Effect of inhaled endotoxin on cardiopulmonary function and E-selectin expression in pigs

Gabrielle Landolt, BVSc, MS; Brett W. Nemke, BS; Benjamin J. Darlen, DVM, MS; Kris T. Kruse-Elliott, DVM, PhD

Objective—To evaluate the effect of controlled exposure to inhaled lipopolysaccharides (LPS) on the pulmonary inflammatory response of anesthetized pigs.

Animals—Forty-seven 8- to 12-week-old domestic pigs.

Procedure—Pigs were anesthetized with pentobarbital, instrumented for measurement of cardiopulmonary function, and randomly assigned to receive saline (0.9% NaCl) solution or 0.25, 0.5, or 1.0 µg of LPS/kg/h for 2 or 6 hours via nebulization through the endotracheal tube. Cardiopulmonary variables were measured, ex vivo neutrophil superoxide production determined, and postmortem assessment for pulmonary neutrophil influx and modulation of adhesion molecule (E-selectin) expression was done.

Results—Mild changes in cardiopulmonary function were observed in response to inhaled LPS in the 2- and 6-hour groups. In pigs inhaling LPS (0.5 or 1.0 µg/kg/h) for 6 hours, there was significant pulmonary neutrophil influx observed postmortem. An increase in expression of E-selectin on pulmonary endothelial cells after 6 hours of LPS inhalation (0.5 µg/kg/h) was also observed. In contrast, there was no significant influx of neutrophils or expression of E-selectin in lungs from pigs inhaling LPS for 2 hours.

Conclusions and Clinical Relevance—Inhalation of LPS resulted in localized pulmonary inflammation characterized by neutrophil influx and increased expression of the endothelial cell adhesion molecule, E-selectin. It may be possible to relate our experimental findings to the clinical consequences of airborne LPS exposure in swine confinement facilities. (Am J Vet Res 2002;63:1302–1308)

Respiratory disease, particularly viral and bacterial infection, is an important cause of economic loss in swine production. The incidence of pneumonia and pleuritis has been correlated to presence of airborne contaminants in swine confinement operations. These contaminants include feed and fecal dust, microbes, molds, and bacterial lipopolysaccharides (LPS) and peptidoglycans. Although the specific role of each of these contaminants is unknown, airborne endotoxin is believed to contribute importantly to the development of respiratory disease in pigs.

Airborne bacterial endotoxins such as LPS are found in a variety of environments. Biologically important amounts have been found in cotton and flax dust, swine production facilities, poultry farms, buildings with contaminated air humidifiers, and cigarette smoke. In small laboratory animals (mice, rats, guinea pigs) and human subjects, inhaled endotoxin causes pulmonary inflammation characterized by increases in pro-inflammatory cytokines (interleukin-8, tumor necrosis factor-α) and influx of neutrophils into alveolar spaces. Recruited leukocytes and their associated inflammatory biochemicals may then contribute to pulmonary pathologic changes and clinical disease.

Pathophysiologic, immunologic, and inflammatory responses to bacterial and viral pathogens are well described for pigs, particularly during systemic exposure. In contrast, the specific effects and inflammatory mechanisms involved in the pulmonary response of pigs to airborne environmental contaminants are poorly understood. However, exposure to airborne microbes, molds, LPS, and peptidoglycans are postulated to contribute to development of pulmonary disease in pigs as well as humans working in swine confinement facilities.

Several past investigations have evaluated the effect of inhaled LPS on the porcine respiratory system. Results of chamber exposures for several days have shown a distinct pulmonary inflammatory response characterized primarily by an increase in bronchoalveolar lavage leukocyte and neutrophil numbers. The inflammatory response to dust or LPS included cosinophilia. Additionally, when LPS was nebulized via face mask to pigs, there was evidence of an increased in vitro bronchial reactivity. These previous investigations did not demonstrate a significant difference between the effect of inhalation of feed dust and inhalation of LPS. Thus, the specific cellular and biochemical inflammatory effects of inhaled LPS on the porcine lung remain to be determined.

Leukocytes, particularly neutrophils, are important to the immune and inflammatory response of lungs to circulating or inhaled pathogens or toxins. Successful recruitment of neutrophils to tissues is dependent on early adhesive interactions between circulating neutrophils and the endothelium. These events are mediated by a number of cell-surface molecules on endothelium and leukocytes. Selectin-mediated margination and rolling of leukocytes along the endothelium is the initial step in leukocyte recruitment, followed by firm adhesion with transmigration into the tissue as the final step. Endothelial cells constitutively synthesize and store P-selectin, which is then rapidly and briefly
expressed on the cell surface in response to a variety of mediators. In contrast to P-selectin, E-selectin expression, in response to cytokines or LPS, requires 4 to 6 hours to reach a maximum, and continues for up to 24 hours. This study was designed to test the hypothesis that controlled exposure to inhaled LPS causes a measurable inflammatory response localized to the lungs in exposed pigs. When LPS is administered IV to pigs, they may develop a shock syndrome characterized by failing cardiac output, systemic hypotension, pulmonary hypertension, profound hypoxemia, and a high mortality rate. In contrast to this, we hypothesized that inhalation of LPS in pigs would not be associated with clinically important adverse systemic effects. Furthermore, because expression of E-selectin helps modulate neutrophil influx to sites of inflammation, we hypothesized that pulmonary neutrophil infiltration and increased expression of E-selectin would be the predominant pulmonary inflammatory response observed following LPS inhalation in pigs.

Materials and Methods

Animal preparation—The Institutional Animal Care and Use Committee of the University of Wisconsin approved experimental protocols. Forty-seven 8- to 12-week-old domestic pigs weighing 17.2 ± 0.3 kg (mean ± SEM) were used in our study. Pigs were anesthetized (25 mg/kg, IV) and maintained under anesthesia (6 to 8 mg/kg/h, IV) with sodium pentobarbital. They were monitored and instrumented as previously described. Briefly, pigs were placed in supine position and mechanically ventilated with room air via tracheostomy at a constant tidal volume (10 mL/kg) and rate (12 to 16 breaths/min, adjusted initially to maintain end-tidal PCO2 of 33 to 35 mm Hg). Catheters in the airway (for measuring intratracheal pressure [Pt]), pulmonary artery (for measuring mean pulmonary arterial pressure [Ppa] and pulmonary arterial wedge pressure), and femoral artery (for measurement of mean aortic pressure [Pma]) were connected to a multichannel recorder. Cardiac output was measured by the thermal dilution technique by use of a cardiac output computer, normalized for body weight, and expressed as cardiac index (CI, mL/kg/min).

Pigs were anesthetized and instrumented as already described, and a loading dose of Lactated Ringer’s solution (10 mL/kg, IV) was administered, followed by a continuous infusion of 10 mL/kg/h for the remainder of the experiment. Pigs were allowed a minimum of 30 minutes to stabilize following instrumentation and fluid loading (ie, until the measured physiologic variables were considered to be within reference range limits).

At the start of the experiment, a nebulizer, which generates particles that usually measure 2 to 3 μm in diameter, was connected to the ventilator. Saline (0.9% NaCl) or LPS solution was aerosolized with a compressor using room air. Airflow was kept constant at 2 L/min throughout the experiment.

Arterial gas tensions were measured every 2 hours (beginning at baseline 0) by use of a blood gas machine, and the values were corrected to blood temperature. Room-air alveolar-arterial oxygen gradient (AaDO2) was calculated. For measurement of superoxide anion (SO) biosynthesis in response to phorbol myristate acetate (PMA) stimulation, neutrophils were isolated over a Percoll gradient by use of blood samples collected into sodium citrate (0.38% final concentration) at 0, 2, 4, and 6 hours. Peripheral blood neutrophils were suspended at a final concentration of 4.5 × 109 cells/ml. The PMA-induced (1 μg/ml) SO release was assessed by measuring the velocity of reduction of cytochrome c at 550 nm in a microplate reader at 37 °C for a 50 minute period. Changes in optical density were converted to molar concentrations by use of an extinction coefficient of 18.5 × 103 L/mole/cm. For measurement of pulmonary arterial pressure [Ppa] and pulmonary artery (for measuring mean pressure [Pa]), the left branch of the pulmonary artery was cannulated with a 12-F Foley catheter, and the lobe was perfused with 1 L of ice-cold saline solution containing 10 U heparin/mL. The major bronchi were dissected away (leaving primarily lung parenchyma), the lung tissue weighed and ground in ice-cold saline solution, and an aliquot stored at −20 °C until further analysis. The MPO activity was determined spectrophotometrically by measuring oxidation of 3,3',5,5'-tetramethylbenzidine in a microplate reader. Changes in absorbance were converted to nmol of H2O2 consumed/min/g wet lung tissue by use of an extinction coefficient of 3.9 × 105 L/mole/cm.

Lung tissue specimens were taken from 3 standardized locations on the right side (cranial, middle, and caudal). Tissues were fixed in buffered 4% formalin and embedded in paraffin. The formalin concentration was initially selected to facilitate dual use of these specimens for standard histologic analysis and immunohistochemistry, however we ultimately elected to use frozen lung tissue for the latter. Embedded lung tissues were cut into 6-μm-thick sections and stained with HE. Histologic sections from the cranial, middle, and caudal areas of the right diaphragmatic lobe were examined. Neutrophil counts were obtained at 1,000× magnification. In each section, 3 microscopic fields selected at random were examined. Because there was no difference between the 3 regions examined, the total for each pig was determined and expressed as total neutrophil count.

Sections of the intermediate lung lobe, skin, liver, and right kidney were embedded in tissue freezing medium. They were immediately frozen in liquid nitrogen and stored at −80 °C until further processing for E-selectin immunohistochemistry. Tissue blocks were cut into 6-to-8-μm-thick cryostat sections and mounted on glass slides. Tissue sections were air dried at room temperature (18 to 20 °C) for 24 hours. Prior to staining, slides were rehydrated in PBS solution. Staining was performed by use of a staining kit, using instructions and reagents recommended by the manufacturer. With the exception of the overnight step, which was performed at 4 °C, all incubations were performed at room temperature (18 to 20 °C). Following rehydration, tissue sections were incubated with serum blocking solution (nonimmune goat serum) for 10 minutes. Slides were subsequently incubated overnight with the primary anti-CD62E monoclonal antibody. After washing in PBS solution, tissue sections were incubated with biotinylated secondary antibody for 10 minutes, and washed in PBS solution, followed by a 10 minute incubation with the enzyme conjugate. Finally, slides were developed with diaminobenzidine chromogen solution and counter stained in hematoxylin prior to mounting. Each staining procedure contained an isotype-matched negative-control antibody. Sections of porcine skin were used as positive control for the antigen. A semiquantitative estimation of the number of positive staining
blood vessels was made for each tissue section. Sections were examined under medium (100X magnification) power, and the number of regions containing vessels with surface-specific positive staining were categorically graded as absent (0), low (1), medium (2), or high (3).

Experimental design—Pigs were randomly assigned to be nebulized for 2 hours with saline (control) or LPS (from *Escherichia coli*) solution at a dose of 0.25, 0.5, or 1 µg/kg/h (n = 6, 8, 6, and 5, respectively); or to be nebulized for 6 hours with saline solution or LPS at 0.25, 0.5, or 1 µg/kg/h (6, 5, 7, and 4, respectively). Given the complexity and duration of these experiments, only 1 experiment (ie, use of 1 pig) was done per day, hence we were careful to randomize treatment group assignment. There were no significant differences between groups with regard to body weight or sex.

Statistical analysis—All results are expressed as mean ± SEM. Data within and between treatment groups were analyzed by 2-way and 1-way ANOVA, respectively. When F was significant (P < 0.05), further differences between means were determined by use of the Tukey Ω procedure. Means were considered significantly different at P < 0.05.

Results

Effects of endotoxin inhalation on cardiopulmonary function—Mild changes in cardiopulmonary variables were observed in pigs treated for 2 hours with inhaled LPS (data not shown). These included small but significant decreases in Pma, systemic vascular resistance, mean Ppa, and pulmonary vascular resistance. In general, these changes were duplicated during the first 2 hours of the experiment in pigs that received 6-hour inhalation treatment. Therefore, only cardiopulmonary data from pigs that received 6-hour inhalation treatment are presented here.

Generally mild-to-moderate alterations in cardiopulmonary function were observed in the 6-hour inhalation treatment groups. There was a significant decrease in CI from baseline values in pigs treated with 0.25 and 0.5 µg of LPS/kg/h at 6 hours and at 2.5, 3.5, and 4.5 to 6 hours, respectively. No significant changes in CI were observed in pigs treated with 1.0 µg of LPS/kg/h. Changes in CI were not associated with parallel alterations in stroke volume (Fig 1).

Mean aortic pressure declined from baseline values at 1.5 to 6 hours in the control group. In all pigs treated with LPS, Pma decreased significantly from baseline values during the latter half of the 6-hour experiment. There were no significant differences between any of the treatment groups at any time. Systemic vascular resistance did not change significantly from baseline.
values with the exception of a decrease at 4 hours in control pigs (Fig 2).

At baseline (0 hour), pigs treated with 1.0 µg of LPS/kg/h had an unexplained, significantly greater mean Ppa compared with control pigs. The mean Ppa was significantly increased above baseline values at 6 hours in control pigs and pigs treated with 0.25 and 0.5 µg of LPS/kg/h. Pigs treated with 1.0 µg of LPS/kg/h had a mean Ppa that was significantly greater than for control pigs from 5.0 to 5.5 hours. Increases in pulmonary vascular resistance were observed at 6 hours and at 4 to 6 hours in pigs treated with 0.25 and 0.5 µg of LPS/kg/h, respectively (Fig 3).

Mean baseline values for Pt were not significantly different between groups (9.7 ± 0.9, 8.6 ± 1.0, 9.6 ± 0.4, and 9.2 ± 0.8 cm H2O in control pigs and pigs treated with 0.25, 0.5, and 1.0 µg of LPS/kg/h, respectively). At 6 hours, Pt measured 10.8 ± 0.8, 10.5 ± 0.9, 12.4 ± 0.7, and 13.0 ± 1.2 cm H2O in control pigs and pigs treated with 0.25, 0.5, and 1.0 µg of LPS/kg/h, respectively. The only significant difference from baseline was for pigs treated with 0.5 µg of LPS/kg/h.

Baseline values for PaO2 and AaDO2 were similar among the 4 treatment groups (Fig 4, respectively). At 6 hours, pigs nebulized with 0.25, 0.5, or 1.0 µg of LPS/kg/h had hypoxemia associated with a significant increase in AaDO2.

Effects of endotoxin inhalation (for 2 or 6 hours) on neutrophil function and E-selectin expression—Baseline values for ex vivo SO release from isolated neutrophils were similar among the four 2-hour treatment groups (0.25 ± 0.06, 0.38 ± 0.06, 0.26 ± 0.02, and 0.39 ± 0.08 nmol/106 cells/min for control pigs and pigs treated with 0.25, 0.5, and 1.0 µg of LPS/kg/h, respectively). At 2 hours, SO release (0.20 ± 0.03, 0.36 ± 0.04, 0.25 ± 0.03, 0.34 ± 0.09 nmol/106 cells/min for control pigs and pigs treated with 0.25, 0.5, and 1.0 µg of LPS/kg/h, respectively) did not differ from baseline values in any treatment group. Similarly, there were no significant changes in ex vivo SO production from 0 to 6 hours in neutrophils isolated from pigs in the 6-hour inhalation treatment groups, and no differences between groups (Fig 5).

Postmortem analysis of lung tissue sections revealed that neutrophil numbers within the lung tissue were not significantly different between groups undergoing the 2-hour treatments (Table 1). In contrast, sig-
significant neutrophil infiltration into pulmonary parenchyma was observed in pigs nebulized for 6 hours with 0.5 and 1.0 µg of LPS/kg/h. In general, neutrophil infiltration was most pronounced around the bronchi.

Porcine skin was used as a positive control tissue for E-selectin staining. Immunoperoxidase staining of porcine skin with the CD62E monoclonal antibody for E-selectin was easily demonstrated as compared with no staining observed with the isotype control. We did not observe any significant change (compared with controls) in E-selectin expression in porcine lung tissue from pigs in any of the 2-hour treatment groups (Table 1). However, expression of E-selectin in porcine lung tissue was significantly increased in pigs inhaling 0.5 µg of LPS/kg/h for 6 hours, compared with time matched for control pigs. The apparent increased E-selectin expression in pigs inhaling 1.0 µg of LPS/kg/h for 6 hours was not significant. Hepatic or renal E-selectin expression was not observed in any treatment groups.

There were no significant differences in lung MPO activity between any of the 2-hour treatment groups (Table 1). An unexpected finding was no evidence of a significant effect of LPS inhalation on pulmonary MPO activity in the 6-hour treatment groups. Similarly, we did not observe any differences in hepatic MPO activity between any of the treatment groups at either time.

Discussion

Our study was designed to test the hypothesis that pulmonary morphologic changes and adhesion molecule expression occur during inhalation of LPS in pigs with only minimal changes in cardiopulmonary function. Although we did observe significant alterations in several measured cardiopulmonary variables during inhalation of LPS, these were mild when compared with past observations of the effect of systemic endotoxemia in a similar porcine model. In addition, the mild cardiopulmonary changes observed in control pigs in our investigation were similar to previous studies that used IV administration of saline solution as a control.

Intravenous administration of LPS for 6 hours leads to severe cardiopulmonary dysfunction characterized by dramatic reductions in cardiac stroke volume, pulmonary hypertension, vasoconstriction, bronchoconstriction, hypoxemia, and up to 40% early mortality. In contrast, the physiologic response to inhaled LPS was not only mild but was also clearly localized to the lungs. These alterations coincided with morphologic changes (eg, pulmonary neutrophil influx). Several previous studies in other species have demonstrated that inhalation of LPS leads to an increase in airway responsiveness and neutrophil influx into the lungs.

Although we observed some mild increases in pulmonary vasoconstriction, we did not observe any differences in peak airway pressures in our study, and whether inhalation of LPS induces an alteration in airway responsiveness in pigs remains to be determined.

Airborne endotoxin-containing microorganisms are ubiquitous, especially in environmental and occupational dusts, and pose a potential source of pulmonary inflammation on inhalation. Reported concentrations of LPS contaminating the aerosol of swine confinement buildings are generally in the range of 10 to 100 ng/m³, although up to 10-fold higher concentrations have occasionally been observed. In pigs that were exposed to nasally nebulized LPS (doses up to 400 µg/kg) for 30 minutes, the pulmonary inflammatory response was similar to what we observed in our investigation were similar to previous studies that used IV administration of LPS at a similar dose.

The neutrophil inflammatory response observed in our investigation was similar to what we observed in our study.
to those that have been observed in lungs collected at slaughter or in pigs exposed to LPS in an environmental chamber. However, it is important to keep in mind that the aerosol environment in a swine confinement facility includes a mixture of agents besides LPS that may also affect the respiratory tract. The specific and interactive role of these various airborne contaminants in the porcine pulmonary inflammatory response remains to be determined in future investigations.

Xing et al demonstrated that alveolar macrophages may play an important role in initiating a neutrophilic response via expression of cytokines, such as tumor necrosis factor-α and interleukin-1. Macrophages and neutrophils are induced to produce reactive oxygen intermediates and proinflammatory cytokines (tumor necrosis factor-α, interleukin-6) by LPS. Furthermore, LPS also stimulates bronchial epithelial cells to release chemotactic factors for neutrophils and upregulate E-selectin expression. Expression of E-selectin may then contribute to neutrophil sequestration in the pulmonary microcirculation with subsequent infiltration and transmigration. Indeed, we observed increased expression of E-selectin that coincided with pulmonary neutrophil infiltration in pigs that inhaled LPS for 6 hours. Expression of E-selectin generally peaks at 4 to 6 hours after the initial stimulus. This may further explain our results for pigs that received 2 hour LPS inhalation treatment, where neither E-selectin expression nor pulmonary neutrophil infiltration were demonstrated.

Successful recruitment of leukocytes to sites of tissue injury is dependent on a regulated interaction between the endothelium and circulating leukocytes. This interaction is mediated through the expression of various cell adhesion molecules. The selectins mediate the first adhesive step, which is characterized by tethering and rolling of leukocytes on activated endothelial cells, platelets, or other leukocytes. Given that E-selectin is expressed on endothelial cells after activation by LPS, subsequent neutrophil-derived inflammatory responses (eg, adhesion and extravasation of neutrophils) may be dependent on previously upregulated expression of E-selectin. Our results indicate that E-selectin is expressed in the pulmonary vasculature of pigs following a 6-hour exposure to nebulized LPS. Because Keelan et al have shown that IV infusion of interleukin-1 induces E-selectin expression in many organs, we speculated that some of the inhaled LPS (especially at the higher doses) would be absorbed over the bronchial and alveolar epithelium and might induce upregulation of E-selectin, indicative of inflammation, in organs other than the lungs. Examination of sections of the liver and kidney revealed no positive immunostaining for E-selectin. This failure to demonstrate E-selectin expression in other organs implies that the effect of inhaled LPS in our model was localized to the lung and not the result of circulating LPS. It is also possible that LPS did not enter the systemic circulation at a concentration adequate to stimulate measurable E-selectin expression in these organs.

Superoxide release by neutrophils is an important activation response of these cells during inflammation. The capacity for SO release can be upregulated, or primed, by prior exposure to various stimuli. We have previously demonstrated this ability in porcine neutrophils from endotoxemic pigs by use of the same ex vivo PMA stimulation assay. Pretreatment of porcine neutrophils with the lipid-derived inflammatory mediator, platelet-activating factor, enhanced release of SO in response to stimulation with phorbol ester in vitro. Additionally, priming of neutrophils by LPS has been demonstrated by use of human neutrophils in an in vitro model. We have also recently demonstrated that porcine neutrophils exposed to LPS in vivo during IV administration of LPS exhibit increased ex vivo release of SO in response to PMA. This indicates an effect of LPS exposure on circulating neutrophils. In contrast, in this investigation, we failed to demonstrate any alteration in PMA-induced SO release ex vivo from neutrophils isolated from pigs following LPS inhalation. This provides further support for the concept that inflammatory effects of inhaled LPS in our model were localized to the lungs.

Previously, we have observed increased MPO activity in lung tissue from pigs given LPS IV. Pulmonary MPO activity serves as an index of neutrophil influx into the lungs. However, we did not observe significant changes in pulmonary MPO activity in response to LPS inhalation in our study presented here. This is despite histologic evidence of pulmonary neutrophil influx. In retrospect, these results are not surprising given that most of the pulmonary neutrophil infiltration we observed was around the bronchi, and processing of tissue for MPO activity assay requires dissection of the bronchi away from the parenchyma. Therefore, our MPO activity assay only accounted for parenchymal neutrophils.

Results of our study demonstrate the novel finding that inhalation of LPS in pigs leads to upregulation of E-selectin, which is accompanied by pulmonary neutrophil infiltration. Furthermore, our results have shown a measurable local response to LPS inhalation in pigs that can be shown at the cellular and biochemical level; for example, neutrophil infiltration and E-selectin expression. Expression of E-selectin during exposure to airborne endotoxin might be part of a priming response and lead to an increased likelihood of leukocyte-endothelial cell interaction on subsequent secondary exposure. Interestingly, recent work has shown that sustained expression of E-selectin expression requires a continuous presence of LPS. Failure to maintain LPS exposure leads to complete disappearance of E-selectin expression by 24 to 48 hours. However, most swine production facilities have an ongoing presence of LPS in the air environment, which might cause persistent expression of E-selectin in the porcine lung. Such an upregulated pulmonary inflammatory response could contribute to the development of a more severe response to a secondary infectious agent and increased morbidity and mortality. Future studies will be needed to explore the role of such a priming response to LPS and evaluate methods to attenuate the effects of inhaled LPS in housed pigs.

Dual Phase Control Respirator, Harvard Apparatus Inc, South Natick, Mass.

AJVR, Vol 63, No. 9, September 2002
References


