Effect of endotoxin on oleic acid lung injury does not depend on priming

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Schuster, Daniel P., James K. Kozlowski, Tim McCarthy, Jason Morrow, and Alan Stephenson. Effect of endotoxin on oleic acid lung injury does not depend on priming. J Appl Physiol 91: 2047–2054, 2001.—Recent studies have demonstrated significant synergistic physiological and biochemical effects between low-dose endotoxin (Etx) administration and oleic acid (OA)-induced canine lung injury. To evaluate whether this interaction depends on Etx priming of some key cell population, we compared the effects of giving low-dose Etx both after as well as before inducing lung injury with OA. In addition to hemodynamic and blood-gas measurements, positron emission tomographic imaging was used to measure edema accumulation and intrapulmonary blood flow distribution. Biochemical measurements of the stable metabolites of prostacyclin and thromboxane were obtained as well as measurements of isoprostanes and reactive sulf-hydryls as evidence for possible concomitant oxidant production. We found that the physiological and biochemical effects of low-dose Etx developed 30–45 min after its administration, regardless of whether Etx was administered before or after OA. No increase in either isoprostane or reactive sulf-hydryl production after Etx and/or OA was detected. These data suggest that the synergistic effect of low-dose Etx and OA-induced lung injury is not due to a priming effect of Etx.

Positron emission tomography; pulmonary edema

The injection of oleic acid (OA) into the pulmonary circulation is one of the most commonly used experimental models of acute lung injury (19). The injury, at least initially, appears to be the result of a direct interaction between OA and pulmonary endothelial cell membranes; i.e., injury is not initiated by inflammatory cells or their products. Nevertheless, small doses of endotoxin (Etx), which by themselves are largely devoid of systemic or pulmonary hemodynamic effects, can markedly alter the physiological expression of lung injury after OA administration, including a failure to develop otherwise characteristic pulmonary hypertension, a failure to redistribute pulmonary blood flow away from edematous lung regions, and the development of systemic hypotension (7). These effects (i.e., effects of low-dose Etx in this model) appear to be mediated by enhanced arachidonic acid release in the presence of upregulated cyclooxygenase-2 (COX-2), which results in increased prostacyclin production (9).

This sequence of events, namely Etx administration followed by OA-induced injury, suggests a priming effect of Etx on some cell population, which then modifies the response to OA. Certainly, there is ample evidence that Etx can prime circulating inflammatory or endothelial cells, thereby greatly enhancing the production of various prostanoids (15, 23, 24, 27, 30). Whether the effects of low-dose Etx on the OA model involve priming effects on inflammatory cells (circulating or resident in the lung) or on other cell populations (e.g., the endothelium) has not been determined.

Priming has been defined as a sequence of events in which a first stimulus influences an intermediate step used by a second stimulus (13). Furthermore, the response to the two stimuli should be different from any sequential response to the same stimulus. By some definitions, the priming signal should be distinct from the second signal (13). Accordingly, we proposed that low-dose Etx (the first stimulus) can influence the regulation of the inducible form of COX-2 (the intermediate step), which, in the presence of acute lung injury (the second stimulus), results in the production of prostacyclin that far exceeds that observed in the absence of low-dose Etx. The physiological and thus clinical consequences of this sequence of events are a dramatic worsening of gas exchange and systemic hemodynamics.

By the above definition, the priming stimulus must come before the second stimulus. It would be expected, then, that reversing the order of the stimuli should alter the expression of their combined effects if priming per se is the operative mechanism. Thus, assuming that the effects of Etx in this model necessarily depend on Etx-induced priming of a critical cell population, we hypothesized that low-dose Etx administered after OA would have little to no effect on prostanoid production, gas exchange, or hemodynamics beyond that caused by OA alone.

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As part of these experiments, we also took the opportunity to more carefully characterize the temporal development of the physiological and biochemical effects of the Etx-OA interaction, including biochemical probes for evidence of oxidant production, which would potentially provide additional clues about the mechanism underlying the Etx effect.

METHODS

Animal preparation. These studies were approved by the Washington University School of Medicine Animal Studies Committee. Twenty-six healthy mongrel dogs (weight range = 18.0–22.6 kg; mean 19.8 ± 1.1 kg) were anesthetized with pentobarbital sodium (25 mg/kg) administered via a forelimb peripheral vein, intubated with no. 9 cuffed endotracheal tube (Mallinckrodt, St. Louis, MO), and ventilated with a Harvard pump respirator (Harvard Apparatus, South Natick, MA) with the following settings: fraction inspired oxygen of 1.0, tidal volume of 15 ml/kg, and rate adjusted to a normal arterial pressure. Instrumentation was performed in a sterile fashion with animals fixed in the supine position. After percutaneous insertion of bilateral femoral 8.5-Fr introducer sheaths (Baxter Healthcare, Irvine, CA), a 7.5-Fr balloon-tipped pulmonary artery catheter (Baxter) and a 110-cm 7.0-Fr pigtail catheter (Cook, Bloomington, IN) were positioned in the pulmonary artery under fluoroscopic guidance for hemodynamic monitoring and blood withdrawal, respectively. An external jugular 6.0-Fr introducer (Cook) was inserted percutaneously, and radionuclides were administered via a 5-cm length of infant feeding tube placed in this introducer sheath. A 20-gauge arterial catheter (Arrow International, Reading, PA) was percutaneously inserted into a femoral artery by using a Seldinger technique for continuous blood pressure monitoring and blood sampling. Catheter patency was maintained using intermittent heparinized saline flushes.

Cardiac output was measured by the thermodilution technique (difference of 2 successive measurements ≈5%) by using a cardiac output computer (American Edwards Laboratories, Irvine, CA). Pressure transducers (Baxter) were calibrated to the center of the lateral chest and connected to a Mennen model 742 monitor (Mennen, Clarence, NY) for monitoring of systemic and pulmonary arterial pressures and periodic wedge pressure measurement. Continuous systemic and pulmonary arterial pressures were recorded with the use of a Macintosh 165B portable computer with AcqKnowledge 3.5.5 software (BIOPAC Systems, Santa Barbara, CA). Blood gases were analyzed by using an Instrumentation Laboratories (Lexington, MA) model 1630 blood-gas analyzer. A transurethral bladder catheter was placed in all animals.

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Table 1. Interventions in the different experimental groups

<table>
<thead>
<tr>
<th>Drug Intervention</th>
<th>PET Imaging</th>
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<tr>
<td>Etx administration 0.5 h before OA (or placebo)</td>
<td>OA administration</td>
</tr>
<tr>
<td>Normal (n = 5)</td>
<td>P</td>
</tr>
<tr>
<td>Etx only (n = 6)</td>
<td>x</td>
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<tr>
<td>OA only (n = 5)</td>
<td>P</td>
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<tr>
<td>Etx→OA (n = 5)</td>
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<td>OA→Etx (n = 5)</td>
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In the OA→Etx group, the first set of positron emission tomography (PET) imaging was obtained before endotoxin (Etx) administration. OA, oleic acid; P, placebo; x, drug administration or PET imaging occurred; n = no. of dogs.

Position emission tomography techniques. Regional pulmonary blood flow and regional lung water content were measured by using positron emission tomography (PET) imaging techniques. In general, PET is used to measure the tissue concentration and distribution of a positron-emitting radionuclide, which in the present study was simply H_215O. The activity data measured with PET, when combined with blood activity (used as a reference) and analyzed with an appropriate compartmental mathematical model, yield tomographic images representative of pulmonary blood flow. In the present study, these measurements were obtained with a Siemens/CTI ECAT EXACT HR plus 962 scanner, a 63-plane positron camera with an axial sampling of 2.43 mm, isotropic resolution of 4.6 mm, and a 15 × 56-cm field of view. Because the pattern of pulmonary blood flow in normal dogs (as measured with PET imaging) is well established in this model (7–9), we only obtained pulmonary blood flow data once, ~2 h after lung injury (Table 1).

The animals were placed in the scanner with the most caudal tomographic slice about 1–2 cm below the level of the dome of the diaphragm. To improve signal-to-noise ratio of the activity measurements, we reduced the data to 21 slices (for each time frame) by combining 6 original planes to form a single transverse slice with an axial sampling of 7.1 mm. Scanner design features, methods for calibration, corrections for activity decay, corrections for photon attenuation, and supporting validation studies of our methods have been described previously (18).

Image analysis. Our methods for image analysis have also been described in detail elsewhere (21, 22). To evaluate the relationship of pulmonary blood flow to anatomic position within the lung, the pulmonary blood flow data in each image pixel are sorted by strips along a lateral-dorsal gradient. Arbitrarily, data were divided into 20 strips stacked vertically in the ventral-dorsal direction so that each strip contained 20–25 pixels, which could then be averaged. By keeping the number of strips per region and the number of tomographic slices per dog constant, we could average bin values across dogs, which allows comparisons between experimental groups. To quantify perfusion redistribution, we calculated and summed the fractional pulmonary blood flow in the six most dorsal strips. Reliably, these bins represent the edematous lung regions after OA injury (7–9). The average value for each group was compared. A significantly reduced fractional blood flow to these six image bins compared with normal, uninjured lungs represents perfusion redistribution away from the edematous lung region.

Experimental protocols. Five groups of five to six dogs each were studied (Table 1). In the normal group, animals were anesthetized and instrumented as described above, but no experimental interventions were performed. In the Etx-only group, 15 μg/kg Escherichia coli Etx (Fisher Scientific, Pittsburgh, PA) was injected into the central venous circulation.
after baseline measurements. After this, no other interventions were performed. This dose of Etx is known to inhibit perfusion redistribution during acute lung injury without significant systemic hemodynamic changes (7, 9, 26, 28). In the OA-only group, the only experimental intervention (other than placebo administration) was 0.08 ml/kg OA infused into the central venous catheter. In the Etx—OA group, Etx was injected via the central venous catheter, followed 30 min later by OA. Finally, in the OA—Etx group, OA was administered after baseline data were obtained. The animals were then watched for ~2 h, after which Etx was administered as above, and the animals were watched for ~1 h more. At the end of each study, animals were euthanized with additional pentobarbital sodium followed by 20 ml of saturated KCl. Time 0 in each group was the time of OA administration (or placebo as appropriate; Table 1).

Biochemical analyses. Blood was drawn at baseline, before each intervention (Etx or OA administration, PET imaging) and at selected times between interventions, which resulted in a blood sample being drawn approximately every 30 min during the experimental observation period. In group OA—Etx, blood was drawn more frequently (approximately every 15 min) for ~1 h after Etx administration. Blood was drawn into tubes containing EDTA (1 mg/ml) and indomethacin (5 μg/ml) and then was centrifuged immediately at 1,800 g for 10 min at 5°C. The plasma was removed and stored frozen at ~80°C until assay.

An enzyme immunoassay was used to measure the stable metabolites of the eicosanoids prostacyclin and thromboxane, 6-keto-PGF1α, and thromboxane B2 (TxB2), respectively. The methods used to make these measurements have been reported previously (7).

Evidence for possible oxidant production was obtained by measuring plasma reactive sulphydryl (RSH) and 8-iso-PGF2α concentrations. A reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was used to determine the plasma oxidant-RSH content, by using previously described methods (11). Because 99% of plasma RSH groups are protein associated, the RSH content was normalized for total protein, as measured by the biuret reaction technique, to account for any protein concentration differences among animals. F2-isoprostanes were quantified in plasma by employing a stable isotope-dilution mass-spectrometric assay as previously described (14). The precision of the assay is ±6%, and the accuracy is 96%.

Statistical analysis. Data are presented as means ± SD. Statistical significance was determined by one- or two-way ANOVA as appropriate (including algorithms for repeated measures when needed) by using the General Linear Models Procedure of the Statistical Analysis System (SAS, Cary, NC). Because of the large number of possible interactions and comparisons with a relatively small number of animals per experimental group, we limited statistical testing either to a comparison of mean values among the groups at the end of each experiment or to a comparison of final values to baseline values within any one group. We accepted P < 0.05 as indicating statistical significance.

<table>
<thead>
<tr>
<th>Table 2. Selected hemodynamic and blood-gas data</th>
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<td>CO, l/min</td>
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<td>Normal</td>
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<tr>
<td>CO2, mmHg</td>
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<tr>
<td>OA only</td>
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Values are means ± SD. Because of the large number of possible interactions and comparisons with a relatively small number of animals per experimental group, we limited statistical testing to either a comparison of mean values among the groups at the end of each experiment, or to a comparison of final values to time 0 values within one group. An additional set of data was obtained in the OA—Etx group at 3.0 h after Etx administration (coincident with the 2nd PET scan in this group). These data were not significantly different from the data in this group at 2.5 h. CO, cardiac output; WP, wedge pressure; PaCO2, arterial partial pressure of CO2. *Significantly different (P < 0.05) compared with time 0 in the same group. †Significantly different (P < 0.05) compared with mean values in the OA-only group.
RESULTS

Hemodynamic, blood-gas, and PET data. In the normal group, cardiac output, systemic and pulmonary artery blood pressures, and blood gases were stable throughout the experimental observation period (Table 2, Fig. 1).

In the other experimental groups, cardiac output fell significantly in each group, but final values were comparable in each case (Table 2). The wedge pressure fell in each experimental group and reached statistical significance in the Etx-only and Etx\textsuperscript{3}OA groups. The pH fell significantly from baseline in each group, and the level reached in the Etx\textsuperscript{3}OA and OA\textsuperscript{3}Etx groups was significantly lower than in the OA-only group (Table 2). PaCO\textsubscript{2} rose significantly from baseline only in the Etx\textsuperscript{3}OA and OA\textsuperscript{3}Etx groups (Table 2). Overall, these hemodynamic and blood-gas changes are similar to previously reported effects of OA (7, 9).

Mean arterial blood pressure fell in all experimental groups (Fig. 1). Note that, after the initial interventions were completed by 0.5 h, the mean arterial blood pressure was stable in all experimental groups for over 90 min. However, in animals given both low-dose Etx and OA, regardless of whether they were given at the beginning or at the end of each study, the fall in blood pressure was greater. Importantly, the additional fall in blood pressure in the OA\textrightarrow{}Etx group did not occur until after administration of low-dose Etx (Fig. 1).

By the end of each study, pulmonary artery pressure increased significantly only in the group given OA alone (Fig. 2). Stated differently, low-dose Etx alone had no effect on pulmonary artery pressure and prevented or reversed the pulmonary hypertension that otherwise developed in OA-treated animals.

Pulmonary injury, assessed from PET images of lung water concentration, was comparable in each group given OA (Fig. 3). There was no evidence from this measurement that low-dose Etx exacerbated the degree of lung injury (Fig. 3).

The distribution pattern of regional pulmonary blood flow was very different among the experimental groups despite the same degree of pulmonary edema (Fig. 4). Animals treated with lung injury developed moderate pulmonary hypertension, which is prevented or reversed in animals also treated with Etx. Values are means ± SD. *Significantly different (P < 0.05) compared with the other groups.

The differences in perfusion pattern in the setting of similar degrees of pulmonary edema had a profound effect on oxygenation (Fig. 5). Low-dose Etx alone had no effect on oxygenation, a finding that is not unex-
The fraction of total pulmonary blood flow (fPBF) going to dorsally positioned (edematous) lung regions (pixel bins on the PET images). The hatched horizontal bar indicates the range of fPBF to these same bins (±1) from the normal group. Values are means ± SD. *OA injury by itself is associated with perfusion redistribution away from these edematous regions (i.e., reduced fPBF compared with the normal distribution; \( P < 0.05 \)). **Animals that received low-dose Etx in addition to OA injury showed either no perfusion redistribution or a nearly complete reversal of perfusion redistribution \( (P < 0.05 \) compared with either the OA-only group or OA→Etx group before Etx administration).

Fig. 4. The fraction of total pulmonary blood flow (fPBF) going to dorsally positioned (edematous) lung regions (pixel bins on the PET images). The hatched horizontal bar indicates the range of fPBF to these same bins (±1) from the normal group. Values are means ± SD. *OA injury by itself is associated with perfusion redistribution away from these edematous regions (i.e., reduced fPBF compared with the normal distribution; \( P < 0.05 \)). **Animals that received low-dose Etx in addition to OA injury showed either no perfusion redistribution or a nearly complete reversal of perfusion redistribution \( (P < 0.05 \) compared with either the OA-only group or OA→Etx group before Etx administration).

Fig. 5. Temporal changes in arterial oxygenation \( (\text{PaO}_2) \) among the different experimental groups. Note that \( \text{PaO}_2 \) is lowest in animals receiving both Etx and OA and that it does not fall to its lowest level in the OA→Etx group until after low-dose Etx is administered. Values are means ± SD. **Significantly different \( (P < 0.05 \) compared with mean data immediately before Etx administration in the OA→Etx group. In addition, mean \( \text{PaO}_2 \) at the end of the study, in all groups given OA, was significantly lower than baseline (significance symbols not shown for purposes of clarity). Mean \( \text{PaO}_2 \) in the Etx→OA group at the end of the experiment was significantly lower than in the Etx-only group and the OA→Etx group before Etx administration. However, once Etx was administered to the OA→Etx group, mean \( \text{PaO}_2 \) also fell, comparable to that in the Etx→OA group.

Fig. 6. Temporal changes in 6-keto-PGF\(_{1\alpha}\) concentrations among the different experimental groups. Note that 6-keto-PGF\(_{1\alpha}\) concentrations are highest in animals receiving both Etx and OA and that the 6-keto-PGF\(_{1\alpha}\) concentrations do not increase in the OA→Etx group until after low-dose Etx is administered. Values are means ± SD. *Mean data in these groups at the end of the experiment were significantly higher than at baseline or in the other groups at the end of the experiment \( (P < 0.05 \).
roration in oxygenation (Fig. 5) and a fall in systemic and pulmonary arterial blood pressures (Fig. 1). The abrupt onset of these physiological changes is graphically illustrated by serial data from one animal in the OA→Etx group (Fig. 7).

Plasma TxB$_2$ concentrations showed no change in either normal or OA-only groups (data not shown). In the other groups, changes in TxB$_2$ concentrations were highly variable. They rose transiently (about double the baseline value) in the Etx-only and Etx→OA groups but were not different from the normal or OA-only groups by the end of the experiment. In the OA→Etx group, the TxB$_2$ concentration after Etx was 448 ± 166 pg/ml blood, which was significantly higher than the concentration in the OA-only group (238 ± 292 pg/ml blood).

Evidence for oxidant production as a possible mediator of the Etx-OA interaction was sought by measuring 8-iso-PGF$_{2\alpha}$ and RSH concentrations in circulating blood. Unfortunately, the RSH measurements could not be performed in the three groups receiving OA because of hemolysis in virtually all these samples. In the Etx-only group, however, there was no evidence of significant oxidant production with this assay (no significant change over time or compared with data from the normal group).

Supporting these limited observations are the serial measurements of 8-iso-PGF$_{2\alpha}$ concentrations. By the end of the experiment, the 8-iso-PGF$_{2\alpha}$ concentrations were slightly but statistically higher only in the Etx→OA group and then only compared with the OA→Etx group (Fig. 8). This tendency was not seen until approximately the last hour of experimental measurements (data not shown). In other words, the kinetics of the increase in 8-iso-PGF$_{2\alpha}$ concentrations were delayed compared with the development of increased 6-keto-PGF$_{1\alpha}$ concentrations.

**DISCUSSION**

In several previous studies, our laboratory has reported a striking synergistic effect between low-dose Etx and OA-induced injury (7–9). At least one set of consequences of this synergism is apparently mediated by COX-2-driven increases in prostacyclin production (9), the results of which are prevention of pulmonary hypertension, prevention of the normally expected redistribution of regional pulmonary blood flow away from edematous lung regions, markedly worsening oxygenation, and the development of systemic hypotension. It is important to keep in mind that the dose of Etx used in this study was at least 30–60 times less than that usually used to cause experimentally-induced septic shock in dogs (3). When administered alone, this dose of Etx has minimal cardiovascular effects, no effect on lung water accumulation, and no effect on gas exchange (Table 2, Figs. 1–5).

The current study extends this previous work by showing that 1) the mechanism for the Etx-OA-injury interaction is inconsistent with the phenomenon of priming as an underlying mechanism, 2) the onset of effects from this interaction follows a predictable time delay of ~30–45 min after both drugs are administered, and 3) the interaction may be independent of (i.e., is not mediated by) increased oxidant production. Priming. In our previous studies, low-dose Etx was used primarily to ablate intrapulmonary perfusion redistribution in an effort to discern its importance to maintaining gas exchange (7–9). Because low-dose Etx has also been used in numerous studies to prevent the development of hypoxic pulmonary vasoconstriction (25, 26, 28), we speculated that the effects of Etx in this setting implied that the mechanism underlying perfusion redistribution in this model was also hypoxic pulmonary vasoconstriction. Regardless of its intended purpose in these previous studies, Etx was always administered before OA administration. When we discovered that the effects of Etx were apparently mediated by COX-2-driven increases in prostacyclin production (9), we further speculated that Etx primed lung cells (most likely the endothelium) to increase COX-2 production, which, in the presence of increased arachidonic acid release secondary to OA-induced lung in-
jury, resulted in markedly increased prostacyclin production.

Clearly, others have shown that Etx, including low doses or concentrations of Etx, can alter cellular responses to a subsequent challenge with higher doses of Etx or to a new exposure to exotoxin, platelet activating factor, or arachidonic acid, among other secondary stimuli, and result in increased eicosanoid production (1, 4, 5, 15, 23, 24, 27, 30). However, these other studies have not altered the sequence of first and second stimuli, so whether they indeed involve priming or synergy is not always clear.

As stated previously, priming has been defined as a sequence of events in which a first stimulus influences an intermediate step used by a second stimulus. Obviously, the temporal relationship between the first and second stimuli is inherent in such a definition. Thus a straightforward test of priming in our case was to simply reverse the order of the two stimuli (low-dose Etx and OA administration). Because the physiological and biochemical consequences were the same regardless of order, the interaction must not depend on Etx priming of a specific cell population.

Temporal relationships. The administration of low-dose Etx ~2 hrs after fully developed and stable lung injury (time periods from 1.0–3.0 h in Figs. 1 and 5) allowed us to better define the temporal development of this interaction. Previously (7, 9), when we administered the Etx just 30 min before OA, the interpretation of subsequent physiological changes was confounded by the progressive changes in edema accumulation, intrapulmonary hemodynamics, and gas exchange that take place during the first 90–120 min in this model. Furthermore, previous biochemical measurements had been obtained only at the time of PET imaging, which resulted in only two to three observations per experiment.

In the present study, the effects of administering low-dose Etx were not immediate, but required a predictable period of 30–45 min before becoming apparent (Figs. 6 and 7). To the extent that the Etx-OA interaction is dependent on COX-2 activation (generally thought to be inducible and not constitutively expressed), this time interval may seem short. However, recent in vitro data have shown that a variety of inflammatory mediators, including lipopolysaccharide, can indeed lead to substantial COX-2 activation in as little as 30 min (16). Furthermore, Ermert et al. (5) have shown that COX-2 may be constitutively expressed in rat lung. Thus COX-2-mediated production of prostacyclin is still a plausible explanation for the low-dose Etx-OA injury interaction.

Biochemical mechanisms. Cyclooxygenase is not thought to be rate limiting in eicosanoid production, so an increase in prostacyclin production, although possibly dependent on upregulation of cyclooxygenase, implies additional release of arachidonic acid. It is possible that OA injury involves increased arachidonic acid release, which, in the presence of upregulated cyclooxygenase, combines to produce increased prostacyclin production. A recent study in rats showed that 6 h of lipopolysaccharide infusion alone had relatively little effect on prostacyclin production, but that concentrations rose rapidly after a source of arachidonic acid was provided (10). Another recent study showed that many of the physiological consequences of OA injury can be prevented by a phospholipase A2 specific inhibitor, presumably by reducing arachidonate release (6). The results of the current study are consistent with these observations.

Because prostacyclin synthase (the terminal enzyme in prostacyclin production) activity is largely limited to endothelium, it is reasonable to assume that these cells must ultimately be responsible for the increased prostacyclin production seen in the current studies. However, the source of increased cyclooxygenase expression could be circulating inflammatory cells, inflammatory cells that have become trapped in the lung as a result of endothelial injury, or endothelium in the lung or elsewhere. If lung endothelium is not the source of increased cyclooxygenase activity, a mechanism for delivering the intermediate product PGH2 must be identified, and examples of transtranscellular transport of eicosanoids have been described (12).

Although OA injury itself does not require inflammatory cells to develop (19), inflammatory cells clearly do become sequestered and migrate into the lung after OA injury. The effects of Etx on endothelium (if any in the context of this study) may be a direct one or may involve intermediate products of inflammatory cells, such as oxygen-free radicals, tumor necrosis factor-α, or platelet-activating factor among others, all of which have been implicated in cascades of events that culminate in increased eicosanoid production. Such participation by inflammatory cells, if required, could help explain some or all of the temporal delay that was observed after Etx administration before physiological and biochemical changes became manifest. In the current study, however, we failed to find any convincing evidence that increased oxidant production was at all involved in explaining the Etx-OA interaction. Indeed, what evidence for increased oxidant production was obtained came only after several hours of exposure to both Etx and lung injury and clearly after the increase in prostacyclin concentrations was already present. Although such inferences from biochemical measurements obtained in plasma are not definitive, they do point away from oxidant production as a likely explanation for the Etx-OA interaction.

Clinical implications. The so-called “two hit” hypothesis is one commonly invoked paradigm used to explain the pathogenesis of acute respiratory distress syndrome (ARDS) and associated other multiorgan system dysfunctions (2). The hypothesis claims that sequential inflammatory insults are often required for organ injury to become manifest. Priming of inflammatory cells by cytokines or other mediators is an intrinsic part of this paradigm. If one keeps in mind that the results of the current study may differ if retested in other species or other models of lung injury, our data do suggest that alternative scenarios could also be important. Our results suggest that small, intermittent, circulating con-
centrations of Etx can substantially affect the physiological consequences of already established and apparently stable lung injury. What appears to be relatively mild noncardiogenic edema can quickly change to a clinical picture of severe hypoxemia and systemic hypotension (changes after Etx in the OA—Etx group in Figs. 1, 5, and 7). Of course, it is not known whether a comparable phenomenon occurs in ARDS, nor, if it does, exactly how low a concentration of Etx would trigger these events.

There are possible therapeutic implications as well. Anti-Etx therapies have been repeatedly found to not affect outcome in sepsis or ARDS (29), but these clinical trials have always focused on the apparently initiating septic event. The results of the present study, however, may indicate that more prolonged treatment may be necessary, perhaps until lung injury has fully or substantially resolved.

In conclusion, we have shown that the previously reported synergistic effect of low-dose Etx and OA lung injury occurs whether the Etx is administered before or after OA, essentially eliminating Etx priming as the underlying mechanism. The physiological and biochemical disturbances that result from this interaction predictably develop ~30–45 min after the second agent is administered. Finally, we failed to find evidence that oxidant production was required to produce these effects.

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