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Are Events After Endotoxemia Related to Circulating Phospholipase A_2?

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Objective
The authors sought to determine whether the signs and symptoms of endotoxemia were related to the endotoxin-stimulated increase in circulating phospholipase A_2 (PLA_2) activity.

Background
Because hypotension and pulmonary injury have been associated with elevated PLA_2 activity in septic shock and PLA_2 levels are reduced with the administration of glucocorticoids, the PLA_2 response to endotoxin was investigated in volunteers pretreated with and without hydrocortisone.

Methods
Carefully screened human subjects were studied under four conditions: (1) saline, (2) hydrocortisone, (3) endotoxin, and (4) hydrocortisone administration before endotoxin exposure. Pulse rate, blood pressure, temperature, and symptoms of endotoxemia were serially measured. Plasma for tumor necrosis factor concentrations and PLA_2 activity was obtained.

Results
After lipopolysaccharide, pulse rate and tumor necrosis factor concentrations rose at 1 to 2 hours; temperature increased maximally at 4 hours. PLA_2 activity reached peak levels at 24 hours. With hydrocortisone pretreatment, a 50% reduction in the concentrations of tumor necrosis factor and PLA_2 occurred. Significant correlations between other variables and PLA_2 activity were not observed. The enzyme identified by monoclonal antibody was the secreted nonpancreatic PLA_2 (SNP-PLA_2).

Conclusions
The results of this study suggest that elevations in circulating SNP-PLA_2 activity and systemic events associated with intravenous endotoxin administration are unrelated.

Circulating lipopolysaccharide (LPS) arising from gram-negative bacteria is associated with a well-described set of effects in humans, including alterations in cardiopulmonary function, leukocytosis, fever, and production of acute phase proteins.[1,2] During experimental endotoxemia, the cardiovascular changes include increased cardiac output, hypotension, and narrowing of the pulse pressure.[1] During septic shock, these events are enhanced and result in pulmonary dysfunction, failure of other organs, and a high probability of death.[3] Initially, these observations were thought to be the results of the direct effects of bacteria or their endotoxins. More recently, it has been discovered that this syndrome is linked to the induction of proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and IL-8.[1,4–8] These cytokines can stimulate the hypothalamic–pituitary–adrenal axis and the acute-phase response. Endogenous lipid mediators have also been related to the clinical sequelae of endotoxemia. Phospholipase A_2 (PLA_2), an enzyme that releases lysophospholipid and fatty acid from phospholipid, has been implicated in the pathophysiology of endotoxemia. Increased levels of arachidonic acid metabolites, including leukotrienes, platelet-activating factor, and prostaglandins, have also been observed after endotoxin administration.[9–17]

Vadas's group[3] and others identified a relationship between elevated levels of secreted nonpancreatic PLA_2 (SNP-PLA_2) and the duration and magnitude of hypotension secondary to septic shock in critically ill patients. In addition, the increased activity of SNP-PLA_2 in plasma was associated with the severity of the adult respiratory distress syndrome after septic shock.[3,18] When PLA_2, obtained from *Naja naja* (cobra snake) venom, was infused in rabbits, hypotension, organ failure, and death resulted. Administration of dexamethasone before the PLA_2 infusion attenuated these effects.[19] Because neither recombinant human SNP-PLA_2 for intravenous administration nor specific blocking agents of SNP-PLA_2 designed for human administration are available, we administered LPS to healthy volunteers and studied the SNP-PLA_2 response with and without hydrocortisone pretreatment to determine if increased SNP-PLA_2 activity correlated with the signs and symptoms of endotoxemia.

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MATERIALS AND METHODS

Subjects

This study was reviewed and approved by the Committee for the Protection of Human Subjects in Research at the Brigham and Women's Hospital. Fifteen male subjects (age range, 20 to 50 years) were evaluated with a medical history, physical examination, serum chemistry and hematologic profile, urinalysis, stool for ova and parasites, chest x-ray, exercise electrocardiogram, and diagnostic antibody testing for hepatitis B surface antigen and the human immunodeficiency virus. Each individual included in this study was informed of the protocol, completed the screening evaluation successfully, and gave written consent before admission to the hospital. Ten volunteers (mean age, 37 ± 3 years; mean weight, 75 ± 3 kg; mean height, 177 ± 3 cm; body surface area, 1.92 ± 0.05 m^2) were screened successfully for this study. All subjects were within ± 10% of ideal weight for height as determined by the 1983 Metropolitan Height-Weight Table.[20]

Study Protocol

Each volunteer was admitted to the Clinical Research Center on four separate occasions. Each admission lasted 3 days. Readmissions were separated by an interval of at least 2 weeks. During the initial 36 hours of observation, the subjects were allowed to drink distilled water *ad libitum* and ate three meals and a snack calculated to provide 30 kcal/kg of body weight, 1 g/kg of protein, and 150 mEq of sodium chloride daily. During this period of acclimatization, activity was restricted to ambulation on the research unit.

On the second study day, two intravenous catheters (Intima, Becton-Dickinson Deseret Medical, Salt Lake City, UT) were inserted into veins of the left upper extremity. The distal catheter was used to obtain arterialized venous blood using the heated-hand technique while patency was maintained with 0.9% sodium chloride (lot numbers 133728 and 155721, Baxter Healthcare, Deerfield, IL). The investigational solutions were delivered through the proximal catheter through which sodium chloride 0.9% was infused at 0.7 mL/kg/hr (Flowgard 6000 volumetric infusion pump serial number 1209248x, Travenol Laboratories, Deerfield, IL). The volunteer consumed nothing by mouth until 2 P.M. After this time, all calories and water for that day were consumed.

The order in which the different treatment regimens would be administered to each subject during each 3-day study was randomly assigned by the research pharmacist. On the day of the study, two intravenous injections were administered at 8:30 A.M. and 9 A.M. The investigators

and subjects were blinded from the substances injected. These included (1) 2 mL of saline 0.9% followed by 2 mL of saline (group NS); (2) 2 mL of saline before reference standard endotoxin 4 ng/kg[21] (*Escherichia coli* 0113, lot EC-5, Bureau of Biologics, Food and Drug Administration, Bethesda, MD; group LPS); (3) hydrocortisone sodium succinate 100 mg (n = 5) or 200 mg (n = 5) in 2 mL of diluent preceding 2 mL of saline 0.9% (group HCSS); and (4) hydrocortisone sodium succinate 100 mg (n = 5) or 200 mg (n = 5) in 2 mL of diluent and then reference standard endotoxin 4 ng/kg (group HCSS/LPS). Thus, in all, six experimental conditions were observed.

On study day 3, final measurements and blood were obtained at 9 A.M. The volunteer was allowed to eat breakfast and was discharged from the hospital.

Measurements

Rectal temperature, blood pressure, pulse, and respiratory rate were measured every 6 hours during the first 36 hours. Body weight was obtained each day after the first morning void. On the second study day, clinical parameters were recorded hourly from 6 A.M. until midnight. The rectal temperature was measured using an indwelling thermistor (Rectal Temperature Probe, Yellow Springs, Inc., Yellow Springs, OH), and blood pressure was recorded with a standard monitoring device (Dinimap, Critikon, Tampa, FL). Pulse rate was observed from an electrocardiographic display. After the insertion of the intravenous catheters, values for blood pressure, pulse, and respiratory rate were allowed to return to basal levels before withdrawal of the baseline blood samples.

During study day 2, symptoms including back pain, myalgias, headache, anorexia, and chills were assessed every 15 minutes for maximal response from 10 A.M. to 1 P.M. Complaints associated with endotoxemia were graded from 0 (absence of symptoms) to 4 (most extreme symptoms), and then, the highest response for each symptom was added to produce a single score for each volunteer during each of the four study groups.[22,23] The duration of symptoms was also recorded and analyzed.

On study days 1 and 3, at 9 A.M., peripheral venous blood was obtained for serum renal and hepatic chemical profile and complete blood count. During the second study day, baseline blood samples were obtained at 75 and 15 minutes before LPS or NS. Then, timed arterialized venous blood samples were obtained for TNF and PLA_2.

Secreted Nonpancreatic PLA_2 and Antibodies

Recombinant SNP-PLA_2 was prepared using a stably transfected CO cell line as previously described.[24] Pancreatic PLA_2 was purified from human pancreatic tissue as described; the final preparation was about 90% pure and completely lacked the apo form of the enzyme.[25] Recombinant SNP-PLA_2 was used to prepare polyclonal antisera by immunizing New Zealand white male rabbits with 20 μg of enzyme in complete Freund's adjuvant (CFA) by injection directly into the lymph nodes as previously described.[26] The animals received intramuscular booster injections with 50 μg/rabbit in incomplete Freund's adjuvant (IFA). The resultant antisera developed neutralizing titers that peaked at 1:2000 dilution for blockage of 1.5 ng of recombinant SNP-PLA_2.

A/J mice (female, 4 to 8 weeks old) were immunized with an intraperitoneal injection of 10 μg/mouse of recombinant PLA_2 in CFA to produce anti-SNP-PLA_2 monoclonal antibodies. The animals underwent booster injections every 2 months with 10 μg of enzyme in IFA. After 8 months, a final intravenous booster of recombinant SNP-PLA_2 (10 μg/mouse) was administered. Fusion of spleen cells to P3X63-Ag 8.653 (ATCC American Type Culture Collection) cells by conventional techniques for hybridoma production[27] led to the isolation of anti-SNP-PLA_2 monoclonal antibodies. One monoclonal antibody, CE9, was chosen for use in a sandwich enzyme-linked immunosorbent assay (ELISA), and another monoclonal antibody, BA11, was found to neutralize SNP-PLA_2 activity. The antibodies were purified from culture supernatants by protein A Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ) affinity chromatography.

ELISA Assay for Serum SNP-PLA_2

Immunlon 2 plates (Dynatech, Chantilly, VA) were coated overnight at 4 C with 0.05 mL of monoclonal anti-SNP-PLA_2 CE9 at 10 μg/mL in 50 mM sodium bicarbonate buffer (pH 9.6). All washes were performed as 6 cycles of washing with 0.1% w/v Tween-20 in phosphate-buffered saline (PBS), and all incubations were done at 4 C. The plates were blocked overnight with 10 mg/mL of gelatin in PBS. The serum samples were diluted in 50 mM Tris HCl pH 7.5, 0.1% w/v Tween-20, 1% v/v fetal calf serum, and 0.05% sodium azide (assay buffer), and 0.05 mL was added to the wells at 4 C for 1.5 hours (because the lower temperature decreases nonspecific PLA_2 bindings). Bound SNP-PLA_2 was detected by incubation with 0.05 mL of a 1:2000 dilution of rabbit antirecombinant SNP-PLA_2 (no. 207) in assay buffer for 1 hour followed by washing. Bound rabbit antibody was quantified by successive incubation with 0.05 mL of 1:2000 dilution of biotinylated goat antirabbit immunoglobulin G (Kappel Organon Teknika, Durham, NC) in assay buffer followed by washing and 0.05 mL of 20 μg/mL of streptavidin-β-galactosidase (Cal Biochem, La Jolla, CA) for 1 hour at room temperature. Bound β-ga-

lactosidase was detected using 4-methylumbelliferyl-β-galactopyranoside (Calbiochem) as the substrate. The substrate was dissolved in dimethyl sulfoxide to create a 50 mM stock solution, which was further diluted to 0.5 mM in 0.1 M PBS, pH 7.5, with 1 mM $MgCl_2$ and 0.5 mM dithiothreitol. The substrate solution (0.1 mL) was added to the plate, developed for 60 minutes at 37 C, and read using a Flow TiterTek Fluoroskan II (ICN Flow, Costa Mesa, CA) fluorescent microplate reader (λ_{ex}, 355 nm; λ_{cm}, 460 nm). A standard curve was generated by plotting relative fluorescence units *versus* the logarithm of the recombinant SNP-PLA_2 concentration, and sample concentrations were determined by interpolation from the standard curve. All samples were measured in duplicate assays.

SNP-PLA_2 Activity Assay

SNP-PLA_2 activity was measured using a novel modification of a colorimetric assay[28] with detergent–phospholipid mixed micelles as the substrate. The lipid substrate assay was prepared by vigorously mixing 35 mg of 1-palmitoyl 2-oleyl phosphatidylglycerol (POPG, Avanti Polar Lipids, Avanti Polar, Alabaster, AL) with 1 mL of solution of 4% w/v Nonidet (Sigma, St. Louis, MO) P40 and 2% w/v sodium deoxycholate. The suspension was heated for 20 minutes at 50 C, diluted with 9 mL of 0.12 M Tris HCl, pH 8.0, 12 mM $CaCl_2$, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 37 C and vortexed. In 96-well plates that had been blocked with a 10 mg/mL of gelatin in PBS solution, 0.05 mL of warm (37 C) substrate solution was mixed with 0.01 mL of enzyme diluted in 0.05 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer pH 7.5 with 0.1 M NaCl, 1 mM $CaCl_2$, 1 mg/mL of bovine serum albumin (not fatty acid depleted, Sigma, St. Louis, MO), and 0.05% sodium azide. After incubation for 20 minutes at 37 C, free fatty acid was determined by the addition of 0.08 mL of reagent A of the WAKO fatty acid kit (NEFA, Biochemical Diagnostics, Edgewood, NY), which also arrests enzymatic activity. The plate was incubated an additional 10 minutes at 37 C followed by the addition of 0.15 mL of reagent B of the same kit. After 5 to 10 minutes at room temperature, the absorbance at 550 nm was read using an ELISA plate reader. Under these conditions, 0.1 optical density represents 2.5 to 2.8 nmol of free oleic acid, yielding a specific activity for the recombinant enzyme of 1.0 to 1.4 mol of fatty acid per milligram per minute. Serum samples contain 50 ± 20 μEq/dL of free fatty acid detected by this assay. A separate sample was included in the incubation period to which substrate solution lacking POPG was added. The serum-derived free fatty acid determined in this manner was subtracted from the total to yield the level of liberated fatty acid arising only from enzymatic hydrolysis. In other assays designed to assess specificity, 10 μL of a 1-mg/mL solution of BA11 (a potent neutralizing monoclonal antibody) in PBS was premixed with 10 μL of sample in 96 well-plates for 20 minutes before assaying for activity. SNP-PLA_2 concentrations were determined by interpolation from a standard curve prepared using recombinant SNP-PLA_2.

TNF Assay

The assay for TNF was described previously.[29]

Data Analyses

The subjects were studied under six conditions (one control and five study conditions, see study protocol). Because the responses of the volunteers to either hydrocortisone 100 or 200 mg with or without LPS were not statistically different, the responses of the individuals receiving hydrocortisone were combined. Thus, comparisons were made between four regimens: NS, HCSS, and LPS with and without hydrocortisone pretreatment. The initial baseline measurement (t = −75 minutes) was considered the basal value for each parameter, and the peak value was defined as the highest value attained during the study period as assessed by repeated-measures analysis of variance. All data points were included in the statistical evaluation. The values for symptom score, temperature, TNF, and SNP-PLA_2 from raw data were analyzed by blocking on subjects in a two-factor (group and time) repeated-measures design. The *post hoc* test used was the Fisher prognostic least-significant-difference analysis (Statistica, Statsoft, Tulsa, OK). Significance was defined as a type 1 error of less than 0.05. NS was used as the control group.

RESULTS

Characterization of Serum SNP-PLA_2 Assays

An ELISA assay for SNP-PLA_2 was developed with a sensitivity range of 0.5 to 200 ng/mL in either serum or buffer. The lower limit of the assay, that is, the zero value plus twice the standard deviation, was 0.5 to 1 ng/mL in buffer. The assay was unable to detect human (data not shown) or porcine pancreatic PLA_2 (Fig. 1A). The SNP-PLA_2 demonstrated considerable affinity for the plastics used in ELISA systems. Gelatin blocking was able to reduce this problem; however, at SNP-PLA_2 concentrations greater than 100 ng/mL, background binding to nonspecific immunoglobulin G_2 was often observed. As a consequence, the SNP-PLA_2 standard curves did not show proper saturation (Fig. 1B). The nonspecific binding of

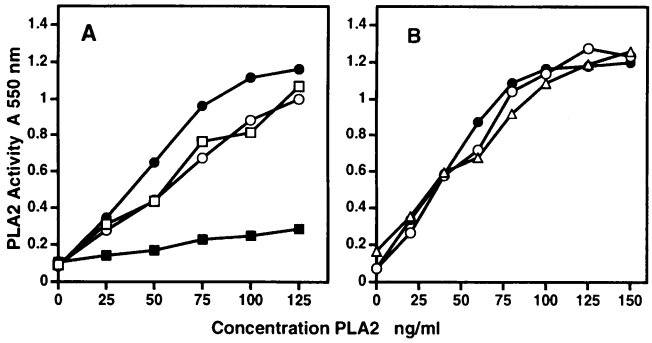

Figure 1. ELISA determination of human nonpancreatic PLA_2. (A) Quantitation of recombinant human SNP-PLA_2 using an antihuman SNP-PLA_2 monoclonal antibody CE9 (solid circles) or a control immunoglobulin G_{2b} monoclonal antibody (open squares) bound to the plate as the capture antibody. Porcine pancreatic PLA_2 binding to the antihuman recombinant SNP-PLA_2 monoclonal antibody is indicated by (open circles). (B) Concentration dependence of recombinant human SNP-PLA_2 in buffer (solid circles), 25% human serum (open circles), and 100% human serum (open triangles).

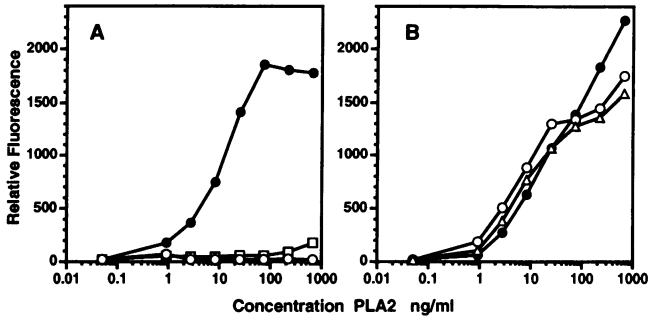

Figure 2. Colorimetric assay for the quantitation of PLA_2 assay. (A) Comparison of the ability of recombinant human SNP-PLA_2 (solid symbols) and human pancreatic PLA_2 (open symbols) activity for their ability to hydrolyze POPG–detergent mixed micelles in the absence (circles) and presence of the antihuman SNP-PLA_2 monoclonal antibody, BA11 (squares). (B) Quantitation of recombinant human SNP-PLA_2 in buffer (solid circles), 25% human serum (open circles), and 100% human serum (open triangles).

SNP-PLA_2 to plastic at high concentrations did not alter the results for SNP-PLA_2 because of the detection with the antirecombinant SNP-PLA_2 polyclonal antibody. Citrated human blood was spiked with varying levels of recombinant SNP-PLA_2 and separated to examine the recovery of SNP-PLA_2. Good recoveries were observed. A number of antibody-based SNP-PLA_2 quantitation assays have been described with similar properties.[30–32]

The activity assay used was a previously described colorimetric assay adapted to determine SNP-PLA_2 enzymatic activity.[24] Phosphatidylglycerol was found to be an excellent substrate for this enzyme, probably as a result of the quality of the mixed detergent–phospholipid micelles formed by this lipid and the negatively charged surfaces. Assays using this lipid were suitable for the detection of 5 to 100 ng/mL of SNP-PLA_2 and were not affected by the presence of serum (Fig. 2). Whole blood was obtained neat or with the addition of citrate, EDTA, or heparin; immediately spiked with 0.1 to 0.5 μg/mL of recombinant SNP-PLA_2; and serum or plasma was prepared. The recovery rate for SNP-PLA_2 was 90%, indicating that plasma preparations using all three anticoagulants yield accurate values of SNP-PLA_2 activity. Neutralization of the activity by monoclonal antibody BA11 was exploited to determine the activity caused by SNP-PLA_2. At high levels of the monoclonal antibody BA11, greater than 90% inhibition of activity was observed. The activity assay described may be suitable for clinical applications because it is sensitive and fast and it discriminates between pancreatic and nonpancreatic SNP-PLA_2. The ability to quantify SNP-PLA_2 activity in the presence of detergent probably avoids the interference caused by various serum components.

Symptoms

The symptoms began 60 to 90 minutes after LPS in a characteristic sequence of malaise, backache, shaking chills, myalgias, and headache. The mean symptom score in this group was 10 ± 2. However, symptoms were significantly attenuated when HCSS pretreatment preceded LPS administration (5 ± 2, $p < 0.05$). Symptoms were not present in the groups that received NS or HCSS. Neither maximal symptom response nor duration of symptoms correlated with peak mean SNP-PLA_2 activity ($r = 0.37$, $p = 0.11$, symptom *vs.* SNP-PLA_2, Fig. 3).

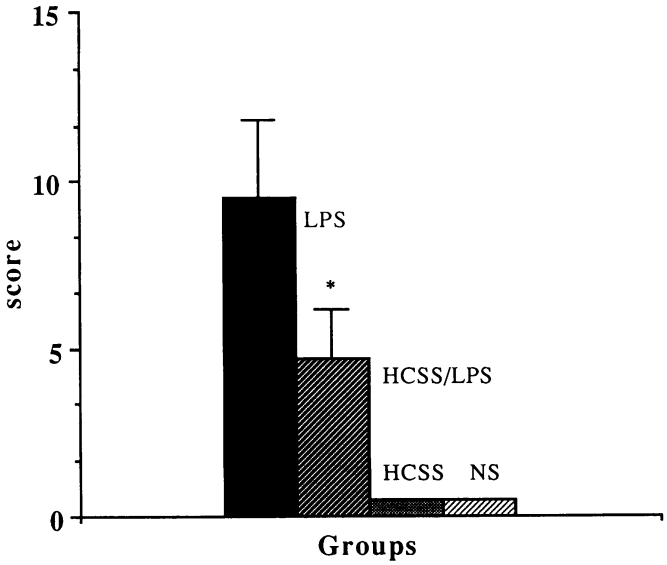

Figure 3. Symptom score of the volunteers. After LPS (black bars), the symptom score was 10; whereas, in volunteers receiving HCSS/LPS, the symptom score was significantly different ($p < 0.05$). The groups receiving NS and HCSS experienced no symptoms.

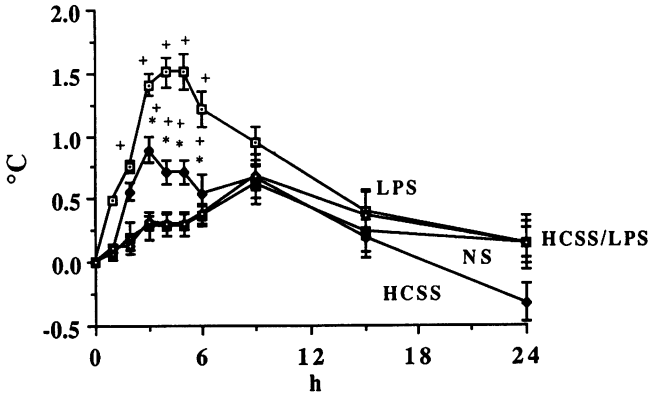

Figure 4. Change in temperature. After LPS, the maximal change in temperature was 1.8 C (+$p < 0.05$ *vs.* all groups), and in volunteers to whom HCSS/LPS was administered, the maximal change in temperature was significantly different from LPS (*$p < 0.05$).

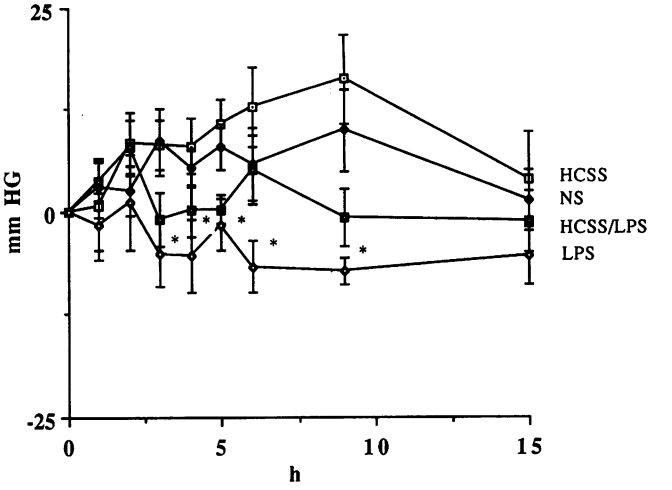

Figure 5. Change in mean arterial pressure. After LPS, the mean arterial pressure was significantly reduced from that in the group receiving NS and HCSS ($p < 0.05$). However, there was no difference in mean arterial pressure in the volunteers receiving LPS and HCSS/LPS.

Clinical Parameters

All measurements of clinical parameters during the first 36 hours of the study were similar in all groups before the administration of any treatment regimen. The rectal temperature ranged between 36.7 and 36.9 C, initial values of pulse rate were between 56 and 58 beats per minute, baseline values for respiratory rate were 16 to 17 respirations per minute, and the basal mean blood pressure varied from 77 to 83 mmHg. After LPS injection, the rectal temperature rose gradually, peaked at 4 hours, and was maximally increased by 1.8 ± 0.1 C. A significantly reduced response was observed when HCSS was administered before LPS (0.9 ± 0.1 C, $p < 0.05$). The NS and HCSS groups were similar but significantly different from the LPS groups ($p < 0.05$, Fig. 4). The maximal pulse rate was present 4 hours after LPS and was significantly attenuated after HCSS pretreatment (LPS, 90 ± 3 beats per minute *vs.* HCSS/LPS, 82 ± 4 beats per minute; $p < 0.01$). There was minimal variation of the pulse rate observed in the control groups. Hourly mean blood pressure and respiratory rate remained constant throughout the study (Fig. 5). Neither, temperature, pulse and respiratory rate, nor mean arterial pressure correlated with SNP-PLA_2 ($r = 0.25$, $p = 0.28$, for mean arterial pressure *vs.* SNP-PLA_2).

TNF

The plasma concentrations of TNF rose from 282 ± 15 pg/mL at baseline to 989 ± 209 pg/mL at 90 minutes after LPS. The decline in TNF level was equally rapid, and baseline values were achieved after 4 hours. When HCSS/LPS was administered, the elevation in TNF was significantly attenuated (571 ± 44 pg/mL, $p < 0.01$) at 90 minutes, returning to control levels before 4 hours. This value for the HCSS/LPS group was 57.7% of the LPS group. In the NS and HCSS groups, the concentrations of TNF did not increase and were similar throughout the study period and significantly different from those in the treatment groups ($p < 0.01$ NS *vs.* HCSS, Fig. 6).

PLA_2

Three hours after LPS injection, PLA_2 activity was detected; PLA_2 increased dramatically and reached maxi-

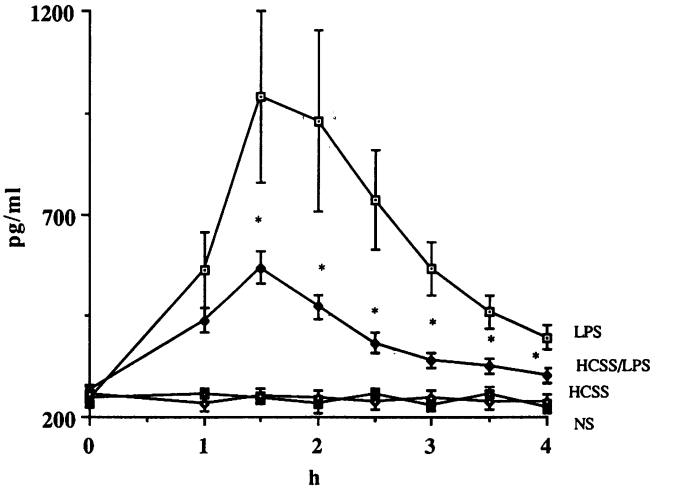

Figure 6. Plasma concentrations of TNF. After LPS, TNF rose to maximal levels at 90 minutes and was significantly different from the other groups ($p < 0.05$). When HCSS/LPS was administered, the TNF response was significantly different from that of LPS ($p < 0.05$). The response of TNF in the groups receiving NS and HCSS were similar.

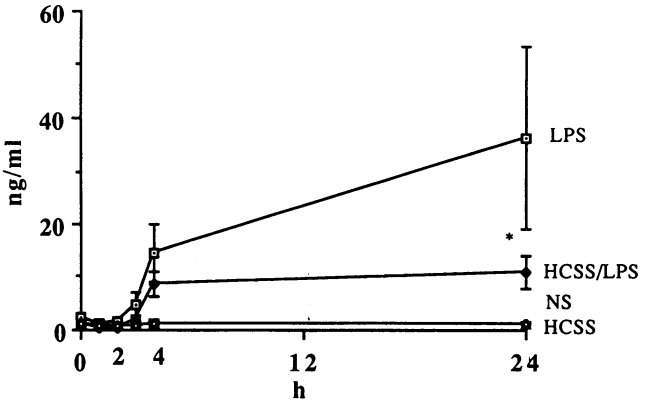

Figure 7. Plasma activity of PLA_2. After the administration of LPS, PLA_2 began to increase at 3 hours, then gradually increased until 24 hours. The difference in the response compared with the other groups was significant. The administration of HCSS/LPS significantly reduced the activity ($p < 0.05$). The groups NS and HCSS produced no significant increase in PLA_2 activity.

mal activity (17.8 ± 4.0 ng/mL) at 24 hours. After HCSS/LPS administration, the initial delay and increase were similar; however, the activity of PLA_2 in this group was only 7.5 ± 2.4 ng/mL at 4 hours and nearly the same at 24 hours (10.0 ± 3.2 ng/mL, $p = 0.1$ *vs.* 4 hours). At 24 hours, the results in the HCSS/LPS group were significantly different from those in the LPS group (56.2%, $p < 0.01$). The NS and HCSS groups had no significant elevations in enzyme activity ($p = 0.57$ NS *vs.* HCSS, Fig. 7). The SNP-PLA_2 activity that resulted from endotoxin treatment could be neutralized by a specific anti-human SNP-PLA_2 monoclonal antibody accounting for the elevated activity of the nonpancreatic enzyme in the blood.

A comparison of the activity and ELISA determinations of SNP-PLA_2 concentrations is shown in Figure 8. There was good correlation between the two values ($r = 0.92$, $p = 0.001$, by linear regression), independent of HCSS pretreatment. There was no relationship established between SNP-PLA_2 and TNF ($r = 0.40$, $p = 0.23$).

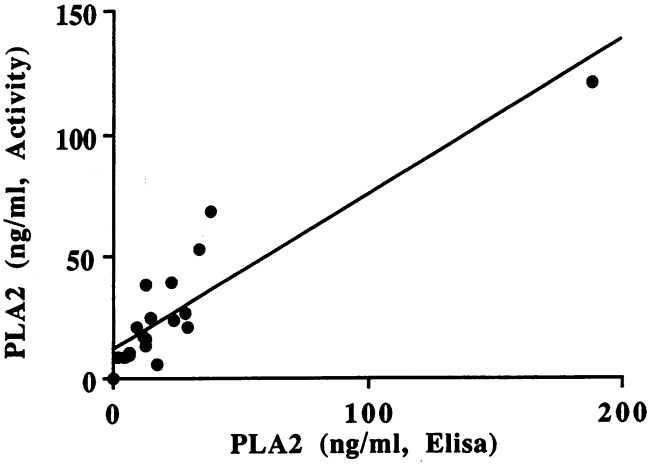

Figure 8. A comparison of assays of PLA_2. The comparison of PLA_2 ELISA and activity assays demonstrated a significant relationship ($r = 0.92$, $p < 0.01$).

DISCUSSION

The administration of LPS initiates systemic inflammatory events, and many aspects of this response are caused by the metabolic products of arachidonic acid and lysophospholipid, that is, leukotrienes, prostaglandins, and platelet activating factor.[9] PLA_2 activity is considered a rate-limiting step in this process, and circulating PLA_2 levels are induced under these conditions. It is not known whether circulating PLA_2 is responsible for increased eicosanoid metabolism, and moreover, there is considerable evidence that intracellular PLA_2 activity is regulated by inflammatory stimuli and is the putative source of precursor arachidonic acid.[33] To investigate the relationship between circulating PLA_2 and its possible pathophysiologic role in systemic inflammatory events, we evaluated the signs and symptoms that occurred after the intravenous administration of LPS to healthy volunteers and the levels of circulating PLA_2.

After intravenous LPS administration, circulating PLA_2 rose at 3 hours and continued to increase at 24 hours; a similar response was found in a previous study.[34,35] The time course of the PLA_2 response is of interest because the rise in plasma concentration was first seen after the resolution of symptoms and the enzyme was at its highest activity 24 hours after the administration of LPS when all signs and symptoms were completely resolved. Similarly, C-reactive protein, an acute-phase reactant, was also increased 24 hours after injection of LPS in humans, paralleling the PLA_2 response.[2,36] The magnitude of the PLA_2 response was 10- to 50-fold lower than that found in patients with septic shock (based on the specific activity of recombinant PLA_2, P. Vadas and J. L. Browning, unpublished data, December 1992, 500 to 2000 ng/mL).[3,30] The difference in the magnitude of SNP-PLA_2 response could be accounted for by the difference in the intensity and duration of the stimuli.

Glucocorticoids were shown to suppress the effects of endotoxin[4,36,37] and are known to decrease the release of PLA_2 when added to cell culture systems or administered to animals before LPS.[18,38] Recent studies also reported that glucocorticoids can inhibit TNF-induced cytosolic PLA_2 activity.[39] A similar response was observed in our subjects. Hydrocortisone attenuated the PLA_2 response, with levels at 24 hours reaching 50% of concentrations observed after LPS alone, and the response of signs and symptoms were similarly reduced.

It has been postulated that steroids regulate PLA_2 by

induction of lipocortins[40] or other plasma proteins, such as the complement protein C3.[41] To examine the role of PLA_2 inhibitory factors in this model, the amount of circulating PLA_2 by ELISA was compared with the amount of protein determined by activity measurements. The PLA_2 concentrations obtained using an ELISA correlated with the concentrations determined by enzyme activity. These analyses confirm that (1) the circulating form of PLA_2 found in this study is the group II enzyme, SNP-PLA_2 (*i.e.*, the enzyme originally characterized in synovial fluid from inflamed joints and platelets) and (2) SNP-PLA_2 is found in the active form in plasma after endotoxemia. The ELISA–activity relationship was valid independent of the intravenous administration of glucocorticoids, which suggests that factors that modulate circulating SNP-PLA_2 activity are unaffected by hydrocortisone pretreatment and, thus, do not contribute to reduced levels of enzyme activity. Moreover, the correlation between SNP-PLA_2 activity and protein levels strengthens the hypothesis that suppression of SNP-PLA_2 activity by pretreatment of LPS with hydrocortisone results from events at the level of gene induction rather than enzyme activity modulation.

The relationship between the signs and symptoms of endotoxemia and mean circulating SNP-PLA_2 levels was examined. In the groups receiving LPS, neither the symptom score nor the duration of symptoms correlated with circulating SNP-PLA_2. In the same groups, the peak mean values for temperature, pulse and respiratory rate, and mean arterial pressure did not correlate with the activity of SNP-PLA_2. In our study, we did not find a relationship between the signs and symptoms of endotoxemia and circulating SNP-PLA_2 activity.

Our results were consistent with the model that glucocorticoids attenuate the response to LPS by decreasing the release of proinflammatory cytokines.[4,36] With administration of hydrocortisone followed by LPS, plasma TNF rose to 60% of peak concentrations of TNF after LPS alone. A proportional reduction in SNP-PLA_2 activity was also observed; after hydrocortisone pretreatment, the 24-hour concentration was 50% of the activity after LPS alone. One explanation for our findings has to do with the interrelationship between glucocorticoids, cytokines, and SNP-PLA_2. The rise in SNP-PLA_2 activity at 3 hours may be in response to the appearance of TNF, which was maximal at 1.5 hours after LPS administration. Elevated concentrations of TNF previously were related in time and duration to events associated with endotoxemia.[5,42] The low dose of hydrocortisone was used to mimic the rise in plasma cortisol in humans after LPS administration. This dose of corticosteroid is 200-fold less than the dose of glucocorticoid administered to patients with septic shock[43–46] or used in experimental models of endotoxemia[47] (Table 1). Because small doses of hydrocortisone significantly reduced the TNF concentration and SNP-PLA_2 activity in our study, the administration of nearly physiologic doses of glucocorticoids may significantly reduce these mediators in patients after activation of the systemic inflammatory response.

Studies reporting pharmacologic modification of the response to LPS suggest that substances other than TNF may induce SNP-PLA_2 in vivo. For example, when LPS is administered after pentoxifylline treatment, the plasma levels of TNF are reduced compared with LPS alone, but the plasma activity of SNP-PLA_2 is similar.[6,34] When LPS is administered with ibuprofen pretreatment, the concentrations of TNF are increased, but the circulating SNP-PLA_2 is unchanged.[32,48] These data suggest that circulating SNP-PLA_2 may be controlled by mechanisms that operate in addition to TNF-stimulated SNP-PLA_2 synthesis. Although one group found a correlation between TNF and SNP-PLA_2 ($r = 0.82$, $p < 0.01$),[34,35] we were unable to verify this relationship despite similarities of subject number and study design ($r = 0.40$, $p = 0.23$). A number of other cytokines, for example, IL-1 and IL-6, have been shown to induce SNP-PLA_2 synthesis *in vitro*, and they may contribute to the SNP-PLA_2 response *in vivo*.[49]

Finally, after the SNP-PLA_2 concentration was elevated, the level was sustained or increased for at least 20 hours in our study. This persistent elevation of SNP-PLA_2 is of interest because injections of the pure enzyme in rats result in rapid clearance from the blood, and sustained concentrations greater that 1 μg/mL of SNP-PLA_2 were not achieved after intravenous injections of up to 1 mg of recombinant enzyme (J. L. Browning, personal communication, December 1992). By contrast, circulating levels of SNP-PLA_2 persisted in our subjects, and higher levels were found in the sera of patients with septic shock. This observation suggests that, after a systemic inflammatory response, the elevated levels of SNP-PLA_2 may be caused by a marked induction of enzyme synthesis and/or release or a reduction in renal clearance.

Although the presence of the enzyme has been associated with hypotension and pulmonary dysfunction during septic shock, in this study, circulating SNP-PLA_2 levels and the signs and symptoms of endotoxemia appeared to be unrelated. Circulating SNP-PLA_2 may not mediate any of the sequelae of sepsis and may be considered a protein with an undefined protective effect, as previously suggested.[49] Conversely, there are a number of explanations for the lack of correlation between the events of endotoxemia and circulating SNP-PLA_2. First, it is possible that the quantity of LPS administered results in a reduction in mean arterial pressure that it is of insufficient magnitude to allow the documentation of a relationship between circulating SNP-PLA_2 and mean arterial pressure. Alternatively, the circulating SNP-PLA_2

Table 1. COMPARISON OF CORTICOSTEROID AND DOSE USED TO TREAT ENDOTOXIN/TNF/SEPTIC SHOCK

Author	Subjects/Cells	Glucocorticosteroid Utilized	Equivalent Hydrocortisone Dose	Result
Vadas and Hay[19]	Rabbits/LPS/PLA_2	Dexamethasone	333 mg/kg ip*	Blocked PLA_2
Nakano et al.[38]	Rats/LPS	Dexamethasone	333 mg/kg ip*	Blocked PLA_2
Shumer[43]	Septic shock	Dexamethasone	100 mg/kg iv	Reduced death
Shumer[43]	Septic shock	Prednisolone	188 mg/kg iv	Reduced death
Sprung et al.[44]	Septic shock	Dexamethasone	200 mg/kg iv	Shock reversal
Sprung et al.[44]	Septic shock	Prednisolone	188 mg/kg iv	Shock reversal
Bone et al.[45]	Septic shock	Prednisolone	750 mg/kg iv	No effect
Hinshaw et al.[46]	Septic shock	Prednisolone	469 mg/kg	No effect
Santos et al.	Volunteers/LPS	Hydrocortisone	1–1.5 mg/kg iv*	50% blocked

* Pretreatment; Note: 25 mg hydrocortisone = 5 mg prednisolone = 0.75 mg dexamethasone; LPS = lipopolysaccharide; PLA_2 = phospholipase A_2.

levels may be too low to have hypotensive effects. Second, the pathologic effects of SNP-PLA_2 may be manifested in synergy with other inflammatory events or substances and may not be initiated in this study. Third, immunohistologic studies found large amounts of SNP-PLA_2 in vascular smooth muscle from patients with septic shock.[16] Circulating SNP-PLA_2 may be a consequence of the synthesis of SNP-PLA_2 in vascular smooth muscle, saturation of these tissues, and spillage of SNP-PLA_2 into the circulation, and thus, it would be a poor indicator of tissue-specific events. Progress in this field will require elucidation of the tissue source of circulating SNP-PLA_2 and a correlation of the increase in activity of tissue SNP-PLA_2 with signs and symptoms of endotoxemia.

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