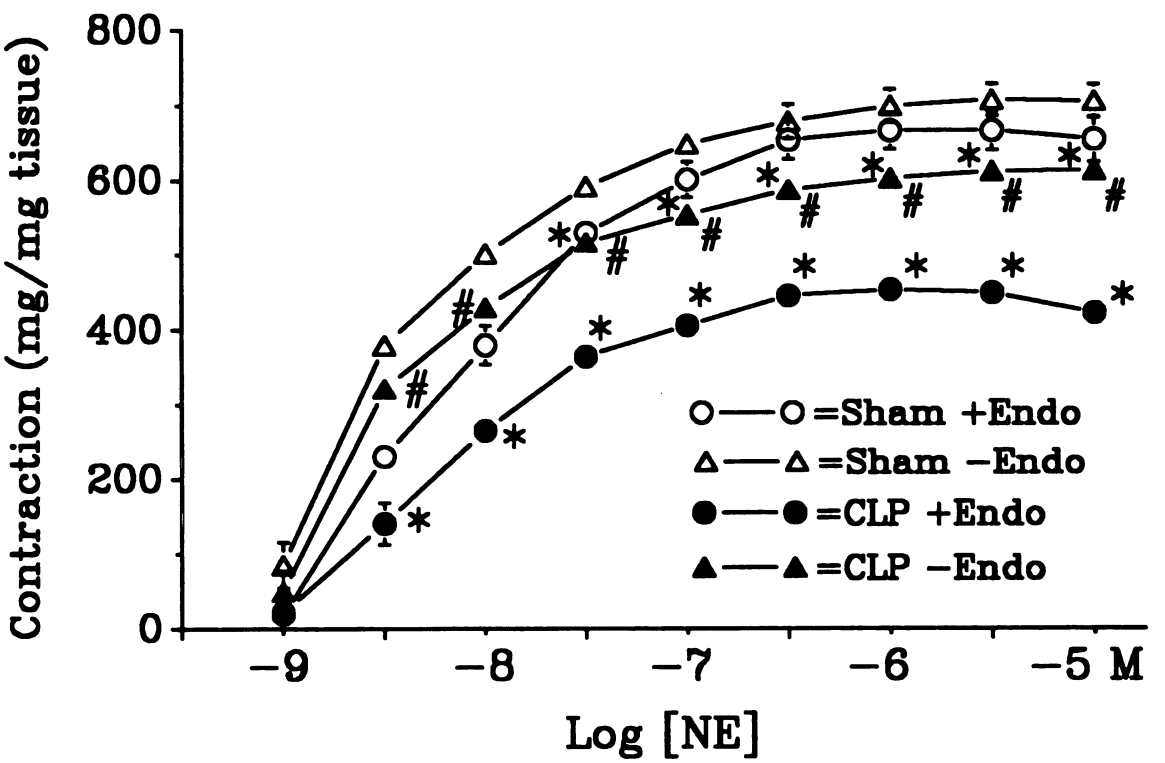
Potassium Chloride-induced vascular contraction:

Aortic rings harvested at 5 hours post-CLP from both septic and sham rats demonstrated no significant alteration in either cumulative dose-response (Figure 9) or peak KCl-induced vascular smooth muscle contractility (Figure 10). This finding was not altered by the presence or absence of intact endothelium in either septic or sham aortic rings.

At 10 hours following CLP-induced sepsis, there is a significant decrease in KCl-induced peak vascular contraction in septic aortic rings with intact endothelium. Endothelium removal, however, completely restored KCl-induced peak contractility of septic aortic rings to sham levels (Figures 11 & 12).

Again at 20 hours following CLP, there is a marked decrease in KCl-induced peak contraction in septic aortic rings with intact endothelium, which is completely restored to sham levels following removal of septic endothelium (Figures 13 & 14).

Figure 5. Cumulative dose-response curve to various concentrations of norepinephrine (NE) in aortic rings at 20 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.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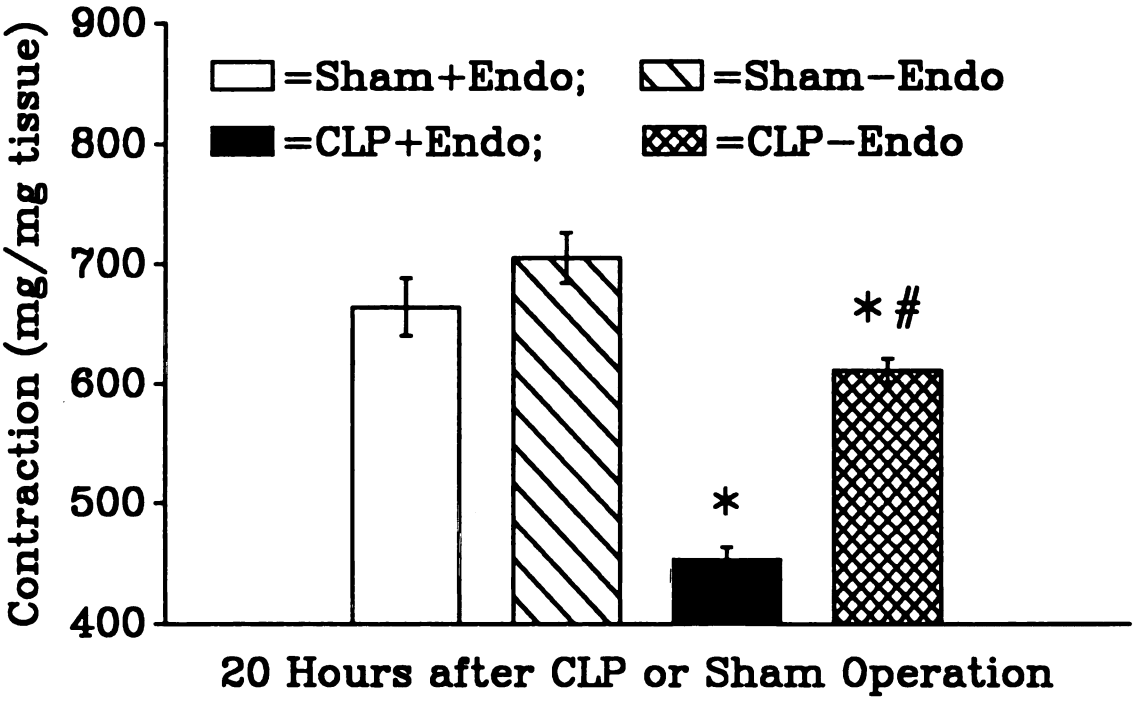
Log [NE]
O—O =Sham +Endo
△—△ =Sham −Endo
●—● =CLP +Endo
▲—▲ =CLP −Endo

Figure 6. The peak vascular contraction induced by 1 X 10-6M NE at 20 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact rings. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.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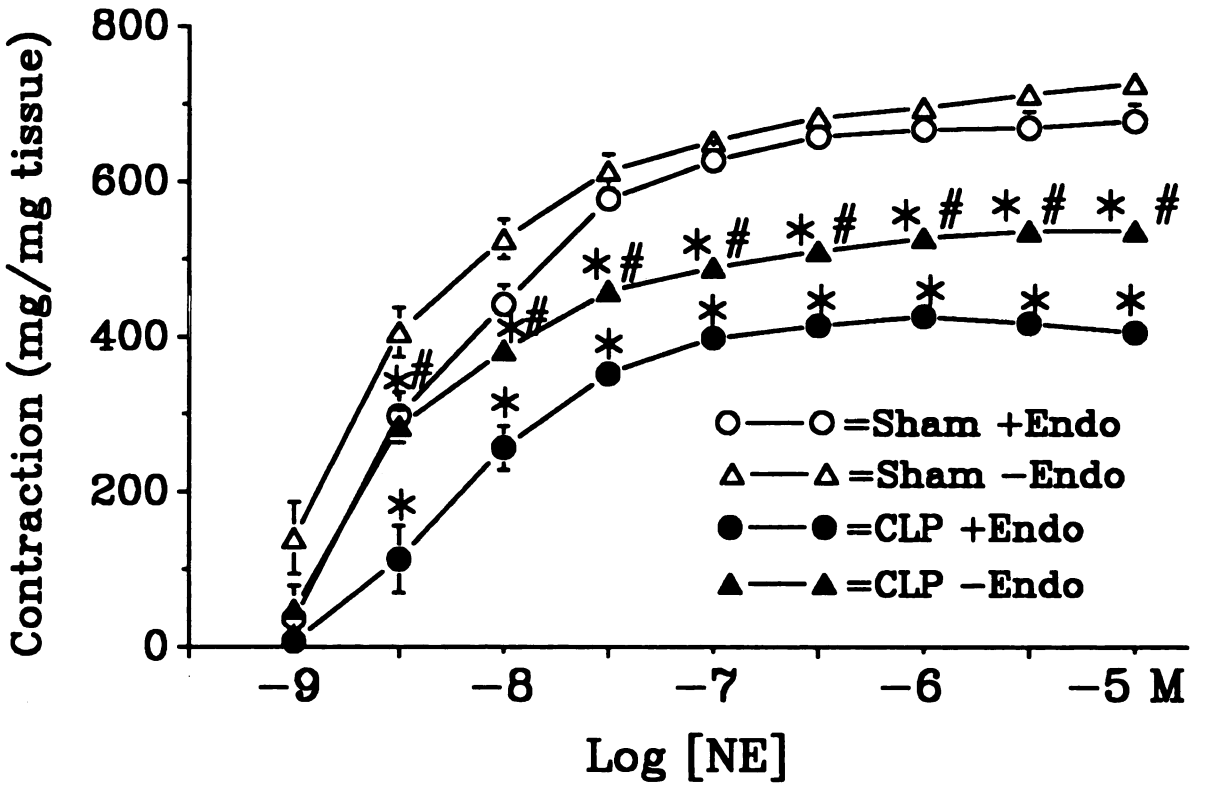
Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
*
*#
20 Hours after CLP or Sham Operation

Figure 7. Cumulative dose-response curve to various concentrations of norepinephrine (NE) in aortic rings at 35 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.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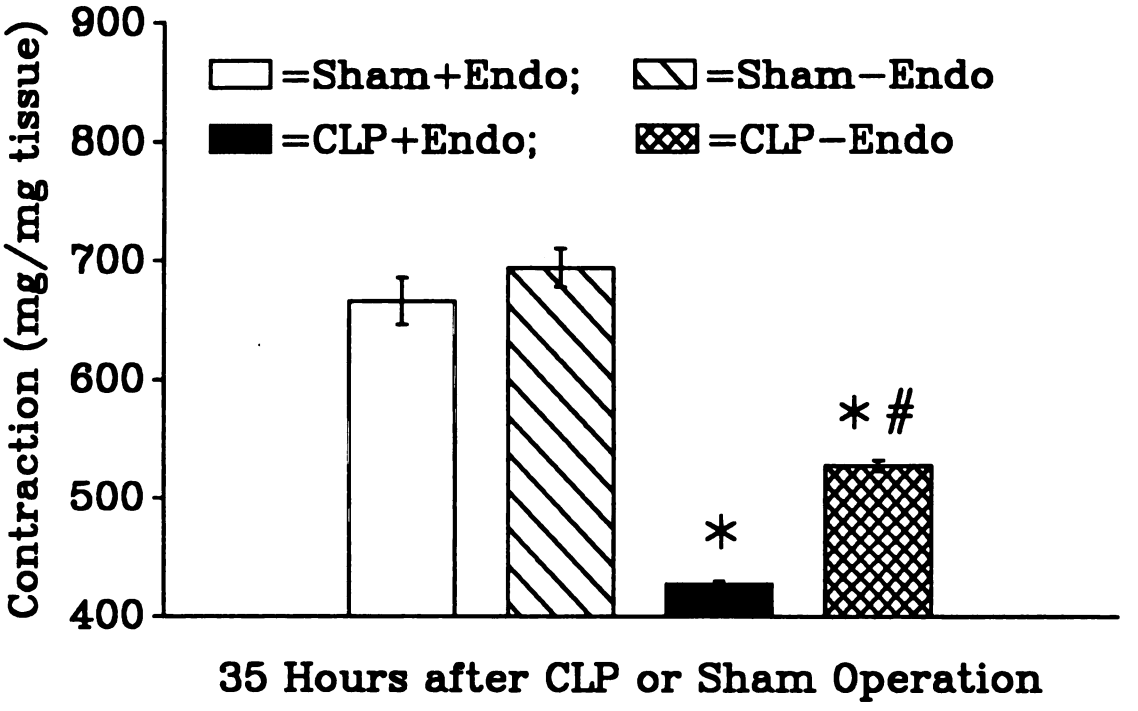
●—● =CLP +Endo
▲—▲ =CLP −Endo

Figure 8. The peak vascular contraction induced by 1 X 10-6M NE at 35 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact rings. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.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Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
*
*#
35 Hours after CLP or Sham Operation

Figure 9. Cumulative dose-response curve to various concentrations of potassium chloride (KCl) in aortic rings at 5 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.

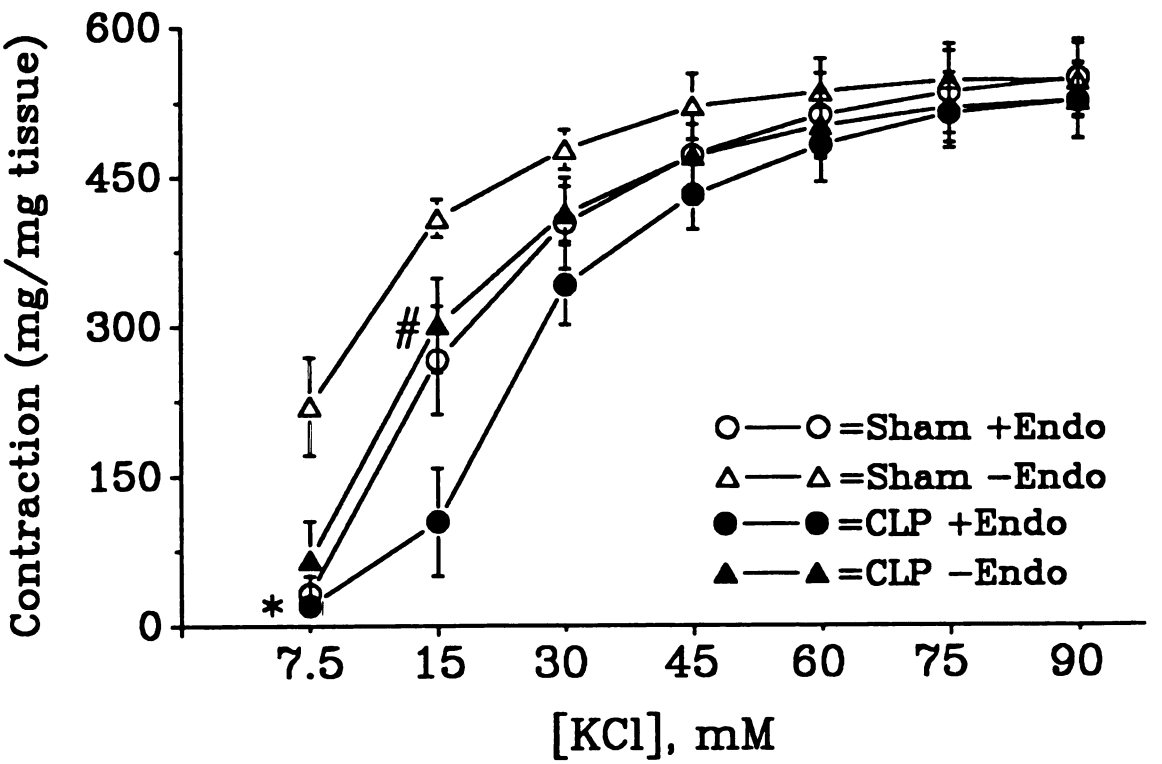

600
450
300
150
0
Contraction (mg/mg tissue)
7.5
15
30
45
60
75
90
[KCl], mM
*
#
O—O =Sham +Endo
Δ—Δ =Sham −Endo
●—● =CLP +Endo
▲—▲ =CLP −Endo

Figure 10. The peak vascular contraction induced by 90mM KCl at 5 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.

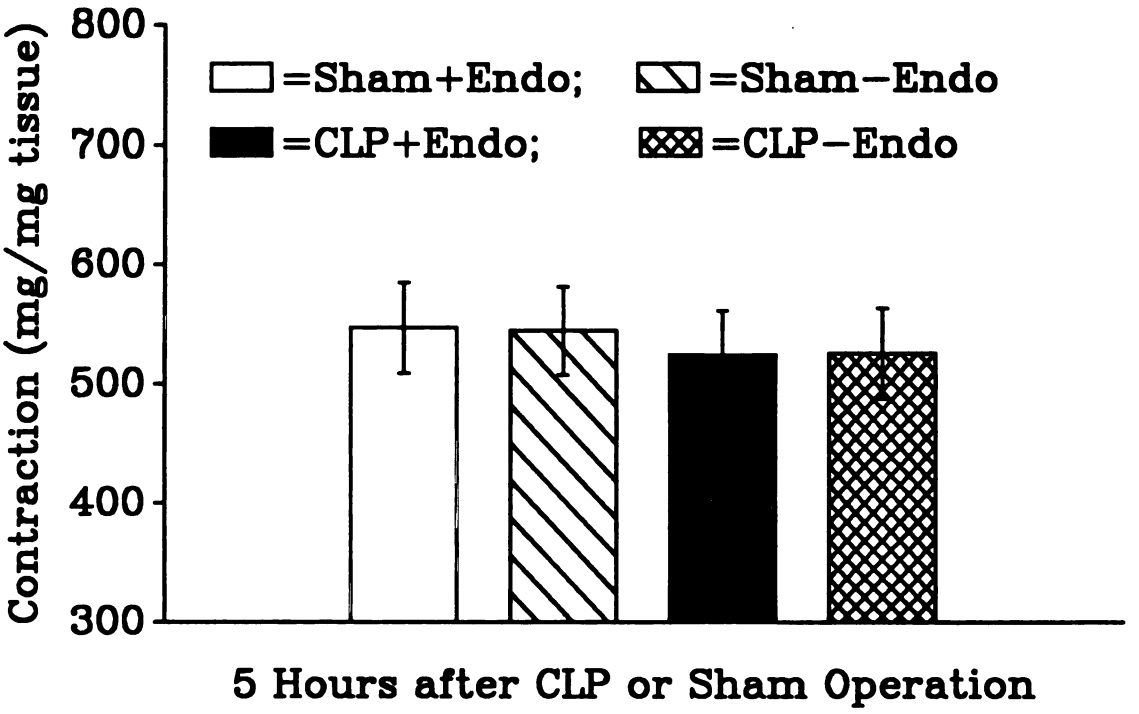
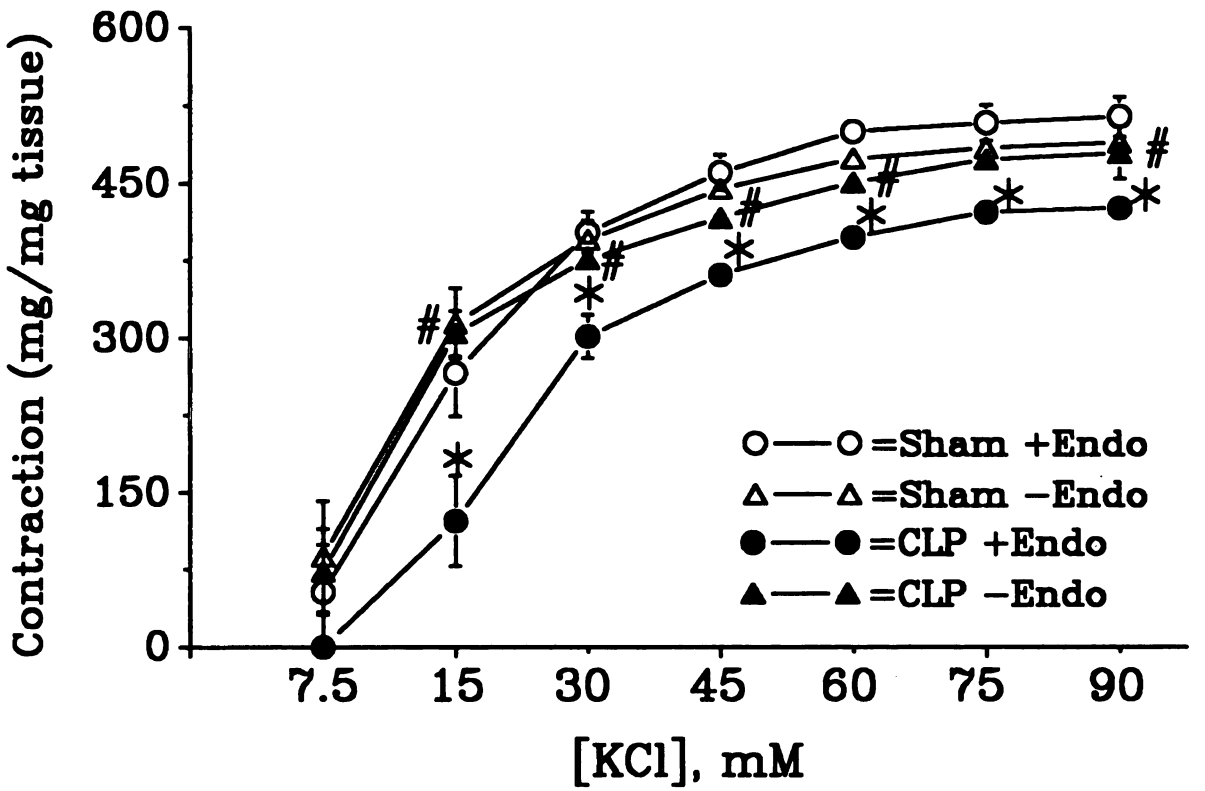

800
700
600
500
400
300
Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
5 Hours after CLP or Sham Operation

Figure 11. Cumulative dose-response curve to various concentrations of potassium chloride (KCl) in aortic rings at 10 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.


600
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Contraction (mg/mg tissue)
7.5
15
30
45
60
75
90
[KCl], mM
O—O =Sham +Endo
Δ—Δ =Sham −Endo
●—● =CLP +Endo
▲—▲ =CLP −Endo
#
*

Figure 12. The peak vascular contraction induced by 90mM KCl at 10 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact rings. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.

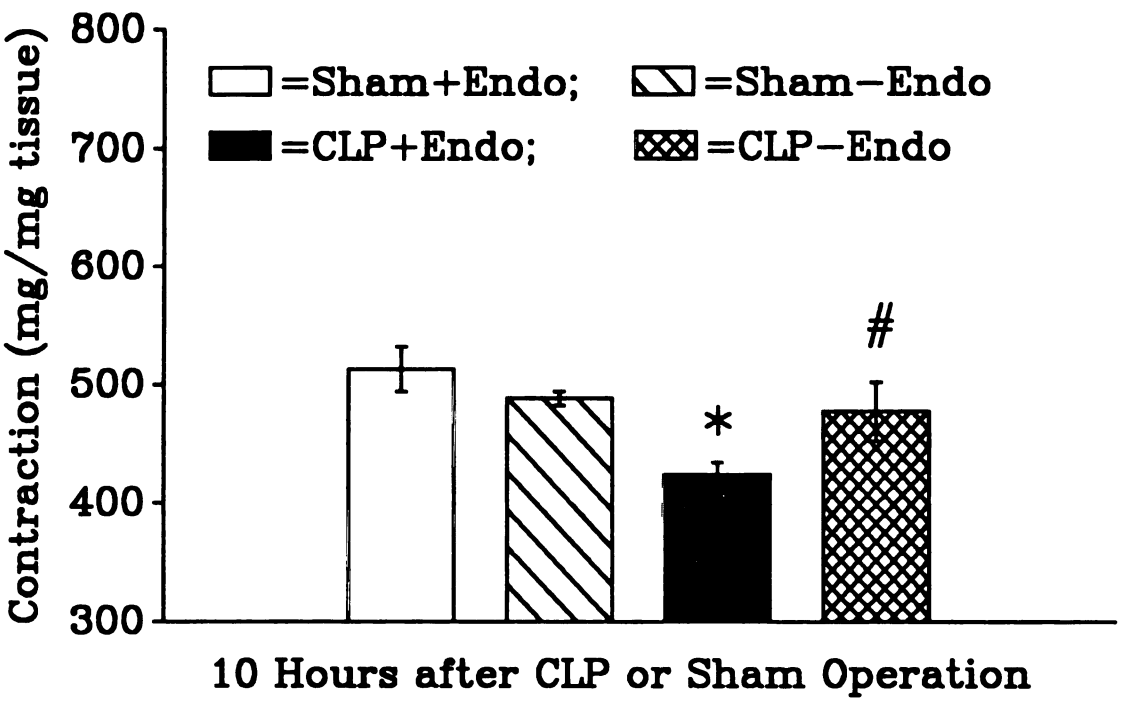

800
700
600
500
400
300
Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
*
#
10 Hours after CLP or Sham Operation

Figure 13. Cumulative dose-response curve to various concentrations of potassium chloride (KCl) in aortic rings at 20 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.

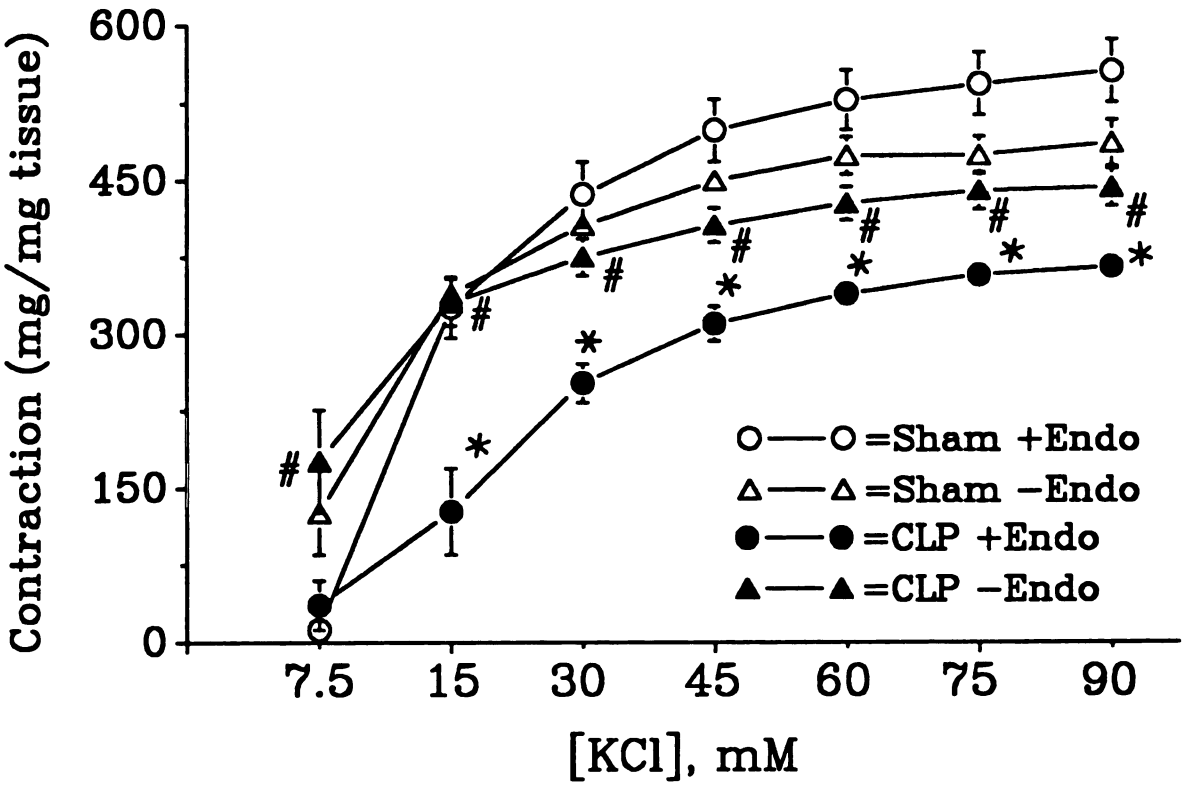
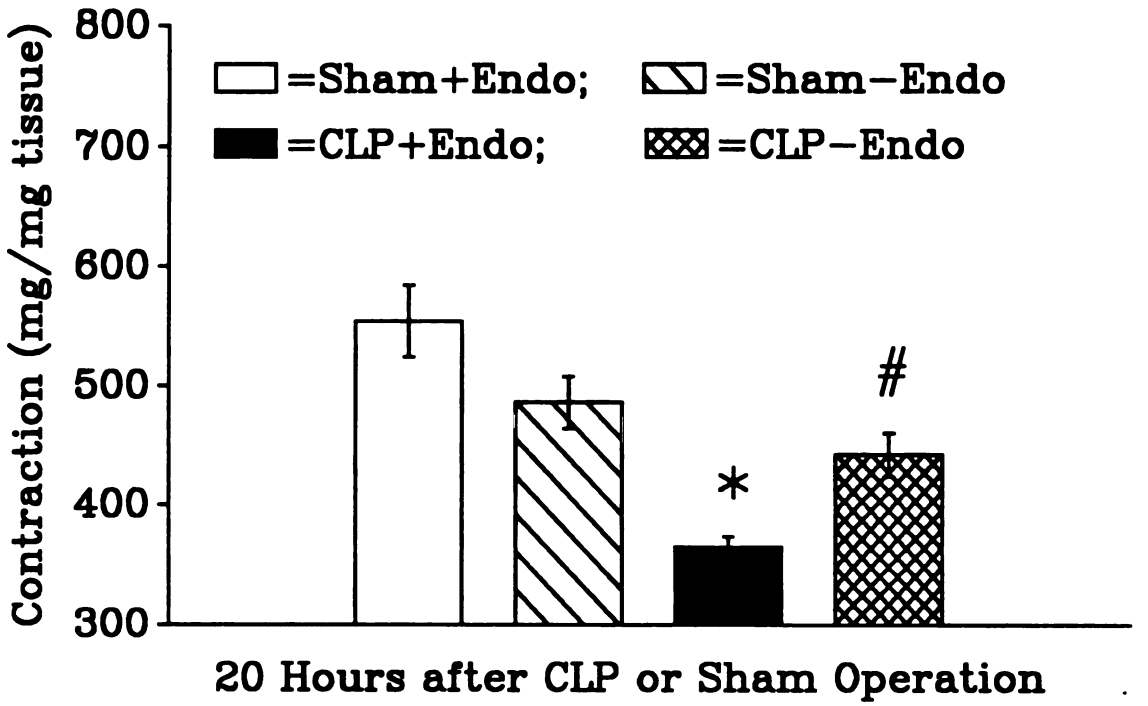

600
450
300
150
0
Contraction (mg/mg tissue)
7.5
15
30
45
60
75
90
[KCl], mM
O—O =Sham +Endo
Δ—Δ =Sham −Endo
●—● =CLP +Endo
▲—▲ =CLP −Endo
#
*

Figure 14. The peak vascular contraction induced by 90mM KCl at 20 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six or seven animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact rings. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.


800
700
600
500
400
300
Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
*
#
20 Hours after CLP or Sham Operation

At 35 hours following CLP, KCl-induced vascular contractility is severely impaired in aortic rings with intact endothelium. In contrast to the restoration of contractility observed at 10 and 20 hours post-CLP, removal of septic endothelium at 35 hours following CLP was significantly improved but not restored to sham levels (Figures 15 & 16).

PART II

ALTERATION OF PEAK VSM CONTRACTILITY TO NOREPINEPHRINE FOLLOWING BLOCKADE OF NITRIC OXIDE SYNTHESIS

At 10 and 20 hours following CLP-induced sepsis, addition of L-NMMA to the organ baths did not significantly alter NE-induced peak contractility in endothelium-denuded aortic rings (Figures 17).

Endothelium denuded vessel rings harvested 35 hours following CLP demonstrated a significant (18%) improvement in peak smooth muscle contractile function following addition of L-NMMA (Figure 17). This augmentation of peak contractile function in these septic vessel rings was fully reversed following addition of excess L-arginine (3mM) to the organ chambers.

Figure 15. Cumulative dose-response curve to various concentrations of potassium chloride (KCl) in aortic rings at 35 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact septic rings. +Endo, endothelium intact rings; -Endo, endothelium-denuded rings.

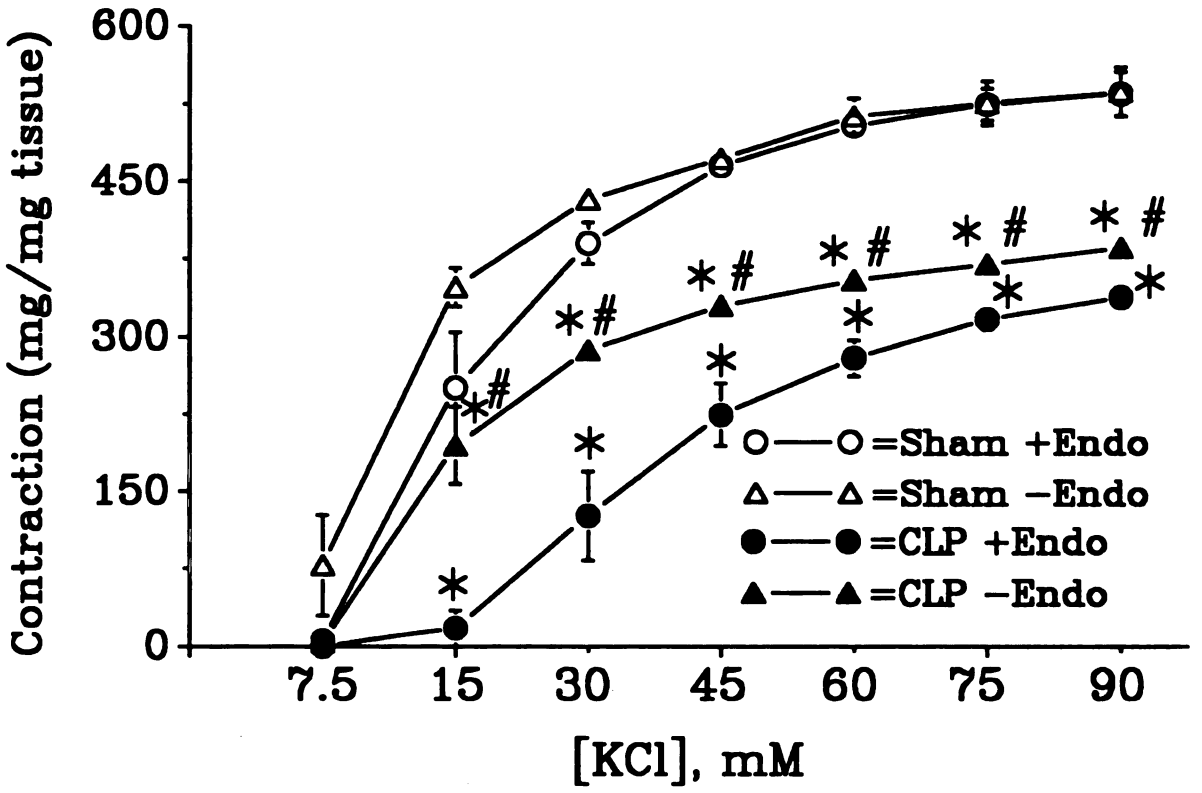

600
450
300
150
0
Contraction (mg/mg tissue)
7.5
15
30
45
60
75
90
[KCl], mM
O—O =Sham +Endo
Δ—Δ =Sham −Endo
●—● =CLP +Endo
▲—▲ =CLP −Endo

Figure 16. The peak vascular contraction induced by 90mM KCl at 35 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were six animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA and Tukey's test. *P < 0.05 versus corresponding sham-operated animals; #P < 0.05 versus corresponding endothelium-intact rings. +Endo, endothelium-intact rings; -Endo, endothelium-denuded rings.

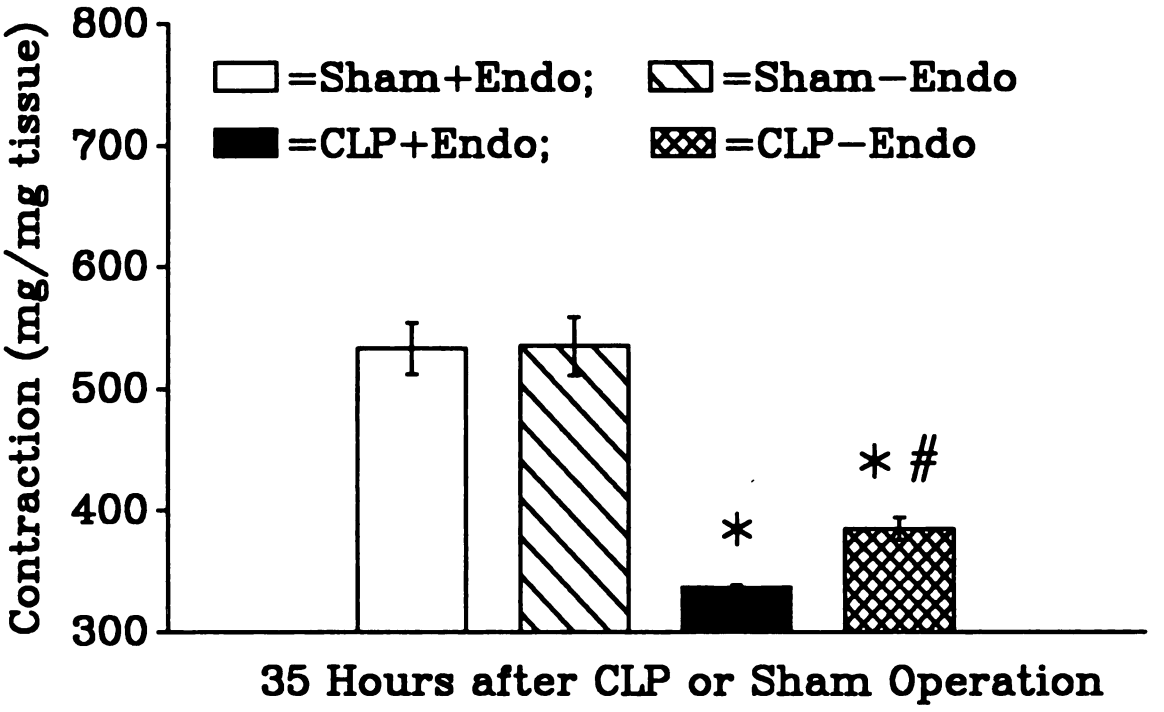
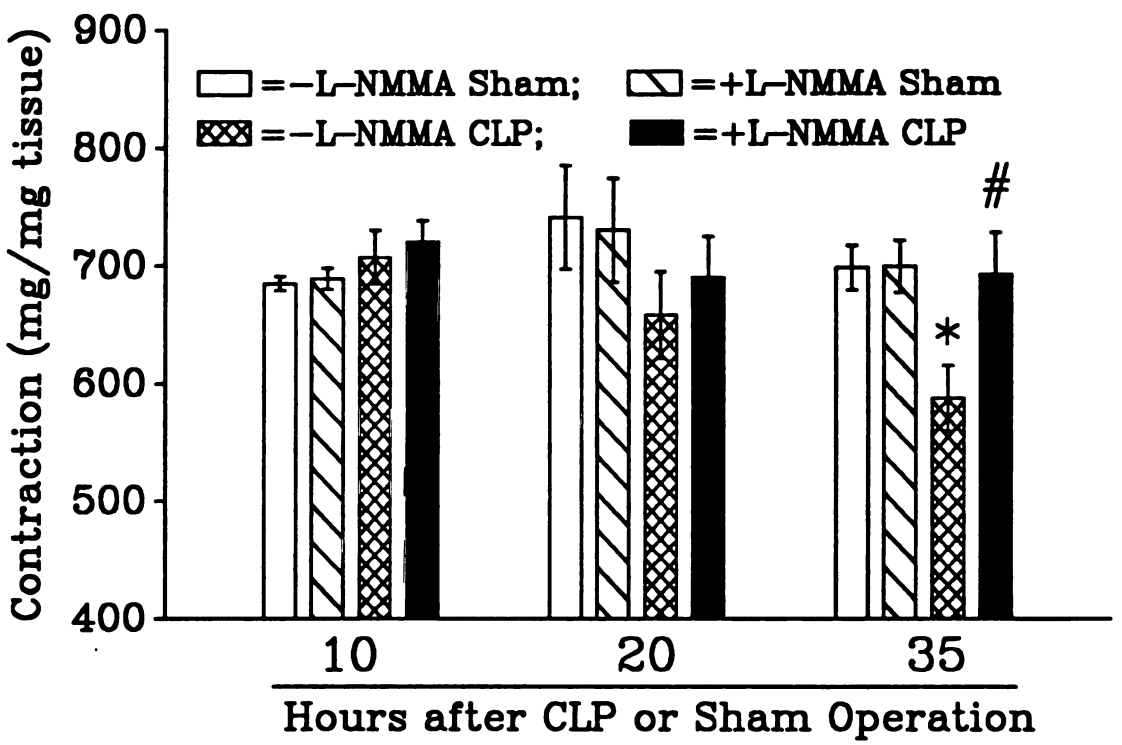

800
700
600
500
400
300
Contraction (mg/mg tissue)
=Sham+Endo;
=Sham−Endo
=CLP+Endo;
=CLP−Endo
*
* #
35 Hours after CLP or Sham Operation

Figure 17. The peak vascular contraction induced by 1 X 10-6M NE at 10, 20 and 35 hours after sham operation (Sham) or cecal ligation and puncture (CLP). There were four to five animals in each group with two aortic rings per rat. Values are presented as mean ± SE and compared by one-way ANOVA. #P < 0.05 versus corresponding sham-operated animals; *P < 0.05 versus corresponding -L-NMMA. +L-NMMA: following addition of N^G-monomethyl-L-arginine. -L-NMMA: without addition of N^G-monomethyl-L-arginine.


900
800
700
600
500
400
Contraction (mg/mg tissue)
=-L-NMMA Sham;
=+L-NMMA Sham
=-L-NMMA CLP;
=+L-NMMA CLP
#
*
10
20
35
Hours after CLP or Sham Operation

DISCUSSION

Rationale for Sepsis Model

The goal of this study was to elucidate the mechanisms of vascular smooth muscle contractile dysfunction from early to late stages of sepsis. Cecal ligation and puncture (CLP) was chosen as the septic model for this study due to its simplicity, and the generation of the 'milieu' of polymicrobial peritonitis.[16] The presence of feculent contamination and necrotic tissue mimics commonly encountered diagnoses such as perforated appendicitis and diverticulitis.[82] Rats subjected to CLP progress through the early and late stages of septic shock in a predictable fashion that is temporally similar to that observed in humans.[16,82] Although *in vitro* incubation with endotoxin has been shown to depress VSM contractility, in this investigation, *in situ* vascular tissue was exposed to the entire gamut of septic mediators present following CLP, for up to 35 hours. The subsequent functional testing of septic vascular tissue, extracted at widely spaced timepoints provides a series of 'snapshots' revealing the state of vascular contractility from early to late stages of sepsis.

Rationale for VSM Contractile Agonists

Norepinephrine was selected as an experimental agonist due to its natural occurrence as a primary alpha-adrenoreceptor-mediated stimulant of VSM contraction. Norepinephrine produces smooth muscle contraction via the hydrolysis of phosphatidylinositol 4,5-biphosphate to produce the second messengers inositol triphosphate and diacylglycerol. Inositol triphosphate mobilizes intracellular calcium stores, while diacylglycerol activates protein kinase C, resulting in myosin light chain phosphorylation and smooth muscle contraction.[83]

To aid in the differentiation between receptor and non-receptor mediated contractile dysfunction, potassium chloride (KCl) was chosen as the second experimental agonist. KCl depolarizes the smooth muscle cell membrane, thus activating voltage-gated L-type channels, which then allow influx of extracellular calcium and subsequent smooth muscle contraction.[83] The use of standardized, yet nonphysiologic concentrations of both NE and KCl was required to elicit maximal (peak) contraction in *ex vivo* vascular tissue.

Discussion of Part I Results

In part one of this study, NE-induced peak vascular contractility decreased significantly at 10 hours after CLP (early sepsis). This depression of NE-induced vascular contraction was completely restored by endothelium removal.

Similarly, KCl-induced peak vascular contraction was depressed significantly at 10 hours post-CLP and was also restored by removal of septic endothelium. However, removal of septic endothelium at 20 hours post-CLP resulted in incomplete restoration of α-adrenoreceptor (NE)-induced peak vascular contraction. Complete restoration of contractility to sham levels in septic endothelium denuded vessel rings utilizing a non-receptor dependent agonist (KCl) implies that some alteration in the alpha-adrenoreceptor-signal transduction chain is at play in the later stages of sepsis (> 20 hours post-CLP). Both NE- and KCl-induced peak vascular contractility remained significantly depressed at 35 hours following CLP, despite removal of septic endothelium.

Discussion of Electron Microscopic Studies

This finding of persistent contractile dysfunction, in the absence of septic endothelial mediators, suggested the possibility that some type of physical damage had been inflicted upon the contractile apparatus of aortic smooth muscle cells exposed to such a prolonged septic insult. This theory, however, was not supported by electron micrographic (EM) analysis of harvested vessel rings.

As depicted in Figures 18 & 19, EM ultrastructural studies of aortic vessel wall cross-sections reveals septic intimal changes such as vacuolization of the endothelial cytoplasm, breakdown of inter-endothelial junctions, and

Figure 18. Electron Micrograph of an aortic ring, presented here in cross section, was obtained from a sham-operated rat. It is representative of normal appearing arterial microanatomy. Note the anatomic relationships between the endothelium (E), basal lamina (B), and vascular smooth muscle (VSM). Also notice the normal appearing junction between adjacent endothelial cells (arrow).

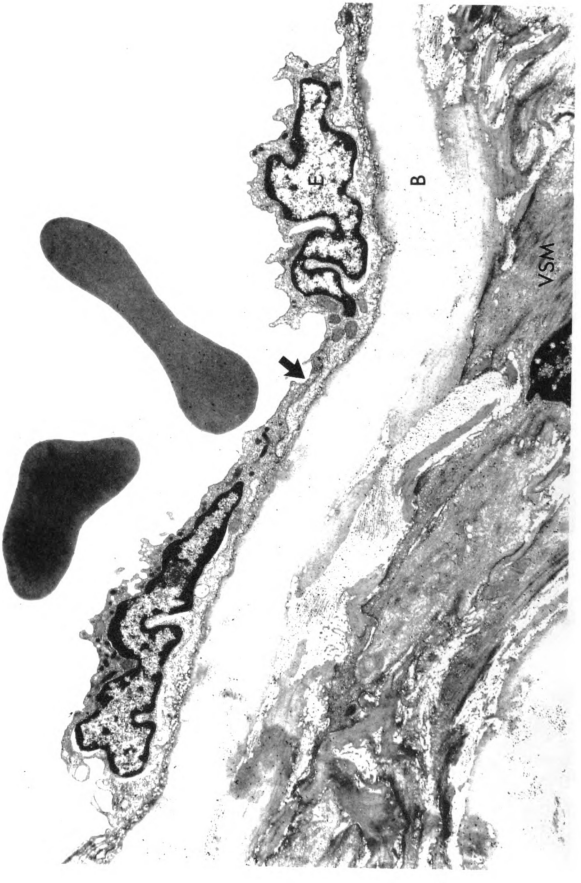

E
B
VSM

Figure 19. Electron Micrograph of an aortic ring, presented here in cross section, was obtained from a septic rat 20 hours post-CLP. There is obvious distortion of normal arterial microanatomy. Note the separation of the endothelium (E), from the basal lamina (B), and vacuolization of endothelial cytoplasm. There is no apparent damage to vascular smooth muscle (VSM), despite exposure to a prolonged septic insult. Notice the breakdown of inter-endothelial junctions (arrows). Also note the presence of leukocyte attachment to the septic endothelium.


E
B
VSM

separation of the endothelium from internal elastic lamina. However, microscopic inspection of vessel wall smooth muscle revealed no obvious loss of structural integrity even at the terminal stages of CLP-induced septic shock. These histological findings are supported by previous microanatomical studies of vessels subjected to both *in vivo*[84,85] and *in vitro*[86,87] septic challenges.

Of further interest, Lee et al., have observed endothelial damage as early as 15 minutes following administration of intravenous endotoxin, as well as, complete restoration of intimal integrity as early as one hour following endotoxemia.[85] The absence of endothelial injury following *in vitro* exposure to endotoxin alone reinforces the concept that multiple blood borne elements (i.e., LPS, inflammatory cells and cytokines) must interact to fully duplicate the physiologic challenges that clinical sepsis presents.[88] The persistence of contractile dysfunction in endothelium-denuded vessel rings, in an *ex vivo* system containing only a physiologic salt solution, implicates the vessel wall, itself, as the source this 'contractile inhibitory factor.'

Discussion of Part II Results

Part II of this study consisted of an investigation into the supposition, that iNOS is the agent responsible for the attenuated contractility of endothelium-denuded vascular tissue observed in the latest stages of sepsis. If iNOS

activation within the vessel wall, during the late stages of sepsis, is responsible for the observed depression of vascular contractility, then blockade of iNOS should result in improvement in maximal (peak) contractile function. The experimental hypothesis that late septic VSM contractile depression is due, in part, to NO produced from iNOS within the vessel wall is supported by the restoration of VSM peak contractile performance following treatment of septic, endothelium-denuded vessel rings with L-NMMA.

As illustrated in Figure 20, L-NMMA is an N^G-substituted L-arginine analogue which has demonstrated potency as a competitive inhibitor of both constitutive and inducible isoforms of NOS [89,90,91]. L-NMMA also inhibits NO release from endothelium[92] and vascular tissues[93,94]. Evidence that a dilatory vascular tone is maintained by a basal release of NO from endothelium is demonstrated by contraction of *ex vivo*[92] and *in vivo*[95-102] vascular tissues by L-NMMA, as well as, the reversibility of L-NMMA induced contraction by addition of L-arginine[93]. Inhibition of acetylcholine-stimulated release of NO from the perfused rabbit aorta, is an effect which is enantiomerically specific and is therefore reversible following administration of L-arginine and not D-arginine.[103] The increase in blood pressure following intravenous infusion of L-NMMA is accompanied by decreased blood flow in the carotid, renal and mesenteric arteries of conscious, chronically instrumented rats.[104] Direct infusion of L-NMMA constricts the brachial arteries[105]

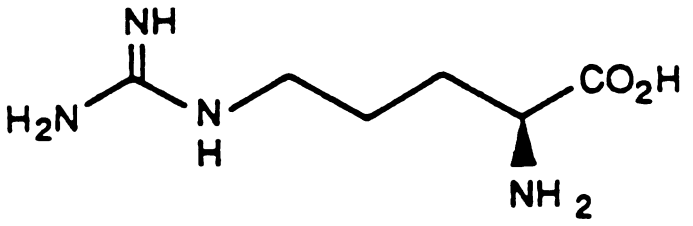
of human volunteers but not the dorsal hand veins,[106] suggesting a greater dependency of human arterial tonicity upon basal NO synthesis. Additionally, both *in vitro*[107] and *in vivo*[106] studies of human venous tissue, exhibit lesser basal NO release and a greater sensitivity to nitrovasodilators when compared to arterial tissue. This differential dependence of mammalian vascular tone upon continuous NO synthesis is supported by the constriction of rodent arterioles, but not venules, following intravenous L-NMMA administration.[108]

Therapeutic Implications

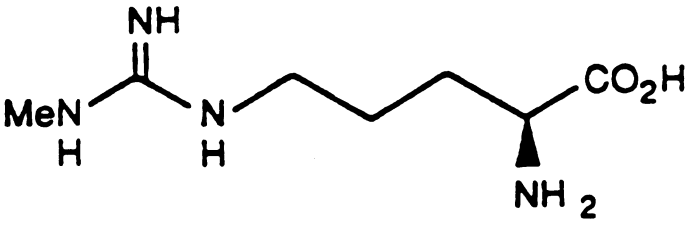
Nitrovasodilators such as nitroglycerin have been administered for the last century in the treatment of various cardiovascular disorders (i.e., angina pectoris).[109] Strong evidence now suggests that NO is the final common effector molecule of all nitrovasodilators which activate soluble guanylate cyclase.[110,111] Of note, inhibition of basal NO synthesis results in increased formation of cGMP and a supersensitive vascular response to exogenously applied nitrovasodilators.[112-117] The long recognized phenomenon of tolerance which develops following chronic nitrovasodilator administration[118] is likely due to desensitization of soluble guanylate cyclase to NO.[119,120] Reduction of arterial vasodilator response to Ach infusion in patients with essential hypertension implies some derangement in NO-mediated vasodilator tone as an etiologic factor in this

Figure 20. Structural formulae of L-arginine and its competitive analogue N^G-monomethyl-L-arginine (L-NMMA).

L-Arg



L-NMMA



disease.[121,122] NO may also contribute to the development of hypertension by inhibition of renin release.[123] Additionally, attenuation of endothelium-dependent relaxation has been demonstrated in both rabbit[124-128] and human[129] atherosclerotic arteries. Also, human atherosclerotic coronary arteries are characterized by reduced basal and stimulated NO release,[130] as well as, a diminished response to Ach administration.[131]

The discovery that glucocorticoids inhibit NOS induction may explain many of the long-observed but poorly understood therapeutic benefits and toxic effects of these compounds. For instance, glucocorticoid administration is beneficial in treatment of endotoxin shock,[132-135] asthma, rheumatoid arthritis and transplant rejection.[136] Conversely, this suppression of NO production may explain why glucocorticoids facilitate the spread of clinical infections.[137]

The demonstration of NO synthesis within the vasculature is equivalent to the discovery of a new and ubiquitous organ which is intimately associated with the homeostasis of all other organ systems. Collectively, the blood vessels and their endothelium comprise the largest organ of the human body with a demonstrated capacity for autocrine, paracrine and possibly endocrine function.[138,139]

Inhibition of NOS has been shown to counteract the profound hypotension associated septic shock in both animals [140] and humans.[22] Alternatively, administration of NO promotors/donors is being investigated as a treatment for

hypertension and atherosclerosis. The vascular smooth muscle relaxing properties of nitric oxide has been utilized in the treatment of impotence,[141] as well as, splanchnic and myocardial reperfusion injury.[142-144] Inhaled nitric oxide gas also shows promise in the treatment of such dire clinical entities as pulmonary hypertension[145] and adult respiratory distress syndrome.[146] Inhaled NO also exhibits bronchodilator effects.[147]

Although the use of NOS inhibitors has exhibited protective and beneficial effects in terms of hemodynamic parameters,[22,25] reports of the effects on NOS inhibitors on end organ function and survival, in various animal models, have been contradictory. Indeed, there is ample evidence that the use of nonselective inhibitors of NOS (i.e., L-NMMA) may result in severe unopposed vasoconstriction with resultant ischemic injury to vital organs.[148-151] Obviously many questions concerning the pharmacology and toxicity of known NOS inhibitors, as well as, the number and properties of yet undiscovered NOS isoforms need to be carefully elucidated in the laboratory setting prior to the conduction of clinical trials.

Ironically, this short-lived, and nonspecific effector molecule has evolved to regulate such diverse functions as control of vascular tone and neurotransmission. Properties such as high reactivity and membrane permeability make NO an ideal antimicrobial and tumoricidal agent. It has been proposed that the capacity of most nucleated cells to induce

NO production may represent a primitive,[152] broad spectrum defense mechanism. Therefore it is possible that activation of iNOS within the vessel wall, in the terminal stages of septic shock, may represent a type of "every cell for itself" phenomenon in which the cell attempts to repel microbial invaders via production of excess NO without regard to physiological consequences such as pathological vasodilation and tissue damage.

SUMMARY AND CONCLUSIONS

SUMMARY

The experimental findings of this investigation can be outlined as follows:

Part I

1. Neither receptor (NE) or non-receptor (KCl) agonist induced vascular contractility was significantly affected 5 hours following CLP; contractile function was not affected by the presence or absence of intact endothelium.
2. At 10 hours post-CLP, there was a marked decrease in NE and KCl-induced peak vascular contractility which was completely restored to sham levels following removal of septic endothelium.
3. At 20 hours post-CLP, depression of NE-induced contractile function was only partially corrected by removal of septic endothelium. Conversely, KCl-induced contraction was completely restored to sham levels following endothelium removal.

4. At 35 hours post-CLP, Both NE and KCl-induced peak contractile function was improved but remained significantly depressed following removal of septic endothelium.
5. Electron microscopic comparison of septic and sham vessel rings revealed no difference in the ultrastructural integrity of vascular smooth muscle.

Part II

6. Treatment with L-NMMA did not significantly affect NE-induced peak contractile performance of endothelium-denuded aortic rings at 10 or 20 hours post-CLP.
7. L-NMMA administration resulted in a significant improvement (18%) in peak vascular contraction of endothelium-denuded aortic rings following 35 hours of CLP-induced sepsis.

Conclusions

Production of endothelium derived septic mediators with resultant depression of *ex vivo* peak vascular contractility occurred as early as 10 hours following CLP. At 20 hours post-CLP, the incomplete restoration of receptor-induced contractility after endothelium removal implies that some alteration in α-adrenoreceptor signal transduction is at play in the later stages of sepsis.

The discovery of continued depression of endothelium denuded vascular tissue to both NE and KCl in the terminal stages of septic shock (35 hours post-CLP) suggests the induction of NOS activity within the vessel wall itself.

The presence of iNOS within the aortic wall was confirmed, albeit indirectly, by a significant augmentation in peak contractility following L-NMMA treatment of endothelium- denuded aortic rings subjected to CLP-induced sepsis for 35 hours. The complete reversal of this L-NMMA contractile augmentation following addition of excess NOS substrate (L-arginine) to the organ chamber provides further evidence that iNOS activation occurs within the vessel wall during the latest stages of sepsis. While complete blockade of all nitric oxide production during sepsis may prove deleterious, it remains to be determined whether the development of agents which selectively inhibit vascular iNOS will be useful in the treatment of septic shock.

APPENDICES

APPENDIX A

APPENDIX A

This work has been presented in part at the following scientific meetings:

Local Presentations

1. "Insights into the mechanism by which vascular smooth muscle function is depressed during late sepsis." Presented March 11, 1993 at the Ninth Annual Research Day Forum of the Michigan State University Department of Surgery, East Lansing, Michigan.
2. "Alterations in vascular smooth muscle function during sepsis." Presented May 6, 1993 at the 42nd Annual Competition for the Frederick A. Coller Traveling Fellowship Awards, Michigan Chapter of the American College of Surgeons, Grand Rapids, Michigan.
3. "Induction of smooth muscle derived nitric oxide produces vascular contractile dysfunction during late stages of sepsis." Presented May 5, 1994 at the 43rd Annual Competition for the Frederick A. Coller Traveling Fellowship Awards, Michigan Chapter of the American College of Surgeons, Dearborn, Michigan.

National Presentations

1. "Insights into the mechanism by which vascular smooth muscle function is depressed during late sepsis." Presented June 16th, 1993 at The Sixteenth Annual Conference on Shock, Santa Fe, New Mexico.

2. "Different mechanisms are responsible for vascular smooth muscle dysfunction during early and late stages of sepsis." Presented November 13, 1993 at The Twenty-Seventh Annual Meeting of The Association For Academic Surgery, Hershey, Pennsylvania.

International Presentation

"Inducible nitric oxide plays a role in producing vascular smooth muscle dysfunction during late sepsis." Presented March 4th, 1993 at The 3rd International Congress on the Immune Consequences of Trauma, Shock and Sepsis Mechanisms and Therapeutic Approaches, Munich, Germany.

APPENDIX B

APPENDIX B

The data for this thesis has been published, in part, within the following scientific journals:

1. Wurster, SH, Wang, P, Ba, ZF, Dean, RE, and Chaudry, IH. Insights into the mechanism by which vascular smooth muscle function is depressed during late sepsis. Circulatory Shock Supplement 1993; 2:39.

2. Wurster, SH, Wang, P, Dean, RE, and Chaudry, IH. Inducible nitric oxide plays a role in producing vascular smooth muscle dysfunction during late sepsis. Intensive Care Medicine (Supplement 1) 1994; 20:S49.

3. Wurster, SH, Wang, P, Dean, RE, and Chaudry, IH. Vascular smooth muscle contractile function is impaired during early and late stages of sepsis. Journal of Surgical Research 1994; 56:556-561.

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