Protein-free phospholipid emulsion treatment improved cardiopulmonary function and survival in porcine sepsis

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IT IS RECOGNIZED that elevated serum cholesterol is a risk factor for overall mortality when it is very high; it is also a mortality risk factor when it is very low (see review, Ref. 18). The benefits of reduced cardiovascular morbidity and mortality by treatment of hypercholesterolemia with cholesterol-lowering drugs are well documented. Low lipid and lipoprotein concentrations are associated with a poor prognosis in several studies of elderly individuals (20, 28, 34). Hypcholesterolemia was associated with development of infectious disorders over a 15-yr period in the Kaiser Permanente study of 15,000 healthy men and women (10) and with reduced hospital survival (35) and poorer clinical outcomes in a surgical intensive care unit (6). These data suggest that circulating lipoprotein may interact with mediators of systemic inflammation or bacterial products.

In fact, there are substantial data indicating that serum lipoproteins may neutralize circulating bacterial toxins. High-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride-rich lipoproteins, very low density lipoprotein (VLDL), and chylomicron remnants have all been shown to bind and neutralize bacterial endotoxin in vitro (3, 8, 33). Bacterial endotoxins are a diverse family of LPS that are shed from the outer membrane of gram-negative bacteria. Serum LPS-binding protein (LBP) presents LPS to CD14 and TLR-4 on cells of the monocyte/macrophage lineage (24, 36), which then initiate release of inflammatory mediators, causing systemic inflammatory syndrome and septic shock (1). Lipoproteins function as alternate neutralizing acceptors of LPS bound to LBP (12, 32, 37). We have shown that LPS neutralization correlates with the amount of phospholipid (PL) in lipid or lipoprotein particles (23). HDL, a primary carrier of PL among lipoproteins, has significant endotoxin-neutralizing capabilities. Infusion of HDL protects against lethal consequences of LPS administration in mice (15) and blocks LPS-induced cytokine production in rabbits (9) and human volunteers (21, 22).

This study tested the hypothesis that prophylactic administration of a protein-free, PL-rich lipid emulsion, which enriches serum lipoproteins with PL and neutralizes endotoxin in vitro, would provide a survival benefit in a porcine model of septic peritonitis (5). Pigs were chosen for this study because their serum lipoprotein distribution and sensitivity of their cardiopulmo-
nary responses to systemic sepsis are closer to humans than are those of rat, rabbit, or dog. Septic peritonitis was modeled by introducing a 90% lethal dose of *Escherichia coli* 0111:B4 encased in a fibrin clot into the abdomen. This allows live gram-negative bacteria to grow and multiply within a protected nidus of infection, colonize the peritoneal cavity, and enter the systemic circulation causing bacteremia, endotoxia, cytokinemia, and cardiopulmonary changes typical of septic shock. In whole human blood, we demonstrated (23) a dose-dependent effect of PRE to retard endotoxin-stimulated tumor necrosis factor (TNF-α) expression. In this study, multiple PRE infusion regimens were tested to establish a similar dose-dependent effect in vivo as well as to gain insight into minimal effective dose levels.

**METHODS**

**Pigs**

Healthy, normal Yorkshire pigs weighing 25–35 kg were obtained from Oak Hills Farms, a certified US Department of Agriculture supplier of laboratory animals. This protocol was reviewed and approved by Rush-Presbyterian-St. Luke’s Medical Center’s Animal Use Committee. Rush’s staff veterinarians carefully monitored these experiments for adherence to National Institutes of Health guidelines for use of animals.

**Experimental Protocols**

**Instrumentation surgery.** Each pig was preanesthetized with ketamine (100 mg/kg) and glycopyrolate (0.016 mg/kg), intubated, and ventilated with isoflurane (1–2%) in oxygen to provide surgical anesthesia. With the use of sterile surgical techniques, ultrasonic crystals (Triton Technology) to measure short-axis diameter and a pressure transducer (Konigsberg Instruments) to measure left ventricular pressure were implanted in the left ventricle. A flow probe (Transonic) was placed around the pulmonary artery to measure cardiac output, and catheters were implanted in the pulmonary artery and aorta. The chest was closed, spontaneous respiration restored, and each animal was transported to postsurgical recovery where it was observed until full recovery.

**Treatment.** After 5–7 days of postsurgical recovery, basal cardiovascular and blood chemistry data were obtained (defined as *day 0*). On the next day, each animal was returned to the lab and animals were treated prophylactically with saline (*n* = 5), placebo (*n* = 7), or PRE (defined as *day 1*). A priming dose was delivered over 15 min followed by continuous intravenous PRE for 48 h. Three treatment groups were used: low (*n* = 7), mid (*n* = 7), and high (*n* = 6); all received a priming dose of 100, 200, or 200 mg PL/kg, respectively, followed by continuous infusion at 25, 25, or 100 mg PL·kg⁻¹·h⁻¹, respectively. PRE or vehicle was infused through the pulmonary artery catheter. Total infusate volume was 2 liters on *day 1* and 1 liter on *day 2*. Animals were observed continually for up to 72 h. Data were collated hourly on *day 1* and daily on *days 2, 3, and 4*. No restraint was necessary during the 48 h of PRE infusion because sepsis reduced physical activity.

**Control treatments: saline/placebo.** Both 0.9% saline (*n* = 7) and a placebo (*n* = 5) were used as control treatments. The placebo solution was prepared by diluting 20% Liposyn 1:64 in 2.6% (wt/vol) glycerol. Lipid content of the placebo solution provides enough opacity to make it visibly indistinguishable from emulsion, but not enough lipid to neutralize endotoxin in human whole blood or to protect mice from a lethal endotoxin challenge (data not shown). Placebo and saline control solutions were administered in volumes equal to volumes received by high-dose group.

**Emulsion.** Fresenius Kabi (Clayton, NC) manufactured PRE used in this study. Soy phosphatidylcholine (Phospholi-70G, American Lecithin) and soy triglyceride (Fresenius Kabi) were mixed (92.5% PC:7.5% TG) and emulsified in 18 mM sodium cholate (New Zealand Pharma), containing 2.6% glycerol wt/vol (Fresenius Kabi) by repeated passes through an APV Gaulin homogenizer at 10,000 PSI. The resulting emulsion, PRE, was sterilized by repeated passages through a 0.22-μm membrane and stored in glass bottles. The final mixture contained 100 mg PL/ml in 18 mM sodium cholate and 2.6% glycerol. PRE contained no protein.

**Emulsion bioassay.** PRE used in this study was tested for bioactivity in a whole human blood TNF-induction system that we have described previously (23). Briefly, a two-way dose-response experiment was carried out by mixing PRE with whole human blood and HBSS to provide final concentrations of PRE PL of 0, 0.1, 0.3, 1.0, 3.0, and 10 mg/ml in 50% whole blood. *E. coli* 0111:B4 LPS (List Biologicals) was added to give final concentrations of 0, 0.01, 0.1, 0.3, 1, 3, 10, and 100 ng/ml. Thirty-six (6 × 6) 1-ml incubations were kept at 37°C for 4 h, centrifuged at 2,000 g for 10 min, and the cell-free supernatants were stored at −70°C for measurement of TNF (Amersham Biosciences ELISA RPN 2148).

**Septic peritonitis.** After recording of basal parameters and delivery of the priming dose, each pig was anesthetized with sodium thiopental, an ultra-short-acting barbiturate. A small (2–3 cm) midline incision was made, and a bacteria laden fibrin-thrombin clot (100–150 ml) was inserted at a dose range of 2–3 × 10¹⁰ colony forming units (cfu/kg *E. coli* 0111:B4. This dose of bacteria was selected to produce a 90% lethal dose peritonitis model. This level of insult was selected after power analysis so that any potential therapeutic effect of PRE infusion would be observable using the smallest number of animals. Animals recovered from thiopental anesthesia within 10 min. Each animal was observed for up to 72 h or until spontaneous expiration (15). Pigs did not receive antibiotic therapy, but did receive 2 liters of normal saline during the first 24 h and 1 liter on each subsequent day.

**Endpoints.** The primary endpoint of this study was mortality. Each animal was observed for 72 h or until spontaneous expiration. An experiment was terminated if cardiac output fell below 1 l/min for 30 consecutive min or if either the principal investigator or monitoring veterinarian (blind- ed to treatment) judged that an animal was in distress with no hope of recovery. Mortality was recorded at 24 and 72 h. Previous experience (5) demonstrated that no animals expired after 72 h and thus can be considered long-term survivors. Secondary endpoints were effects of treatment on serum TNF-α levels and measures of cardiac dynamics and cardiovascular status.

**Cardiodynamic data acquisition and analysis.** Measured variables recorded were left ventricular pressure (LVP), pulmonary artery blood flow (PAQ), pulmonary and arterial blood pressures (PAP and AOP), and short-axis diameter (D). From these measured variables, the following were calculated using software developed in this laboratory (4, 5); ΔD/ΔP max and minimum cardiac output (l/min by integration of PAQ signal), systemic and pulmonary vascular resistance, heart rate (HR), peak LVP, end systolic diameter (ESD), end-diastolic diameter (EDD), and %diameter shortening (%DSh = EDD − ESD/EDD). Cardiodynamic data were obtained every 15 min after implantation of

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bacteria-laden fibrin clot continuing for 6 h each day. These data were collated and indexed by time. Time 0 was defined to be all basal values. Data obtained on the day of implant were indexed by hour, 1–6. Data obtained on following days were indexed in 24-h intervals (24, 48, and 72 h).

Samples and Measurements

Blood and serum. Samples (10 ml) were obtained before PRE infusion (basal), 15 min (after the priming dose), 1, 2, 3, 6, 24, and 48 h after clot implant, and 1, 2, 3, and 4 h postinfusion (49–53 h after clot implant.) Blood was drawn from a central arterial line, allowed to clot, and immediately spun in a refrigerated centrifuge. Aliquots of serum were allowed to clot, and immediately spun in a refrigerated centrifuge. Aliquots of serum were collected (750 l) and analyzed for cholesterol, PL, and triglycerides. Serum endotoxin levels were measured using an enzymatic test kit (Sigma Diagnostics, St. Louis, MO). PL was measured using an enzymatic method based on measurement of choline content of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine (Wako Chemical, Dallas, TX); these PLs comprise ~95% of total serum PLs.

Lipoproteins. Lipoproteins: VLDL, LDL, and HDL were separated by size-exclusion chromatography. Serum (200 l) was injected onto an AKTA FPLC apparatus (Amersham Pharmacia Biotech), fitted with two Superose 6 size exclusion columns in series, and eluted with phosphate-buffered physiological saline (pH 7.0) at 0.5 ml/min (16). Fractions were collected (750 l) and analyzed for cholesterol, PL, and triglyceride as described above. Ex vivo lipoprotein remodeling studies were done by incubating serum (200 l) with saline, emulsion (final PL = 440 mg/dl) and/or BODIPY (Molecular Probes) R595 (List Biological Labs, prepared as described by Yu and Wright (38) (final PL = 1 mg/ml) at 37°C for 30 min. Lipoproteins were separated by flotation from 1.24 g/ml NaBr in a Beckman TL100 rotor at 100,000 rpm for 3 h at 20°C. Native gradient gel electrophoresis was done on 4–30% Isosafe gradient gels from IsoLab (Akron, OH) as described by Nichols et al. (19). BODIPY-PLS and protein (SYPRO Orange) were imaged in a Molecular Dynamics Fluorimagex.

Endotoxin. Serum endotoxin was measured by a kinetic limulus amebocyte lysate method (CXY9002, Coamatic Endotoxin, Associates of Cape Cod, Falmouth, MA) according to the manufacturer's instructions. A Molecular Devices Thermomax microplate reader and SoftMax Pro software, version 2.6.1, were used to collect kinetic data (OD450/20 s for 20 min), estimate reaction lag times, and calculate unknowns. Samples were thawed, diluted 1:10 or more as needed, and heated to 75°C for 5 min to unmask bound endotoxin. Unknowns were analyzed with and without addition of purified E. coli 0111:B4 LPS (List Biologicals) as an internal standard. Recovery of an internal standard averaged 89 ± 23% and was not affected by PL concentration of serum samples. Serum endotoxin levels are reported in nanograms per milliliter.

RESULTS

PRE contained 92.5% PL and 7.5% TG in a solution of 18 mM sodium cholate and 2.6% glycerol. In whole human blood, PRE (300 mg/dl as PL) increased concentration of LPS required to give 50% maximal TNF-α production by 10-fold (from <1.0 to >10 ng/ml). Stored PRE samples retained this level of bioactivity when retested after PRE batches were used in pigs. Preliminary studies in healthy pigs confirmed that PRE could be given to healthy pigs at doses up to 1,200 mg·kg⁻¹·2 h⁻¹ without significant toxicity.

Survival and Serum PL Concentrations

Emulsion was administered before (prophylactic) induction of septic peritonitis as a primed continuous intravenous infusion (CIVI) to maintain normal or supernormal serum PL concentrations during peritonitis. Serum PL levels in placebo (control) and PRE-treated animals are shown in Fig. 1, top. Serum PL in control pigs fell from basal [70 ± 25 mg/dl (n = 12)] to a low of 33 ± 1 mg/dl between 6 and 24 h postinfection. PRE treatment was successful in augmenting endogenous serum PL in all treatment groups. After priming and 4 h of CIVI, it was 164 ± 61 mg/dl in low-dose group (prime 100 mg/kg, CIVI 25 mg·kg⁻¹·h⁻¹), 313 ± 120 mg/dl in mid-dose group (prime 200 mg/kg, CIVI 25 mg·kg⁻¹·h⁻¹), and 585 ± 354 mg/dl in high-dose group (prime 200 mg/kg, CIVI 100 mg·kg⁻¹·h⁻¹) compared with 67 ± 21 mg/dl in controls. Because both mid-dose and low-dose groups received PRE at 25 mg·kg⁻¹·h⁻¹, their serum PL levels were similar between 4 and 48 h. The profile of serum PL concentrations was more complicated in the high-dose group and may reflect saturation of clearance between 8 and 24 h followed by a period of accelerated acute phase clearance. Serum PL
concentrations returned to baseline within 24 h after halt of CIVI in all treated groups.

The Kaplan-Meier survival plot is shown in Fig. 1, bottom. All control and treatment groups received similar bacterial loads (overall mean $2.33 \times 10^{10} \pm 0.33 \times 10^{10}$ cfu, between-group ANOVA: $P = 0.711$). This bacterial load produced a low survival rate in the combined control group. Only one of seven saline- and one of five placebo-treated pigs survived to 72 h for a total survival rate in the combined control groups of two of twelve or 16.7%. Survival rates in the treatment groups were low dose four of seven (57.1%, $P = 0.051$ vs. control), mid dose six of seven (87.5%, $P = 0.04$); and high dose six of six (100%, $P = 0.001$).

Serum TNF-α and Cardiopulmonary Responses to Sepsis and Treatment

Time courses of appearance of TNF-α in serum and cardiopulmonary indicators of the evolution of shock are shown in Fig. 2. Induction of septic peritonitis was
followed by an increase in serum TNF-α concentrations in all animals that peaked between 1 and 1.5 h and returned to baseline by 3–4 h in surviving controls and all treated animals. Treatment lowered peak TNF-α concentrations by 68% from 2,251 ± 1,014 to 720 ± 355 pg/ml (P < 0.05). Cardiac output began to fall in control and treated animals shortly after the spike in serum TNF-α (Fig. 2B). The initial decline in cardiac output was preceded by sharp increases in systemic vascular resistance (Fig. 2C) and pulmonary vascular resistance (Fig. 2D) coincident with peak serum TNF-α levels. A continuing decline in cardiac output in controls is coincident with a second spike in pulmonary vascular resistance and a decline in ejection fraction (%diameter-shortening; Fig. 2E). Although pulse rate (Fig. 2F) increases in both groups, it is not sufficient to preserve cardiac output in the presence of relatively high vascular resistance that develops in untreated animals. Cardiac output recovered to basal or super-basal levels on day 2 in surviving treated animals. These differences in cardiopulmonary function were clearly reflected by clinical signs of sepsis. Increased pulmonary vascular resistance was accompanied by development of pulmonary edema and noticeably labored breathing. Increased systemic vascular resistance and disseminated intravascular coagulation were evident by subcutaneous discoloration and hypothermia. Two survivors in the control group demonstrated these clinical signs for at least 48 h. Neither surviving control animals ate spontaneously; both had to be hand fed and watered. In contrast, treated animals rarely exhibited even mild subcutaneous discoloration or labored respiration. By 4 h nearly all treated animals had rosy pink skin tones characteristic of normo- or hyperperfusion. All surviving treated animals were drinking and eating spontaneously by 48 h, if not sooner.

Effect of Emulsion on HDL and Binding of Fluorescent Endotoxin

PRE PL was found to associate preferentially with serum to HDL (Fig. 3) at doses up to 200 mg·kg⁻¹·2 h⁻¹. Ex vivo mixing studies of emulsion and human serum demonstrated a size shift of HDL on native gradient gel electrophoresis (NGGE) consistent with binding of additional PL. Fluorescent BODIPY-LPS added to serum was bound to normal and PL enriched HDL after NGGE (Fig. 3).

PL Distribution

Distribution of PL across lipoprotein fractions is shown for representative animals pre- and postsurgery and after 2 h of treatment in Fig. 4. Before surgery, most serum PL is in the HDL fraction in healthy animals. In the postsurgical acute phase, total serum PLs were reduced 30–40%, but the relative distribution of PL in VLDL, LDL, and HDL fractions was not changed. Treatment with placebo had no effect on distribution of PL between VLDL, LDL, and HDL compared with pretreatment or saline-treated controls. Infusion of emulsion preferentially increased HDL PL levels in the low-dose group. In the mid-dose group, peak broadening suggests increasing size heterogeneity in all lipoprotein fractions, and small increases in peak area for VLDL and LDL indicate more PL mass in these lipoprotein fractions. In the high-dose group, the capacity of serum lipoproteins to accept PL was clearly reduced.
saturation: most PL eluted as large particles in VLDL size range. This large-particle fraction contained less triglyceride per unit PL compared with VLDL in the pretreatment samples, suggesting enrichment with PL vesicles or a lipoprotein-x-like particle (7, 14). The interpretation is consistent with the decreased PL peak area in the LDL and HDL fractions in the mid- and low-dose groups, which suggests that native lipoproteins and apolipoproteins had remodeled into the larger particles (29, 30).

Serum Endotoxin

Serum endotoxin was measured during the 2nd h of septic peritonitis. Median endotoxin level in controls was 112 EU/ml (25% and 75% confidence limits: 4.22–500) compared with a median of only 3.0 EU/ml (25% and 75% confidence limits: 0.9–17.4) in the combined treatment groups (P < 0.001). Serum endotoxin levels tended to decrease with increased PRE dose, but large within-group variation of the serum endotoxin measurements and small numbers of animals within groups left the dose-group comparisons short of statistical significance (P < 0.07). Further analysis revealed that serum endotoxin concentrations were strongly related to serum PL concentration during the initial 2 h of treatment (see Fig. 5).

Dose-Response Relationships

There was a strong dose-response relationship between serum PL and mortality (Fig. 6). Controls had the lowest PL concentration and the highest mortality. Serum PL concentrations increased across all treatment groups, and increased serum PL was associated with decreased mortality. For individual animals, survival was predicted best by serum PL concentration (at 0–4 h) and bacterial load (P < 0.001 and 0.049, respectively; Cox proportional hazard test). Neither serum endotoxin nor TNF-α added significantly to the Cox proportional hazards model in stepwise multivariate analysis. Only 1 of 15 animals with serum PL concentrations >200 mg/dl died compared with 13 of the 17 animals with serum PL <200 mg/dl.

DISCUSSION

This study demonstrated the novel role of PL as a defense against bacterial toxins. These data confirm that serum lipoproteins participate in host defense. Mortality in this fatal model of septic peritonitis was inversely related to serum PL concentration. Elevation of serum PL was also associated with decreased serum TNF-α and preserved cardiopulmonary function. These data highlight the role of PL, but do not exclude a function for other serum proteins and lipoprotein components that have been shown to interact with LPS. PL was infused as an emulsion into the circulating blood volume, where it combined with LBP, HDL, LDL, and VLDL that were present in serum and formed remodeled particles. Our ex vivo mixing studies show that remodeled PL-enriched particles bind LPS.

This experiment employed a severe model of sepsis in which live gram-negative bacteria reproduced...
within a protected nidus of infection. From this protected peritoneal reservoir, bacteria and endotoxin enter the systemic circulation for at least 48 h, causing bacteremia, endotoxia, cytokinemia, and cardiopulmonary changes typical of septic shock (5).

However, a number of features of clinical sepsis common in hospitalized populations could not be modeled. Clinical septic shock is often preceded by a period of several days of systemic inflammation and hyperdynamic sepsis. These animals were uniformly young and healthy with no other concurrent disease or trauma other than surgery 1 wk before induction of sepsis. In this model, the time of onset of infection was known and there were no prior or subsequent complications. It remains to be determined whether raising serum lipoprotein (PL) concentrations will be of benefit in the more complicated clinical setting.

Two observations illuminate the mechanisms of PRE’s actions in this model. First, prophylactic administration of emulsion delivered PL preferentially to HDL in low- and mid-dose groups, yet optimal suppression of TNF-α and the survival benefit were most evident in the mid- and high-dose groups where much or most of the PL was associated with particles other than HDL. This suggests that the overall serum PL concentration is more important than the concentration of PL in any particular lipoprotein fraction.

The second finding bearing on mechanism was the absence of any accumulation in serum of endotoxin bound to HDL as had been reported in studies of reconstituted HDL in endotoxia (22). Lower serum endotoxin concentrations could be due to either faster clearance of free and lipoprotein-bound endotoxin or slower entry from the peritoneum to the plasma compartment. Although it cannot be excluded that PRE treatment reduced endotoxin input by blunting hypotension and bowel ischemia, reducing translocation of bacteria and endotoxin from the gut reservoir, we believe it is more likely that treatment with PRE enhanced removal of endotoxin from the circulation.

Serum endotoxin is cleared by the liver and excreted into the bile (2, 17, 26). Infusion of a triglyceride-rich, apolipoprotein-E-containing emulsion has been shown to direct hepatic endotoxin uptake away from Kupffer cells to parenchymal cells and out into bile (27). The combination of PL and cholic acid, used in PRE, may accomplish a similar redirection of endotoxin through the liver. HDL PL and cholesterol are cleared into the liver via hepatic scavenger receptor BI (SR-BI) and then excreted into bile (11, 13, 31). Intravenous infusion of HDL stimulates SR-BI-mediated biliary secretion of HDL PL, and coadministration of cholic acid with HDL increases secretion further (25).

The small number of animals in each dose group of this study limited our ability to detect dose-dependent differences in measures with high variability, such as serum TNF and endotoxin levels as well as many cardiopulmonary parameters. Two-way ANOVA comparing vehicle vs. combined treatment groups revealed a significant effect of treatment in TNF and several cardiovascular variables (Fig. 2). However, this was underpowered to detect difference between vehicle and multiple-dose groups.

Limitations of Study

The primary endpoint of this study was survival in a fatal model of septic peritonitis. The number of animals studied to satisfy this primary endpoint was insufficient to support statistically meaningful multiple comparisons by dose group of secondary endpoints, such as cardiac output, vascular resistances, and circulating endotoxin and cytokine levels. Because of high methodological and biological variability of serum endotoxin and cytokine data, preliminary power calculations estimate that ~20 animals per group would be necessary to detect a PRE dose effect, if it exists. To detect a PRE dose effect would require a different study design in which lethality in placebo-treated animals was 50% or less. In this design, sufficient numbers of placebo-treated animals would be alive at 24, 48, and 72 h to compare with the surviving PRE-treated pigs. Thus analysis of secondary endpoints, although scientifically quite interesting and relevant, is beyond the proposed scope of the present study.

In conclusion, prophylactic administration of a protein-free, PL-rich lipid emulsion provided positive survival benefit in a near-fatal porcine model of septic peritonitis. PRE decreased serum endotoxin and TNF-α levels, preserved cardiopulmonary function, and increased survival in a dose- and concentration-dependent manner. Systemic PL concentrations at the time of clot implantation of ~150, 300, and 600 mg/dl PL achieved a 57%, 88%, and complete (100%) survival, respectively. PL-rich emulsion may prove beneficial in treatment of hospitalized patients where serum cholesterol and PL levels are frequently low, suggesting clinical studies of PRE are warranted.

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