

A Synthetic Lipopolysaccharide-Binding Peptide Based on the Neutrophil-Derived Protein CAP37 Prevents Endotoxin-Induced Responses in Conscious Rats

DANIEL J. BRACKETT,^{1,2,3*} MEGAN R. LERNER,^{1,3} MELISSA A. LACQUEMENT,⁴ RONG HE,⁴
AND H. ANNE PEREIRA⁴

Departments of Surgery,¹ Anesthesiology,² and Pathology,⁴ University of Oklahoma Health Sciences Center, and
Department of Veterans Affairs Medical Center,³ Oklahoma City, Oklahoma 73190

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The lipid A component of lipopolysaccharide (LPS) derived from *Escherichia coli* has been implicated as a significant mediator in the development of circulatory and metabolic dysfunction and lethality associated with sepsis. A synthetic peptide corresponding to amino acid residues 20 through 44 of the neutrophil-derived 37-kDa cationic antimicrobial protein (CAP37 P₂₀₋₄₄) possesses lipid A binding characteristics which may be useful in attenuating in vivo responses induced during circumstances of endotoxemia, including sepsis. The *E. coli* LPS to be used in the in vivo study was shown to be attenuated by CAP37 P₂₀₋₄₄ in a dose-dependent manner in the in vitro reaction with *Limulus* amoebocyte lysate. Intravenous infusion of CAP37 P₂₀₋₄₄ (1.5 or 3.0 mg/kg of body weight) with *E. coli* LPS (250 µg/kg over 30 min) into conscious, unrestrained rats prevented LPS-induced hyperdynamic and hypodynamic circulatory shock, hyperlactacidemia, and leukopenia in a dose-related fashion. CAP37 P₂₀₋₄₄ (0.2, 1.0, and 5.0 mg/kg) administered intravenously to conscious, actinomycin D-sensitized rats following a lethal dose of LPS neutralized LPS toxicity, resulting in dose-dependent 7-day survival rates of 30, 50, and 80%, respectively. CAP37 P₂₀₋₄₄ (5.0 mg/kg) significantly inhibited the endotoxin-induced increase in circulating tumor necrosis factor alpha in sensitized rats. These data demonstrate that CAP37 P₂₀₋₄₄ has the capacity to abolish in vivo biological responses to LPS that are relevant to human sepsis and to significantly neutralize the toxicity of circulating *E. coli* LPS.

Lipopolysaccharide (LPS), or endotoxin, is a major component of the outer membrane of gram-negative bacteria that is released upon the death or rapid growth of the bacteria (9, 14, 37, 59). LPS is a significant mediator of many of the responses manifested during the development of gram-negative septic shock (13, 51, 63, 70, 71). Intravenous infusion of endotoxin into normal human subjects elicits cardiovascular (31, 65, 67), cytokine (10, 30, 33, 34), and coagulation (64) responses that are characteristic of symptoms observed in septic patients. Furthermore, there is substantial evidence that the toxic biological activity of endotoxin is associated with the lipid A portion of this molecule (16, 17, 42, 49). These data suggest that direct neutralization of the lipid A component could significantly modify the course and affect the outcome of gram-negative septic shock.

A human polymorphonuclear leukocyte granule-derived protein with a molecular mass of 37,000 Da has been identified that possesses antimicrobial activity against gram-negative bacteria (57, 58). This cationic antimicrobial protein (CAP37) also has significant LPS and lipid A binding activities (46). The amino acid sequence of CAP37 has been established (48), and its gene has been cloned (36). Evaluation of an array of overlapping synthetic peptides based on the amino acid sequence of the native CAP37 protein demonstrated that a peptide corresponding to residues 20 to 44 (CAP37 P₂₀₋₄₄) mimicked the bactericidal activity of the protein (46). A dose-dependent inhibition of the antibiotic activity of CAP37 P₂₀₋₄₄ was produced by preincubation with either LPS or lipid A. This finding strongly suggested that the bactericidal and lipid A binding

domains of CAP37 are the same and that amino acids 20 through 44 may represent a principal domain which binds to the lipid A component of the endotoxin molecule. It is highly probable that this binding is required for the bactericidal properties of the peptide.

The impetus for this report was to extend our studies, which demonstrated the in vitro LPS binding ability of CAP37 P₂₀₋₄₄, to determine the capacity of CAP37 P₂₀₋₄₄ to neutralize in vivo LPS activity. The efficacy of the peptide was determined by its effect on the physiological responses induced in a hyperdynamic model of endotoxic shock in the conscious, unrestrained rat and on survival in an endotoxin-sensitive lethality model. The hyperdynamic model, which was developed to mimic the human response to sepsis, permits documentation of comprehensive cardiovascular parameters and provides access to arterial and venous blood without the bias created by anesthesia or the stress elicited by restraint. The characteristics of the model are critical since the establishment of interventional efficacy for endotoxin-evoked cardiovascular responses requires documentation that cardiac output and systemic vascular resistance responses have been significantly modified (6, 8). The endotoxin-sensitive lethality model was specifically chosen to permit evaluation of toxicity with low concentrations of endotoxin (47). Data from these studies imply that CAP37 P₂₀₋₄₄ may possess the potential to bind lipid A under in vivo conditions, thereby diminishing or abolishing the capacity of LPS derived from gram-negative *Escherichia coli* to evoke responses in the intact animal.

MATERIALS AND METHODS

Synthesis of peptides based on the CAP37 sequence. Peptides were synthesized with an Applied Biosystems model 430A peptide synthesizer (0.1- or 0.5-mmol scale) by solid-phase synthesis (32) using the phenylacetamidomethyl copolystyrene and copolydivinylbenzene resins and the *tert*-butyloxycarbonyl-

* Corresponding author. Mailing address: Department of Surgery, Box 26901, University of Oklahoma HSC, Oklahoma City, OK 73190. Phone: (405) 271-2108. Fax: (405) 271-1194.

TABLE 1. Sequences of peptides synthesized for use in this study^a

Peptide	Initial amino acid	Sequence	Final amino acid
CAP37 P ₂₀₋₄₄	20	N Q G R H F C G G A L I H A R F V M T A A S C F Q	44
CAT-G P ₂₀₋₄₇	20	I Q S P A G Q S R C G G F L V R E D F V L T A A H C W G	47
ELAS P ₂₀₋₄₄	20	L R G G H F C G A T L I A P N F V M S A A H C V A	44
CAP37 P ₁₈₀₋₂₀₂	180	V C E G L A H G V A S F S L G P C G R G R G P D	202

^a Boldfaced residues are amino acids that are identical for CAP37 P₂₀₋₄₄, CAT-G P₂₀₋₄₇, and/or ELAS P₂₀₋₄₄.

protected amino acids (Bachem) as previously published (46), essentially according to the manufacturer's protocol.

A peptide based on amino acid residues 20 to 44 was synthesized by previously reported methods (46). A control peptide based on amino acid residues 180 to 202 of CAP37 (CAP37 P₁₈₀₋₂₀₂) and lacking bactericidal and LPS binding activity was also synthesized. Two other peptides, corresponding to amino acids 20 to 47 of cathepsin G (CAT-G P₂₀₋₄₇) and amino acids 20 to 44 of human neutrophil elastase (ELAS P₂₀₋₄₄), were synthesized. To preserve maximum sequence identity between the cathepsin G and CAP37 peptides, amino acids 22 to 24 were inserted in the cathepsin G peptide, making the resultant peptide three amino acids longer than the elastase and CAP37 peptides (46). The sequences of these peptides are illustrated in Table 1.

LAL assay. The *in vitro* neutralization of LPS activity by CAP37 P₂₀₋₄₄ was assessed by the *Limulus* amoebocyte lysate (LAL) chromogenic assay (Bio-Whittaker, Walkersville, Md.) essentially according to the manufacturer's protocol. *E. coli* lipopolysaccharide serotype 0127:B8 (Sigma, St. Louis, Mo.) was solubilized at a concentration of 5 mg/ml by sonication in endotoxin-free water containing 0.1% triethylamine for 7 min at 54 to 57°C. LPS was prepared at concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, and 50.0 ng/ml. CAP37 P₂₀₋₄₄ and ELAS P₂₀₋₄₄ were prepared at concentrations of 10, 50, 125, and 250 µg/ml in endotoxin-free water. ELAS P₂₀₋₄₄ and CAP37 P₂₀₋₄₄ have 13 identical residues; due to this strong sequence homology, ELAS P₂₀₋₄₄ was used as a reference peptide for evaluation of the endotoxin binding capacity of CAP37 P₂₀₋₄₄ in these studies. The appropriate combinations of peptide and endotoxin were placed in a 96-well microtiter plate (Corning), mixed, and then incubated at 37°C for 15 min. Fifty microliters of the LAL reagent was added to each well, and the solutions were incubated for 11 min at 37°C. Substrate solution (100 µl) was then added to the appropriate wells, and following incubation at 37°C for 6 min, 50 µl of stop solution was added to each well and the absorbance was determined spectrophotometrically at 405 nm (Thermomax microtiter plate reader; Molecular Devices). Each reaction was performed in duplicate, and reported values, expressed as percent inhibition of endotoxin activity, were calculated from the results of three assays executed on separate days.

Hyperdynamic model. (i) Animal instrumentation. Fifty male Sprague-Dawley rats (SASCO, Omaha, Neb.) with a mean weight (\pm standard deviation) of 300 \pm 25 g were instrumented as described in detail in previous reports (6, 8). Following induction with the rapidly metabolized inhalational anesthesia isoflurane (5%), the animals were intubated, connected to a rodent respirator delivering 2.5% isoflurane, and prepared for placement of catheters. A combination thermocouple-catheter was implanted via the right carotid artery with the tip of the thermocouple at the level of the aortic arch just distal to the aortic valve for measurement of thermomodulation cardiac output curves (CardioMax II-R; Columbus Instruments, Columbus, Ohio). This catheter was used for continuous monitoring of aortic blood pressure and heart rate and for sampling of arterial blood. The tips of two catheters were positioned immediately proximal to the right atrium via the right external jugular and cephalic veins for measurement of central venous pressure, rapid injection of room-temperature saline (100 µl) to generate thermomodulation curves for calculation of cardiac output, and 30-min infusions of 1 ml of either saline, endotoxin (*E. coli* 0127:B8 lipopolysaccharide B; 250 µg/kg of body weight; Sigma), or CAP37 P₂₀₋₄₄ (1.5 or 3.0 mg/kg). Catheters were guided under the skin and exited at the neck just below the base of the skull. Incision areas were anesthetized (lidocaine) prior to surgery and again before suturing. The animals were allowed to recover from anesthesia and placed in monitoring cages which permitted unrestrained movement throughout the study period. At the end of each study, the catheters were checked visually to ensure proper placement.

(ii) Experimental protocol. The animals were randomly divided into five groups with 10 rats in each group. Each group received the simultaneous delivery of one of the following 30-min intravenous infusion strategies: (i) saline and saline, (ii) saline and 3 mg of CAP37 P₂₀₋₄₄ per kg, (iii) endotoxin (250 µg/kg) and saline, (iv) endotoxin and 1.5 mg of CAP37 P₂₀₋₄₄ per kg, and (v) endotoxin and 3.0 mg of CAP37 P₂₀₋₄₄ per kg. Equal infusion volumes (total volume, 1.0 ml) were delivered in all groups at a constant rate by using infusion pumps (Razel, Stamford, Conn.). The infusions were initiated after 60 min of recovery followed by blood sampling and 30 min of baseline hemodynamic measurements to ensure normal, stable physiological parameters. Arterial blood (200 µl) was sampled 30 min before and 60 and 240 min after the infusion was begun for measurement of hematocrits, glucose and lactate concentrations (2300 STAT

PLUS; Yellow Springs Instruments, Yellow Springs, Ohio), and circulating-leukocyte counts. Blood was replaced with equivalent volumes of saline. The cardiac index (cardiac output adjusted for every 100 g of body weight), systemic vascular resistance [(mean arterial blood pressure minus central venous pressure) divided by cardiac output], and cardiac stroke volume (cardiac output divided by heart rate) were calculated for the control period and the 4-h study period (6, 8). Animals were euthanized with pentobarbital at the end of the study.

Lethality model. (i) Mortality protocol. Actinomycin D-sensitized rats were used in the lethality experiments to test the potential of CAP37 P₂₀₋₄₄ to neutralize the toxicity of endotoxin. This model was developed based on a previously described methodology (47) for testing interventional effectiveness for attenuation of endotoxin toxicity (24, 43, 73). Six groups comprising a total of 63 animals were anesthetized with isoflurane, and a cannula was placed in the jugular vein of each rat as described in the previous section. Forty-eight hours later, each animal received an intravenous bolus infusion of the combination of actinomycin D (Calbiochem-Novabiochem Corporation, La Jolla, Calif.) and endotoxin (*E. coli* 0127:B8 lipopolysaccharide B; Sigma) in endotoxin-free saline at concentrations of 800 and 2.5 µg/ml/kg, respectively. The endotoxin challenge was followed by bolus infusion of either saline, CAP37 P₂₀₋₄₄ at a concentration of 0.2, 1.0, or 5.0 mg/kg, or control peptide CAP37 P₁₈₀₋₂₀₂ or CAT-G P₂₀₋₄₇ at a concentration of 5.0 mg/kg. Equal infusion volumes were delivered in all groups. Each of the groups receiving peptide comprised 10 animals, and the saline control group contained 13 animals. An additional three control animals were given actinomycin D without endotoxin. Lethality was recorded hourly for the first 48 h and daily thereafter for 7 days. Animals alive 7 days after the endotoxin challenge were considered to be permanent survivors.

(ii) Measurement of TNF- α . Tumor necrosis factor alpha (TNF- α) was measured in two separate groups of animals (eight per group) subjected to the actinomycin D-endotoxin procedure described above. One group was given CAP37 P₂₀₋₄₄ at a concentration of 5.0 mg/kg following the actinomycin D-endotoxin challenge, and the other group received an equal volume of saline following the challenge. Arterial blood (250 µl) was collected before and 90 min after the administration of the experimental agents and was replaced with an equal volume of saline. The 90-min collection time was chosen since it has been demonstrated that this is the approximate length of time necessary to achieve maximum blood TNF- α concentrations following induction by endotoxin (7, 21, 24). Plasma samples from the collected blood were stored at -70°C until assayed. TNF- α concentrations were determined for all samples on the same day by using, as instructed by the manufacturer, an anti-mouse TNF- α -based enzyme-linked immunosorbent assay which cross-reacted with rat TNF- α (Genzyme, Cambridge, Mass.).

Data analysis. Repeated-measures analysis of variance was used to evaluate the hemodynamic, respiratory, and blood parameters (40, 75). When significant ($P < 0.05$) main or interactive effects were found, simple contrasts between groups or within a group (relative to time zero) were made by using the appropriate unpaired or paired *t* test or Duncan's new multiple range test (post hoc) (19, 23). The chi-square test was applied to test for differences in lethality between experimental groups.

RESULTS

CAP37 P₂₀₋₄₄ inhibits endotoxin-induced LAL activity. The results of the LAL assay established a dose-related effect of CAP37 P₂₀₋₄₄ on the neutralization of endotoxin at concentrations from 0.25 to 1.0 ng/ml (Fig. 1A). The highest concentration of CAP37 P₂₀₋₄₄ (250 µg/ml) essentially abolished the capacity of endotoxin to react with LAL as demonstrated by 96% inhibition compared to the control reaction, even at an endotoxin concentration of 1.0 ng/ml. Forty-five percent inhibition occurred with CAP37 P₂₀₋₄₄ at 125 µg/ml at an endotoxin concentration of 1.0 ng/ml. The specificity of CAP37 P₂₀₋₄₄ in the LAL assay was determined by including as a reference peptide ELAS P₂₀₋₄₄ at 250 µg/ml. ELAS P₂₀₋₄₄ was

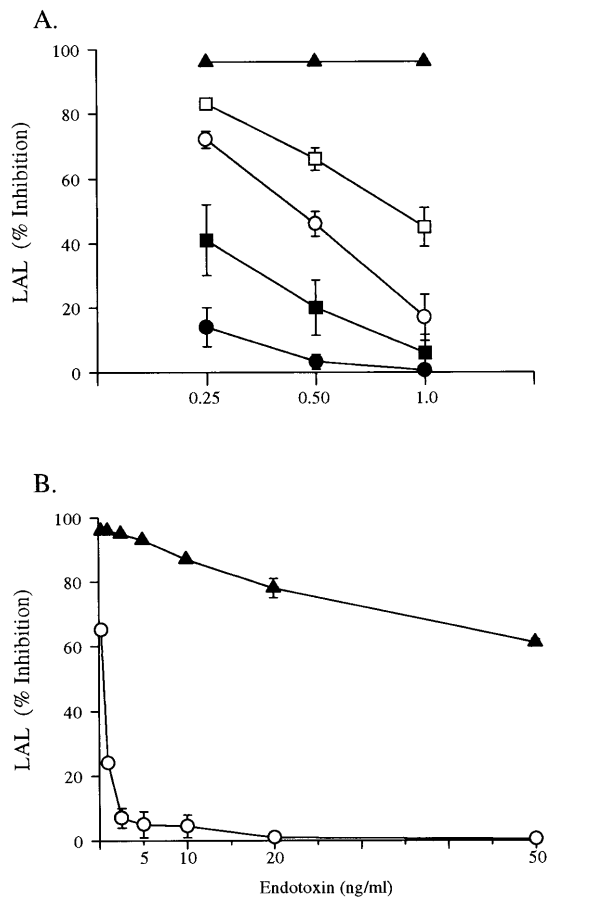


FIG. 1. (A) The dose-response relationship between CAP37 P₂₀₋₄₄ inhibition of endotoxin activity and increasing concentrations of endotoxin (0.25 to 1.0 ng/ml) as determined by the LAL assay. (B) The capacity of CAP37 P₂₀₋₄₄ (250 μg/ml) to inhibit the activity of endotoxin through an extended concentration range (0.25 to 50.0 ng/ml) as determined by the LAL assay. ELAS P₂₀₋₄₄ was used in both studies as a reference peptide since it possesses approximately 50% homology to CAP37 P₂₀₋₄₄. Symbols representing the reactions for each set of peptide concentrations are as follows. ●, CAP37 P₂₀₋₄₄, 10 μg/ml; ■, CAP37 P₂₀₋₄₄, 50 μg/ml; □, CAP37 P₂₀₋₄₄, 125 μg/ml; ▲, CAP37 P₂₀₋₄₄, 250 μg/ml; and ○, ELAS P₂₀₋₄₄, 250 μg/ml. Each symbol and associated bars indicates the mean ± the standard error of the mean of the results of three assays performed on separate days.

active with the lowest concentration of LPS but was clearly less active than CAP37 P₂₀₋₄₄ when the LPS concentration was increased to 1 ng/ml (17 versus 96% inhibition) (Fig. 1A). To further explore the ability of CAP37 P₂₀₋₄₄ to inhibit endotoxin-induced LAL activity, a more extensive dose-response curve was generated (Fig. 1B). CAP37 P₂₀₋₄₄ inhibited *E. coli* LPS activity by over 90% at concentrations up to 5.0 ng/ml and by 61% at 50 ng/ml. The inhibition of LPS activity by ELAS P₂₀₋₄₄ at these concentrations was negligible.

CAP37 P₂₀₋₄₄ prevents endotoxin-induced in vivo responses.

(i) Cardiovascular responses. Infusion of endotoxin evoked an immediate increase in the cardiac index, which reached an apex 30 min after the infusion was initiated and was significantly higher than that of the control group infused with saline (Fig. 2A). The infusion of saline did not result in changes in the cardiac index or in any of the other measured parameters throughout the experimental protocol. Ninety minutes after the beginning of the endotoxin infusion, the cardiac index had decreased to a value significantly lower than that of the control group and remained significantly lower for the remainder of

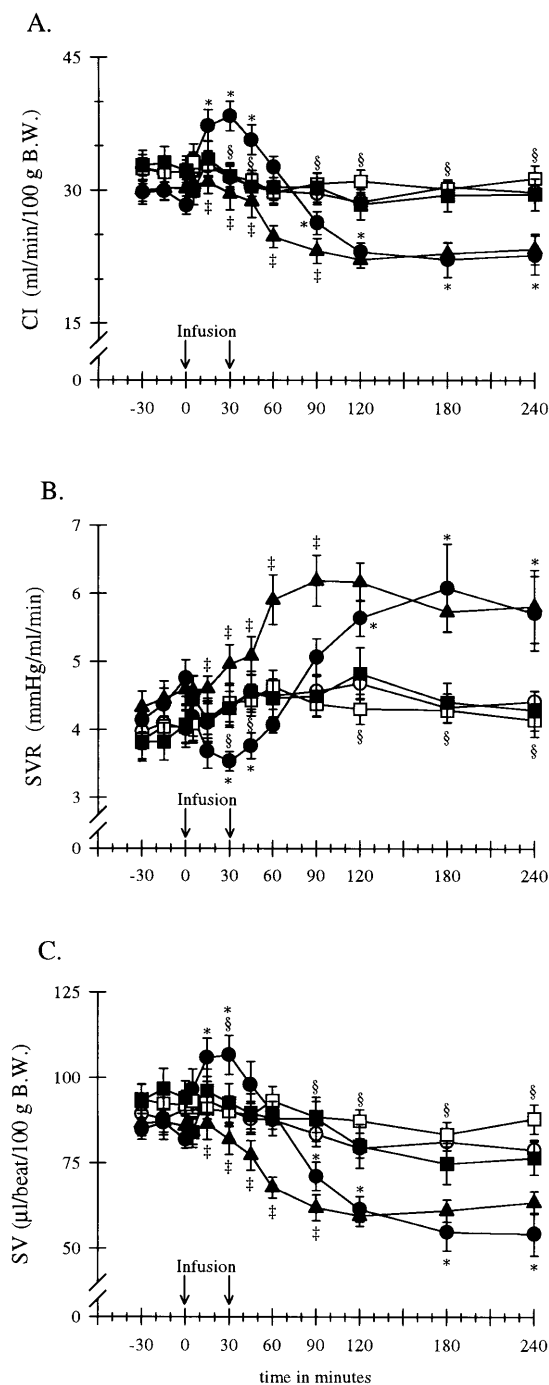


FIG. 2. The dose effect of CAP37 P₂₀₋₄₄ on cardiac index (CI) (A), systemic vascular resistance (SVR) (B), and cardiac stroke volume (SV) (C) responses induced by a 30-min infusion of endotoxin (ETOX) at 250 μg/kg. The arrows on the abscissa designate the ETOX infusion. Symbols representing the responses for each group are as follows: ○, saline plus saline; □, saline plus CAP37 P₂₀₋₄₄ at 3.0 mg/kg; ●, ETOX plus saline; ▲, ETOX plus CAP37 P₂₀₋₄₄ at 1.5 mg/kg; and ■, ETOX plus CAP37 P₂₀₋₄₄ at 3.0 mg/kg. Statistically significant differences ($P \leq 0.05$) are denoted as follows: *, saline group compared to the ETOX plus saline group; †, ETOX plus saline group compared to the ETOX plus CAP37 P₂₀₋₄₄ (1.5 mg/kg) group; and §, ETOX plus CAP37 P₂₀₋₄₄ (3.0 mg/kg) group compared to the ETOX plus saline group. B.W., body weight.

the 4-h monitoring period. Systemic vascular-resistance changes in response to the endotoxin infusion occurred concurrently with changes in the cardiac index but were in the opposite direction (Fig. 2B). The systemic vascular resistance decreased significantly in response to endotoxin, reaching the nadir of its decrease at 30 min, but returned toward the baseline over the next 45 min, and then values significantly increased over the control group were maintained for the remaining 2 h of the 4-h monitoring period. The responses of cardiac index and systemic vascular resistance to the slow infusion of endotoxin during the early episode of endotoxemia are indicative of hyperdynamic reactions, with a subsequent transition to a sustained cardiovascular condition characteristic of hypodynamic shock.

Cardiac stroke volume measurements mimicked the responses of the cardiac index to endotoxin (Fig. 2C). When CAP37 P₂₀₋₄₄ at the higher concentration (3 mg/kg) was infused simultaneously with endotoxin, there was complete elimination of the endotoxin activity responsible for the induction of the hyperdynamic and hypodynamic changes in cardiac index and systemic vascular resistance (Fig. 2A and B). The effect of endotoxin on cardiac stroke volume was eliminated as well (Fig. 2C). The infusion of 3 mg of CAP37 P₂₀₋₄₄ per kg alone did not evoke responses in these three parameters at any time point. Infusion of endotoxin and CAP37 P₂₀₋₄₄ at 1.5 mg/kg, 50% less than that used for the effective intervention described above, eliminated the hyperdynamic responses to endotoxin but did not affect the occurrence or the magnitude of the hypodynamic responses.

Mean arterial pressure (Fig. 3A) and heart rate (Fig. 3B) measurements were essentially unchanged in response to the array of infusions and were statistically the same for all groups across the entire experimental protocol. Respiration rates were also unaffected by endotoxin and were the same for all five groups (data not shown).

(ii) Humoral responses. Endotoxin infusion elicited an increase in blood lactate concentrations at 60 min that was significantly higher than those of both control groups (Fig. 4A). This increase suggests an early shift from aerobic to anaerobic metabolism during the hyperdynamic response induced by endotoxin. Blood lactate concentrations of both control groups were stable throughout the entire experimental period. Infusion of either the high or low concentration of CAP37 P₂₀₋₄₄ completely prevented the endotoxin-induced increase in blood lactate concentrations, and the values were the same as the values for the control groups. The endotoxin-induced metabolic disturbance appears to be associated with the hyperdynamic phase in this model since the lactate concentration had returned to normal at 240 min under hypodynamic conditions and since hyperlactacidemia was not present at any time when the hyperdynamic response was prevented by either concentration of CAP37 P₂₀₋₄₄.

Infusion of endotoxin caused a slight (10%) but statistically significant increase in whole-blood glucose concentrations compared to those of the control group 60 min after the beginning of the infusion and returned to control values at 4 h (Fig. 4B). The hyperglycemic response to endotoxin was blocked by either concentration of CAP37 P₂₀₋₄₄, which indicates an association with the endotoxin dynamics that were involved with both the disturbance in lactate metabolism and the cardiovascular hyperdynamic response. There was not a significant change or difference in hematocrit values between groups throughout the 4-h study period (data not shown), indicating that vascular integrity was maintained and that this concentration of endotoxin did not cause loss of intravascular fluid.

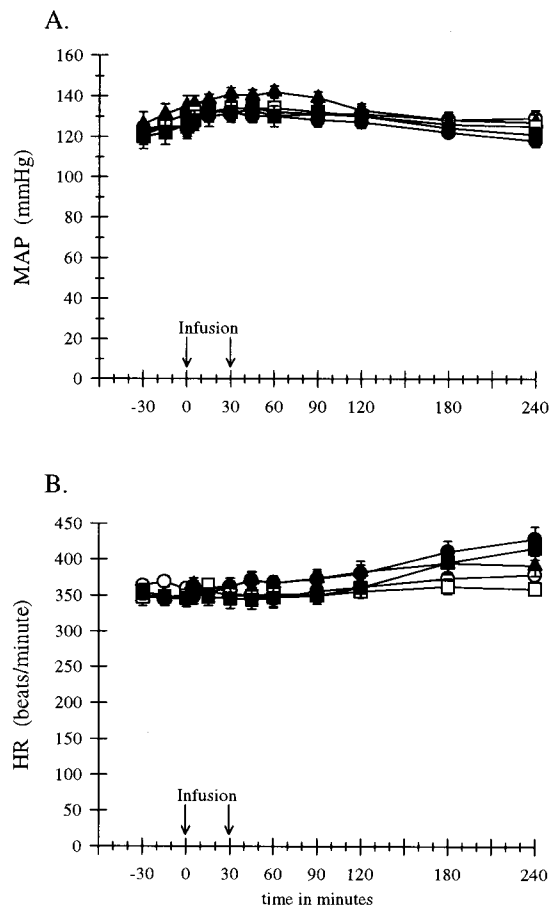


FIG. 3. The dose effect of CAP37 P₂₀₋₄₄ on mean arterial pressure (MAP) (A) and heart rate (HR) (B) responses induced by a 30-min infusion of endotoxin (250 μ g/kg). Symbols representing the responses for each group are as follows: \circ , saline plus saline; \square , saline plus CAP37 P₂₀₋₄₄ at 3.0 mg/kg; \bullet , ETOX plus saline; \blacktriangle , ETOX plus CAP37 P₂₀₋₄₄ at 1.5 mg/kg; and \blacksquare , ETOX plus CAP37 P₂₀₋₄₄ at 3.0 mg/kg. The indicators of statistical differences are described in the legend to Fig. 2.

(iii) Cellular responses. Total circulating-leukocyte counts were significantly decreased 60 min after the initiation of endotoxin infusion, and the decrease persisted for the duration of the 4-h monitoring period (Fig. 4C). The higher concentration of CAP37 P₂₀₋₄₄ significantly inhibited the decrease of leukocytes elicited by endotoxin throughout the study; the circulating-leukocyte counts for this group were significantly higher than those of the endotoxin group and the same as those of the control groups. In contrast to the inhibitory effects on other endotoxin-evoked responses, the lower CAP37 P₂₀₋₄₄ concentration had no effect on the capacity of endotoxin to reduce the number of circulating leukocytes and the counts were significantly lower than those of both control groups.

CAP37 P₂₀₋₄₄ neutralizes endotoxin-induced toxicity. Intravenous administration of endotoxin at a dose of 2.5 μ g/kg to conscious, actinomycin D-sensitized rats resulted in 100% lethality within 24 h. The mean survival time was 11.3 h for the control group treated with only the vehicle used for delivery of peptides in the other five groups (Table 2). When CAP37 P₂₀₋₄₄ at a concentration of 5 mg/kg was intravenously infused following administration of endotoxin, toxicity was virtually eliminated. All 10 of these animals were alive at 48 h, and 8 of the 10 survived for 7 days and were considered to be perma-

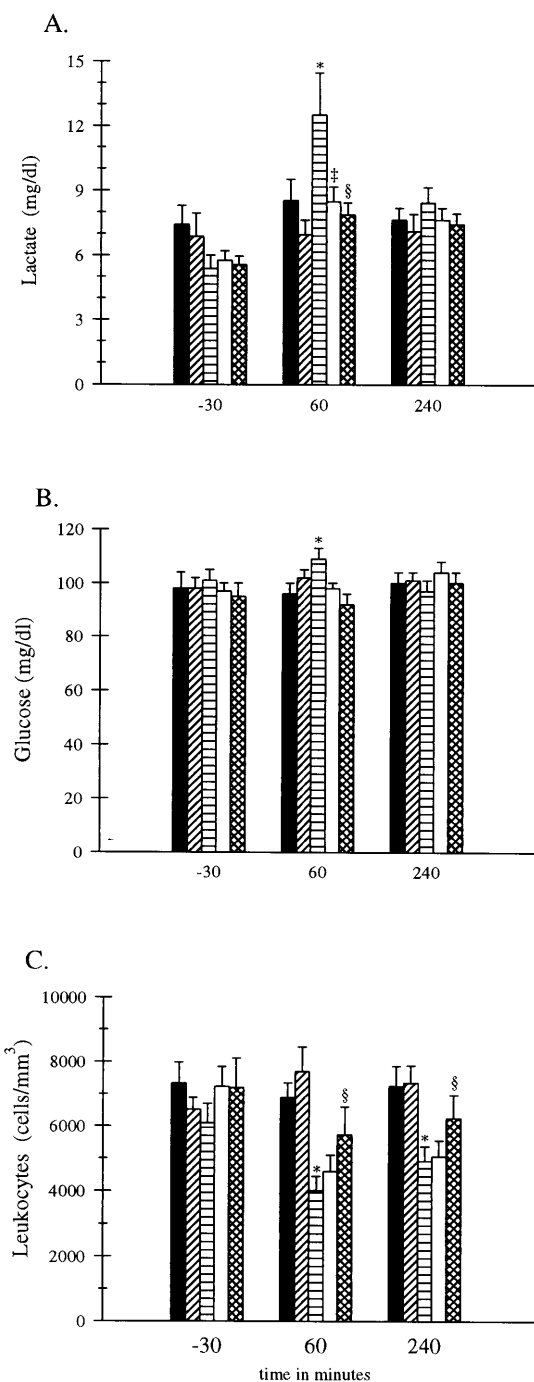


FIG. 4. The dose effect of CAP37 P₂₀₋₄₄ on lactate (A), glucose (B), and circulating leukocyte (C) concentrations 30 min before and 60 and 240 min after the initiation of a 30-min infusion of endotoxin (250 μ g/kg). The groups associated with the different columns are as follows: saline plus saline, solid; saline plus CAP37 P₂₀₋₄₄ at 3.0 mg/kg, diagonal stripes; ETOX plus saline, horizontal stripes; ETOX plus CAP37 P₂₀₋₄₄ at 1.5 mg/kg, open; and ETOX plus CAP37 P₂₀₋₄₄ at 3.0 mg/kg, cross-hatched. Each column and the associated bar represent the mean \pm the standard error of the mean for 10 animals. Indicators of statistical differences are described in the legend to Fig. 2.

ment survivors. Further experiments demonstrated a dose-dependent relationship between CAP37 P₂₀₋₄₄-induced protection and endotoxin toxicity. When CAP37 P₂₀₋₄₄ was given at 1.0 mg/kg after endotoxin administration, 9 of 10 rats survived

TABLE 2. Effect of CAP37 P₂₀₋₄₄ on 7-day survival of actinomycin D-sensitized rats given a lethal dose of endotoxin

Group	Percent survival at time:				
	24 h	48 h	3 days	5 days	7 days
Vehicle	0	0	0	0	0
CAP37 P ₁₈₀₋₂₀₂ ^a	20	10	10	10	10
CAT-G P ₂₀₋₄₇ ^a	20	10	10	10	10
CAP37 P ₂₀₋₄₄					
0.2 mg/kg	30	10	10	10	10
1.0 mg/kg	90	60	60	50	50
5.0 mg/kg	100	100	80	80	80

^a Control peptide; delivered at 5.0 mg/kg.

for 24 h, six survived for 3 days, and 5 were 7-day survivors. The infusion of 0.2 mg/kg protected 3 of 10 rats at 24 h compared to none in the vehicle group; only one survived for 48 h and was still alive at 7 days. The lethality rates were identical in the two control groups, treated with either CAP37 P₁₈₀₋₂₀₂ or CAT-G P₂₀₋₄₇ at 5.0 mg/kg, and had essentially the same effect as the 0.2-mg/kg concentration of CAP37 P₂₀₋₄₄. Two of 10 animals in each of these groups were alive 24 h after the endotoxin challenge; only one in each group survived 48 h, and these were 7-day survivors. Administration of actinomycin D alone, without endotoxin, had no observable effect over the 7-day monitoring period.

CAP37 P₂₀₋₄₄ attenuates endotoxin-induced increases in circulating TNF- α . Baseline plasma concentrations of circulating TNF- α were essentially the same in the group given CAP37 P₂₀₋₄₄ after the actinomycin D-endotoxin challenge described above and in the group given saline after actinomycin D-endotoxin (Fig. 5). The CAP37 P₂₀₋₄₄ concentration of 5.0 mg/kg was selected due to its effectiveness in attenuating lethality (Table 2). There was a greater than 500% increase in the TNF- α concentrations 90 min after the toxic infusion in the group of animals treated with saline. Even though the TNF- α concentrations were increased at 90 min in the group treated with CAP37 P₂₀₋₄₄, these concentrations were approximately 50% of the 90-min values in the saline-treated group, demon-

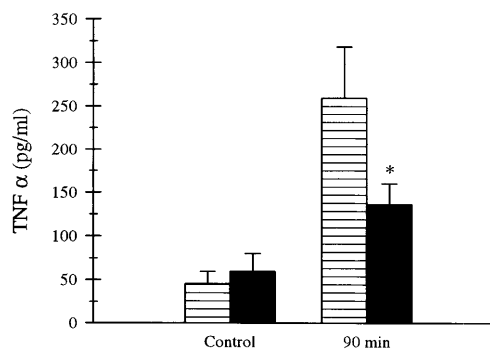


FIG. 5. The capacity of CAP37 P₂₀₋₄₄ to inhibit the increase in circulating TNF- α elicited by endotoxin in conscious rats. The group given endotoxin plus saline is represented by the column with horizontal stripes, and the group given endotoxin plus CAP37 P₂₀₋₄₄ (3.0 mg/kg) is represented by the solid column. Each column and the associated bar represent the mean \pm the standard error of the mean for eight animals. The asterisk denotes a statistically significant difference ($P \leq 0.05$) between groups.

strating significant but not complete inhibition of TNF- α induction by endotoxin.

DISCUSSION

The data derived from these experiments demonstrate that a synthetic peptide consisting of amino acid residues 20 through 44 of the neutrophil-derived protein CAP37, i.e., CAP37 P₂₀₋₄₄, possesses the capacity to neutralize endotoxin activity in both *in vitro* and *in vivo* environments. Measurement of endotoxin activity, as detected by its interaction with LAL, was completely blocked by peptide CAP37 P₂₀₋₄₄. Importantly, the transient hyperdynamic response and the subsequent hypodynamic shock state induced by endotoxin in conscious, unrestrained rats were both prevented by the interaction of CAP37 P₂₀₋₄₄ with endotoxin. Moreover, the toxic potential of circulating endotoxin was essentially neutralized by CAP37 P₂₀₋₄₄. These actions of CAP37 P₂₀₋₄₄ in all three experimental situations were accomplished in a dose-dependent manner. Our data support the concept that CAP37 P₂₀₋₄₄ is a candidate for further evaluation as a therapeutic agent in the treatment of certain gram-negative infections and possibly sepsis.

Lipid A, the component of endotoxin essential for the basic reaction in the LAL assay (16), is recognized as the biologically active component of endotoxin (16, 52, 53). The capacity of CAP37 P₂₀₋₄₄ to inhibit the *in vitro* biological reaction of endotoxin in the LAL assay suggested that CAP37 P₂₀₋₄₄ possessed the characteristics necessary to significantly affect integrative biological responses to endotoxin under *in vivo* conditions. However, inhibition of *in vitro* LPS reactions does not necessarily establish the potential of an LPS-binding reagent to neutralize *in vivo* biological activity (18, 44); furthermore, there is not a compulsory relationship between binding to lipid A and neutralization of the *in vitro* or the *in vivo* activity of the molecule (44, 74). Differences in the neutralization characteristics of the various anti-LPS reagents may be due to affinity specificity for discrete and separate lipid A epitopes or substructures (28, 66) which provide distinct binding sites associated with specific lipid A-evoked responses (15, 45). The data from our study demonstrate that the LPS-binding characteristics of CAP37 P₂₀₋₄₄ meet the criteria required to provide effective neutralization of the *in vivo* responses evoked by endotoxin.

In the conscious, freely moving rat model used in this study, the properties of endotoxin necessary to evoke hyperdynamic and hypodynamic responses, as documented by measurement of cardiac output, stroke volume, and systemic vascular resistance, were completely neutralized when animals were infused with CAP37 P₂₀₋₄₄. This particular model of endotoxemia was developed to manifest a hyperdynamic response (increased cardiac output and decreased systemic vascular resistance) similar to the initial stage of shock found in human sepsis and has been the model chosen for other studies of sepsis hemodynamics (1, 22). However, we believe that this is the first study to exploit the characteristics of this nonlethal sepsis model in the conscious animal, eliminating depression or alteration of cardiovascular and metabolic function and compensatory responses associated with the use of anesthesia (5, 55, 56). The hyperdynamic response in this particular conscious-rat model of sepsis is transient, occurring during the first hour, and is immediately followed by a hypodynamic response characterized by decreased cardiac output and stroke volume and increased systemic vascular resistance. These characteristics permit the evaluation of both types of endotoxin-induced cardiovascular dysfunction in one model.

Both the hyperdynamic and the hypodynamic responses in-

duced by endotoxin were prevented by the 3.0-mg/kg concentration of CAP37 P₂₀₋₄₄, while a completely different inhibitory effect was obtained when the concentration of the CAP37 P₂₀₋₄₄ infusion was reduced by one-half. However, this was not a typical dose-response effect in which the responses would be expected to have similar directional and temporal patterns with a significant difference in the magnitude of the response. In this study, the lower concentration of CAP37 P₂₀₋₄₄ completely abolished the early hyperdynamic episode found with the higher concentration, but unlike the higher concentration, the lower CAP37 P₂₀₋₄₄ concentration had no effect on the occurrence or magnitude of the hypodynamic response. The elimination of the hyperdynamic response revealed that the hypodynamic response may be dependent on mechanisms that are separate and independent of the dynamics involved in the hyperdynamic phase of this model of endotoxemia. The initiation of this response occurred approximately 45 min after the beginning of the endotoxin infusion and was near its maximum amplitude within 15 min; both events occurred 60 min earlier than the hypodynamic response evoked by endotoxin alone. However, the beginning of the endotoxin-induced cardiovascular response associated with the hypodynamic episode in the group that received the lower concentration of CAP37 P₂₀₋₄₄ coincided with the reversal of the hyperdynamic phase in the untreated endotoxin group. It is possible that the factors responsible for the hypodynamic response were induced at the same time in both groups and are involved in the reversal of the hyperdynamic phase in the group receiving only endotoxin. This dose-related phenomenon appears to be due to some variable other than simply the amount of available, active, circulating endotoxin since the concentration of endotoxin required for induction of the hypodynamic response has been shown to be greater than the amount required to evoke the hyperdynamic response (22), not less as occurred in this study.

Mean arterial blood pressure, heart rate, and central venous pressure were essentially unchanged for all groups throughout the study. The capacity to maintain blood pressure and heart rate in all of the groups in the presence of 30 to 40% changes in cardiac output and systemic vascular resistance provides evidence that the integrity of the cardiovascular compensatory system was intact. The absence of changes in arterial blood pressure and heart rate in the groups in the presence of profound changes in the principal parameters that determine the delivery of blood emphasizes the importance of comprehensive cardiovascular measurements in studies designed to generate data for evaluating interventional efficacy. An effect or the lack of an effect on blood pressure alone is insufficient to make definitive conclusions regarding the cardiovascular system since normal blood pressures can occur in the presence of either high or low cardiac output depending on the status of the vascular system. Other studies using a similar model (1, 22) have reported changes in these parameters which may have been due to the influence of anesthesia on the cardiovascular system and the effectiveness of compensatory responses during cardiovascular challenge (5, 55, 56).

Endotoxin evoked a significant elevation in blood lactate concentrations 60 min after the beginning of the infusion, suggesting inadequate tissue perfusion and a shift from aerobic to anaerobic metabolism that was temporally associated with the hyperdynamic response. Support for this association is derived from results demonstrating the concurrent prevention of the hyperlactacidemic event and the hyperdynamic response by both the higher and lower concentrations of CAP37 P₂₀₋₄₄. The prevention of hyperlactacidemia and the hyperdynamic response were the only parameters measured in this study that were affected by both concentrations of CAP37 P₂₀₋₄₄. Endo-

toxin-induced hyperlactacidemia did not appear to be related to the hypodynamic response since lactate concentrations had returned to normal values during the latter portion of the study, when the response was fully developed. Additionally, hyperlactacidemia did not occur at all in the group receiving the lower concentration of CAP37 P₂₀₋₄₄, even though the hypodynamic response was present. Therefore, in this model of endotoxemia, there appears to be a relationship between hyperlactacidemia and the factors involved in the hyperdynamic response and no correlation with the hypodynamic episode.

Comparison of circulating-leukocyte concentrations revealed an additional dose-dependent effect of CAP37 P₂₀₋₄₄. Endotoxin infusion induced a significant decrease in the concentration of leukocytes that was sustained throughout the study, indicating an increase in the marginating leukocyte pool. The effect of endotoxin on circulating-leukocyte concentrations was not altered by CAP37 P₂₀₋₄₄ at the lower concentration but was significantly attenuated with the higher CAP37 P₂₀₋₄₄ concentration at both the early and late measurement points. The importance of this inhibitory action on the apparent endotoxin-induced leukocyte-endothelial cell interaction should not be underestimated since this reduces the potential for leukocyte migration into the interstitial tissue, where cellular injury may occur (3, 20, 38, 68). A role for leukocytes in the pathology associated with sepsis and endotoxemia in experimental animals (25, 26, 41, 61) and septic patients (29, 50, 72) has been indicated.

The effect of CAP37 P₂₀₋₄₄ on the toxicity of endotoxin was remarkable. A highly lethal dose of endotoxin was used in this study, resulting in the death of all animals within 24 h, with a mean survival time of 11.3 h. Endotoxin lethality was completely blocked for the first 48 h after endotoxin challenge and was prevented in 80% of the animals at the end of 7 days by the 5.0-mg/kg concentration of CAP37 P₂₀₋₄₄. Importantly, these data demonstrate that CAP37 P₂₀₋₄₄ is capable of effectively binding the toxic portion of circulating endotoxin in the presence of whole blood and its constituents. The antagonistic effect of CAP37 P₂₀₋₄₄ on lethality was dose related as demonstrated by the reduction in 7-day survival to 50 and 10% when the CAP37 P₂₀₋₄₄ concentration was reduced to 1.0 and 0.2 mg/kg, respectively. It is important to note that endotoxin-induced death continued beyond 3 days in the CAP37 P₂₀₋₄₄-treated animals. These results suggest that monitoring animals for 24 h to 3 days, as is often reported in the literature, is not adequate to demonstrate permanent survival and to support interventional efficacy. However, this is not to say that data from 24-h to 3-day monitoring periods are not important. Significant attenuation of lethality within a 3-day monitoring period certainly indicates a beneficial interventional influence on parameters that determine long-term survival and may provide a window of time for other therapeutic support.

In addition to the profound inhibitory influence of the higher concentration of CAP37 P₂₀₋₄₄ on the toxicity of endotoxin, circulating TNF- α concentrations were also markedly reduced, albeit not completely abolished. There are abundant data implicating TNF- α as an essential mediator of sepsis (2, 4, 11, 60, 69); however, the results reported here demonstrate that complete prevention of TNF- α synthesis is not necessary to significantly alter or prevent the course of endotoxin-initiated biological events and lethality. Two possible explanations may be considered to address this disparity: either lethal levels of TNF- α were not reached (39), or TNF- α alone is not responsible for lethality evoked by endotoxin and other potent endotoxin-induced mediators are involved in the development of the septic syndrome (12, 27, 35, 54, 62). It was not within the scope of this study to generate conclusions regarding these two

possibilities; however, it is probable that both are involved to some degree in the results presented in this report.

The data generated by this study demonstrate that CAP37 P₂₀₋₄₄ significantly inhibits or prevents (i) the *in vitro* biochemical reaction between endotoxin and LAL; (ii) the *in vivo* endotoxin-induced cardiovascular, metabolic, and immunologic responses; and (iii) endotoxin-evoked lethality. The effectiveness of these neutralizing actions was dependent on the concentration of CAP37 P₂₀₋₄₄. These results support the conclusion that CAP37 P₂₀₋₄₄ effectively binds the lipid A portion of *E. coli* LPS *in vitro* and, when introduced into the cardiovascular system under *in vivo* conditions, possesses the capacity to bind the biologically active, toxic component of circulating endotoxin. This study has provided the justification and database for the initiation of future studies from our laboratories to evaluate (i) the efficacy of CAP37 P₂₀₋₄₄ when this peptide is delivered for extended time periods after the introduction of endotoxin or bacteria into the circulatory system and (ii) the potential for combining CAP37 P₂₀₋₄₄ with antagonists of cytokines and other mediators known to be induced by endotoxin and active during the development of sepsis in a chronic septic situation. Only if positive results are obtained from studies such as these will it be appropriate to suggest that CAP37 P₂₀₋₄₄ may be worthy of consideration as a potential therapeutic tool in the treatment of sepsis.

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