Influence of Fructose-1,6-Diphosphate on Endotoxin-Induced Lung Injuries in Sheep

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Background. Fructose-1,6-diphosphate (FDP) is reported to have a salutary effect in endotoxin shock and sepsis. This investigation describes the effect of FDP on pulmonary and systemic hemodynamics, lung lymph protein clearance, and leukocyte count in sheep infused with Escherichia coli endotoxin.

Materials and methods. Anesthetized sheep (n = 18), some of which underwent thoracotomy to cannulate lymphatic nodes, were used in this study. After stabilization, all sheep received E. coli endotoxin, 5 µg/kg i.v. infusion over 30 min. Concomitant with the endotoxin infusion, half of the animals were randomly selected to receive an i.v. bolus of FDP (10%), 50 mg/kg, followed by a continuous infusion of 5 mg · kg⁻¹ · min⁻¹ for 4 h; the rest were treated in the same manner with glucose (10%) in 0.9% NaCl.

Results. Pulmonary artery pressure (PAP) and resistance in the glucose group increased from 20.8 ± 1.6 to 36.7 ± 3.2 mmHg (P < 0.007) and from 531 ± 114 to 1137 ± 80 dyn · s⁻¹ · cm⁻⁵, respectively (P < 0.005). Despite an increase during endotoxin infusion, these parameters in the FDP group returned to control values. There were no differences in left ventricular pressures, cardiac output, heart rate, and arterial oxygen tension between the groups. In the glucose group, lymph protein clearance was higher (P < 0.01) and blood leukocyte count was lower (P < 0.02). The wet/dry lung weight ratio (g/g) for the glucose group was 5.57 ± 0.04 and for the FDP-treated group 4.76 ± 0.06 (P < 0.0005).

Conclusion. FDP treatment attenuated significantly the characteristic pulmonary hypertension, lung lymph protein clearance, and pulmonary vascular leakage seen in sheep infused with endotoxin. © 2007 Elsevier Inc. All rights reserved.

Key Words: endotoxin; sheep; fructose-1,6-diphosphate; lung lymph protein clearance; pulmonary arterial pressure; leukocytes; wet/dry weight ratio.

INTRODUCTION

Lung injury induced by Escherichia coli endotoxin is characterized by pulmonary hypertension and increased microvascular permeability, resulting in the formation of pulmonary edema [1–4]. Experimental studies have reported salutary effects of fructose-1,6-diphosphate (FDP) in shock of different etiologies, including canine endotoxin shock [5–8]. FDP reduces the mortality rate in experimental sepsis and ameliorates hematologic and histological alterations [9]. In clinical shock resulting from trauma or sepsis, hemodynamic and pulmonary function parameters are improved with FDP treatment [10]. Furthermore, in a recent study FDP was shown to prevent α-naphthylthiourea (ANTU)-induced pulmonary edema [11]. It is generally accepted that non-cardiogenic pulmonary edema is caused by an acute inflammatory process in which the major participating factor is increased capillary permeability. Damage to capillary endothelium is suggested to be caused largely by oxygen free radicals released from activated and sequestrated neutrophils on the lung [12–15]. FDP inhibits respiratory burst and oxyradical generation of activated human and canine neutrophils [16–18]. Although the role of the neutrophils in the genesis of permeability edema appears to be predominant, relevant studies also suggest the involvement of immune cells in the process [19–21]. FDP has been shown both in vitro and in vivo to possess immunosuppressive properties [22–24]. On the basis of these reports, we proposed to study the effects of FDP on pulmonary

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hemodynamics, lung lymph protein clearance, peripheral blood leukocytes count, and lung water content in sheep infused with *E. coli* endotoxin.

**MATERIALS AND METHODS**

Eighteen sheep (63.5 ± 4.56 kg) were fasted overnight, but permitted free access to water. Anesthesia was induced with thiamylal sodium (Surital) (35 mg/kg i.v.) and maintained with the same anesthetic as required. The animals were placed in left lateral decubitus position and mechanically ventilated with ambient air via endotracheal tube with a Harvard respiratory pump. Tidal volume and respiratory rate were adjusted as required during the experimental period so that arterial blood gases remained within the physiological range. Under fluoroscopic guidance, 8 F radiopaque catheters were positioned in the left ventricle, thoracic aorta, pulmonary artery, and superior vena cava via carotid arteries and jugular veins, respectively. Large-bore polyethylene catheters were inserted in the femoral artery and vein. Patency of the catheters was ensured by flushing with diluted heparin in 0.9% NaCl (5 U/mL). In 14 sheep, right thoracotomy was performed, through which the effluent duct from the caudal mediastinal lymph node was catheterized and the tail of that node ligated in 12 animals.

Left ventricular, pulmonary arterial, and aortic pressures were measured with Statham (P23Db) transducers and monitored on a DR-8 Electronics for Medicine recorder (White Plains, NY), as was the electrocardiogram (EGK). These parameters were monitored constantly and recordings were made at specified time intervals. The cardiac output was measured by the dye dilution method (indocyanine green [Cardiogreen], Hyntton, Westcott & Dunning, Inc., Baltimore, MD) with a Lexington cardiac computer (Model RDL, Lexington Instruments Corp., Waltham, MA).

Arterial blood gases and pH were measured with a Radiometer ABL-3, and hematocrit level was determined using heparinized capillary tubes. Initially, leukocyte count and differential were performed with the Coulter and the hemocytometer. However, the results obtained with the hemocytometer showed substantial variation. Thus, presented results are those obtained with the Coulter. This part of the study was performed in a certified clinical pathology laboratory. Lymph and plasma protein levels were determined with a digital refractometer (Digital Refractometer, PR-I, ATACO Co. Ltd., Tokyo, Japan).

Endotoxin (*E. coli*, batch 0111:84) purchased from Difco laboratories (Detroit, MI) was dissolved in 0.9% NaCl. Fructose-1,6-diphosphate sodium salt (Esafosfina) was obtained from Biomedica Foscam SPA (Rome, Italy). Sodium thiamylal (Surital), heparin (Upjohn, Kalamazo, MI), 10% dextrose and 0.9% NaCl (Trocenor; Baxter, Deerfield, IL), and Cardiogreen were obtained from the hospital pharmacy. The experimental procedure was as follows. After the surgical preparation, the animals were heparinized intravenously (50 U/kg), and 2 h later received a supplemental dose (25 U/kg). All sheep then received endotoxin, 5 μg/kg i.v. infusion over 30 min. Along with the endotoxin, half of the animals were randomly selected to receive a slow i.v. bolus (50 mg/kg) of 10% FDP, followed by a constant infusion of 5 mg · kg⁻¹ · min⁻¹ throughout the experimental period. The other animals received the same volume i.v. bolus and constant infusion of 10% dextrose in 0.9% NaCl. Hemodynamic parameters, arterial oxygen tension, hematocrit, and lung lymph protein clearance were measured at the time intervals indicated below (Results). After 4 h of endotoxemia, the sheep were euthanized with saturated KCl. The major blood vessels were cut, and the lungs were inflated and exsanguinated, freed from the airways, and weighed. Tissue samples were taken from the upper and lower lobes, peripherally and anteriorly near the hilum (≥6 from each animal); weighed on a Sartorius research balance (R180D) to the nearest 0.1 mg; and placed in the drying system, through which dry air under partial vacuum at 60°C was constantly circulated. The samples were weighed after 48 h and every 12 h thereafter until the weight was constant in two consecutive determinations. The ratio of wet to dry lung weight (wet/dry ratio) served as an index of pulmonary edema. Despite the fact that this method does not account for differences in the intravascular blood content of the lungs, studies suggest that it is a good indicator for pulmonary edema [11, 25–27]. The wet/dry ratios in the glucose group were similar to that reported for untreated sheep injected with *E. coli* endotoxin [28]. It should be pointed out that in two FDP-treated and two dextrose-treated sheep, the experiments were performed without opening the thorax. We did encounter difficulty with lymph collection. In two animals, the catheters dislodged and could not be repositioned, and in one animal the lymphatic vessel was perforated during the experiment. In two sheep, the attempt to catheterize the lymphatic vessel was unsuccessful. The lymph data presented are for four FDP-treated and five control sheep.

These studies were conducted according to the National Institutes of Health policy guidelines on animal care, and the protocol was approved by the Institutional Animal Care and Use Committee.

**Statistics**

For each measured parameter during the control and experimental periods, the means ± SEM were calculated. We analyzed the data with a model that takes into consideration the fact that the repeated observations on the same animal are correlated.

SAS PROC MIXED (SAS Institute Inc., Cary, NC) was applied in the analysis of the repeated measures data set. The models include fixed effects of treatment and time and their interaction and random effects in lab animals. We compared various covariance structures, including compound symmetry, unstructured, first-order autoregressive, and spatial power covariance. This last structure gave the best fit for the relationship of the repeated observations within animals.

Although a comparison between groups of the different time points was a hypothesis of interest, we recognize that there were many comparisons and caution the reader that we are reporting unadjusted *P* values.

Differences between and within groups for some data (such as the wet/dry lung ratio hematocrit) were detected by paired or unpaired two-tailed Student’s *t*-test as appropriate. Values of *P* < 0.05 were considered significant for both methods used in the data analysis.

**RESULTS**

There were no significant differences between the groups in any parameters measured before infusion of the endotoxin. During the administration of endotoxin, pulmonary artery pressure (PAP) increased substantially in both groups (Fig. 1). After the endotoxin infusion was completed in the FDP group, PAP declined until the end of the study. In contrast, PAP continued to increase progressively in the control group (Fig. 1). The pulmonary vascular resistance (PVR) response to endotoxin was somewhat delayed, but it followed the same pattern as that of PAP (Fig. 2). Left ventricular end diastolic pressure (LVEDP) remained unchanged in both groups (Fig. 3). In both groups, there was a significant decrease in mean arterial pressure (MAP) for approximately 1.5 h after endotoxin infusion was started; MAP remained unchanged thereafter (Fig. 3). Cardiac output (CO) increased significantly in both groups after endotoxin infusion, but began to decline after t = 90 min (Fig. 3). The other systemic hemody-
namic data are also presented in Fig. 3. Pulmonary function, as assessed by determination of arterial oxygen tension, did not differ significantly between the two groups during the first 3 h. Throughout this period, arterial PO$_2$ ranged from 86 to 95 mmHg in both groups. At $t = 3.5$ and 4 h, PO$_2$ was lower in the control group ($74 \pm 5.9$ and $73 \pm 6.4$ mmHg), whereas there was a small increase in the FDP group ($104 \pm 10.1$ mmHg [$P < 0.25$] and $98 \pm 7.9$ mmHg [$P < 0.02$], respectively). At $t = 4$ h, arterial pH was lower in the FDP group ($P < 0.03$) Although initially some hemoconcentration was noted in both groups; later the hematocrit was higher in the control group ($t = 0$ versus $t = 4$ h at 4 h ($P < 0.005$)). Lung lymph protein clearance was significantly higher in the control group ($P < 0.0005$).

**DISCUSSION**

This investigation describes the effects of FDP on sheep infused with endotoxin. The sheep all appeared healthy before the study, were kept in the same environment, and were of comparable body weight (glucose group 63.4 ± 4.36 kg; FDP group 63.9 ± 4.76 kg). The baseline values of all measured parameters were similar in both groups.

Reports in the literature have consistently emphasized the opinion that noncardiogenic pulmonary edema in humans is a frequent complication in Gram-negative sepsis [28–30]. Data in support of this opinion have been
derived from animals injected with endotoxins, bacteria, and suspected mediators that produce changes in the lung similar to those observed in human disease [1–4, 12, 26, 27, 31–34].

Although the precise mechanisms by which endotoxin or sepsis induces lung injury are not well understood, studies establish a reasonable theoretical basis for the mediation of such injury by activated leukocytes [12–15]. Activated neutrophils, which sequestrate in the lungs, can release a number of deleterious agents capable of causing microvascular injury (i.e., lysosomal enzymes and oxygen free radicals) [12–15]. Entrapped in the lungs, the activated neutrophils generate oxyradicals that in turn alter the permeability of the microvasculature to plasma protein. Thus, the target cells in endotoxin-induced lung injury are postulated to be neutrophils. Experimental models using oxyradical-generating systems or conditions do produce pulmonary injury. It is of particular importance to emphasize that interventions, such as oxygen free radical scavengers or inhibitors and leukocyte depletion, attenuate or prevent formation of noncardiogenic edema [35–38]. In the present study, there was a marked decline in leukocyte counts in the peripheral blood in both groups during and after endotoxin infusion. This observation indicates acute trapping of leukocytes in some organs (most probably in the lung); after t = 90 min, however, leukocyte counts in the FDP group began to increase and by the end of the study approached control values (control versus t = 4 h NS, paired). In the control group, leukopenia persisted throughout the study (control versus 4 h, P < 0.02, paired). This observation may be explained by several recognized actions of FDP. FDP is reported to attenuate leukocyte adherences in postischemic reperfused tissues [39]. Furthermore, it has been effective in attenuating postischemic reperfusion injury in kidney, intestine, and brain tissue [40–42]. As in all of these conditions, neutrophils contributed further to the development of additional tissue injury by the generation of oxyradicals and release of a number of deleterious agents, such as thromboxane and lysosomal enzymes. In vitro, FDP has also been reported to inhibit the respiratory burst of canine and human stimulated neutrophils and therefore the generation of free radicals [16–18]. Based on these observations, it is reasonable to assume that such a mechanism is in part responsible for FDPs attenuation of pulmonary vascular injury during endotoxia in sheep.

In addition to the involvement of the neutrophils in endotoxin-induced lung injury in sheep, the important role of the macrophages in the process has recently been emphasized [20, 21]. The characteristic pulmonary hypertension and increased microvascular permeability to endotoxin are abrogated if the animals are subjected to intravascular macrophage depletion or administration of detergent [20].

Whether FDP attenuated the synthesis and the action of proinflammatory mediators such as TNF-α or IL-1 in the present study is open to speculation. Tamaki et al. [24] demonstrated that FDP not only preserved phagocytosis, but also inhibited secretion of TNF-α and IL-1β and production of nitric oxide. However, evidence exists that FDP can modify the activity of the immune cells [22]. In vitro studies have shown that T lymphocyte proliferation and IL-2 expression are inhibited by FDP, whereas in vivo studies alone and in combination with CyA have reported prolonged cardiac allograft survival [22]. Interestingly, FDP inhibits IL-1 and IL-6 and regulates NFkB and AP-1 [23]. It is possible that FDP modified the behavior of the pulmonary macrophages in the present study.

**FIG. 4.** Effect of endotoxin infusion on lung protein clearance. The protein clearance is expressed as the ratio of the experimental to the baseline protein clearance (protein clearance/baseline clearance). (■), FDP (n = 4); (●), control (n = 5). *P < 0.006, **P < 0.0005 for the differences between the groups.

**FIG. 5.** The response of leukocytes (WBC [counts/μL]) in sheep to endotoxin. (■), FDP (n = 8); (●), control (n = 8). *P < 0.05, **P < 0.02, ***P < 0.005 for differences between the groups.
FDP is a naturally occurring high-energy intracellular metabolite that is intimately linked to the regulation of many metabolic pathways [43]. The use of FDP to enhance energy production from the glycolytic pathway in ischemic and hypoperfusion states has been addressed in a number of studies [5–8, 10]. This agent has been shown to have a salutary effect in renal, intestinal, and brain ischemia [40–42]. FDP treatment of animals subjected to endotoxin, hemorrhagic, traumatic, and compound 48/80-induced shock resulted in significantly reduced mortality [5–8]. In patients with ARDS as a complication of sepsis, trauma, gastric aspiration, and other conditions, FDP treatment has been shown to improve pulmonary function and pulmonary and systemic hemodynamics [10].

Various agents have been used as experimental tools in the investigation of the pathogenesis of permeable pulmonary edema; ANTU is one such agent [32]. The well-known efficacy of this compound in inducing acute respiratory failure resulting from injury to the lungs in animals has become useful in an animal model for the study of pulmonary microvascular injury and in screening for therapeutic interventions [33, 34].

In a recent study, FDP was shown to prevent ANTU-induced pulmonary edema in dogs [11]. It is important to note that treatment in that study was initiated 30 min after the administration of the pneumotoxic agent (ANTU). In a similar study, Martin et al. [44] reported that administration of FDP immediately after administration of a single dose of ANTU (5 mg/kg) afforded pulmonary protection in half of the dogs in the study. The partial protection in this study could be attributed to the dosage of FDP used. In the previously described study [11], the dogs received a total of 600 mg/kg, whereas in the Martin et al. [44] experiments, the dogs received a total of 11.25 mg/kg of FDP. Such a large discrepancy in the FDP dosages could account for the partial lung protection observed [44].

The endotoxin infusion in the present study did not affect systemic hemodynamics to the same degree in the control group as observed in canine endotoxin shock [5]. The differences in species and endotoxin dosage might account for this discrepancy. In the present study, 5 µg/kg was infused, whereas the endotoxin dose in the canine experiments was 1 mg/kg [5]. PAP and PVR were similar in the two groups at 90 min of endotoxin, but after 2-4 h, during the permeability phase, there was an increasing difference between the two groups. In the control group, the changes in PAP and PAR followed a parallel course characterized by continuous increase, which attained significance by 1.5 and 2.5 h, respectively. The results are similar to those reported by other investigators [2–4, 26]. Our data and those reported by others show that endotoxin causes pulmonary hypertension, but the mechanism has not been defined. Therefore, we cannot state that FDP has a vasodilating effect, because in sham-operated animals (dogs), it has no effect on pulmonary pressure and resistance [11]. Does FDP antagonize the effect of endotoxin? This premise is unlikely, because PAP increased during infusion in both groups, and leukocyte blood count decreased in the same manner. Therefore, it is speculative to assume that FDP might have antagonized the endotoxin effect, because clinical observations indicate that FDP improved pulmonary hemodynamics and function when noncardiogenic edema, as a result of sepsis, was already present [10].

Another beneficial effect of FDP observed in this model was the significantly attenuated lung lymph protein clearance at physiological left atrial pressures. Also, the wet/dry ratio of the lungs was lower in the FDP-treated animals than in the controls. These observations support the argument that the integrity of the pulmonary microvasculature was possibly protected by FDP from the noxious agents released by activated neutrophils [12–15]. Further, FDP might have provided additional lung protection by down-regulating cytokine expression and secretion (e.g., TNF-α, IL-1β and IL-6) [23, 24]. FDP prevented alteration of pulmonary hemodynamics, decreased lung lymph protein clearance, and progressively increased the peripheral blood leukocyte counts in sheep infused with endotoxin. These results indicate that FDP may act by decreasing pulmonary microvascular permeability. Although the precise mechanism by which FDP prevented endotoxin-induced pulmonary edema is unknown, we cautiously postulate that this pulmonary protection was achieved, at least in part, by inhibiting the generation of oxynitrals by the neutrophils.

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