Nitric Oxide Opposes Phorbol Ester-Induced Increases in Pulmonary Microvascular Permeability in Dogs

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ABSTRACT

In addition to its effects on vascular tone, nitric oxide (NO) has been suggested to function as a participant in fluid homeostasis affecting interactions between the endothelium and circulating inflammatory cells. The role of NO in the increased microvascular permeability of acute lung injury, however, remains controversial. We investigated the hypothesis that NO opposes increases in pulmonary vascular permeability after phorbol myristate acetate administration, i.e., in a model of neutrophil-dependent acute lung injury. In anesthetized dogs, phorbol myristate acetate (10 μg/kg, i.v.) had no effect on pulmonary arterial pressure (Ppa) or extravascular lung water. After pretreatment with the NO synthesis inhibitor, NG-nitro-L-arginine methyl ester (10 mg/kg, i.v.; 5 mg/kg/hr), an identical dose of phorbol myristate acetate resulted in a 20 ± 8 mm Hg (P < 0.01) increase in pulmonary arterial pressure and a 186 ± 86% (P < 0.01) increase in extravascular lung water. To determine if the pulmonary edema was related to increases in microvascular pressure or to changes in the microvascular permeability coefficient, experiments were performed in isolated blood-perfused dog lungs. The addition of phorbol myristate acetate (4.2 × 10^{-8} M) to the perfusate was without effect on microvascular pressure or pulmonary capillary filtration coefficient. However, after NG-nitro-L-arginine methyl ester (100 μM), phorbol myristate acetate resulted in increases in both microvascular pressure and permeability coefficient that were prevented by pretreatment with L-arginine (1 mM). These data support the hypothesis that endogenous NO opposes increases in pulmonary vascular permeability as well as microvascular pressure in this neutrophil-dependent model of acute lung injury resulting in preservation of the endothelial barrier to the passage of water and solutes and prevention of the formation of pulmonary edema.

In humans, the adult respiratory distress syndrome occurs in association with conditions as disparate as aspiration of gastric contents, head injury, sepsis, hemorrhagic shock and severe trauma (Ashbaugh et al., 1967). Etiology notwithstanding, the pathophysiological changes associated with ALI are decreased lung compliance, reduced arterial oxygen tension and nonhydrostatic pulmonary edema (Anderson et al., 1982). Efforts to identify those mechanisms responsible for development and perpetuation of the increased pulmonary vascular permeability of ALI have been directed toward definition of the role of complement (Henson et al., 1982), leukocytes (Henson et al., 1982; Gie et al., 1991), platelets (Binder et al., 1980), coagulation/embolism (Barrie and Malik, 1982), fibrinolysis (Haynes et al., 1980), oxygen metabolites (Faintone and Ward, 1985), cytokines (Royall et al., 1989), products of arachidonic acid metabolism (Lonigro et al., 1990) and, more recently, NO (Abdih et al., 1994; Berishia et al., 1994; Kavanaugh et al., 1994; Guidot et al., 1995) as pathogenic factors. Despite these efforts, a comprehensive description of the mechanisms of enhanced microvascular permeability associated with ALI has not yet emerged.

Functional and anatomical integrity of the vascular endothelium is critical for control of the movement of water and solutes between the vascular lumen and the interstitial space. In the lung, dysfunction of the endothelial barrier can result in increased vascular permeability and pulmonary edema. In addition, dysfunction of the endothelium results in adherence of PMNs (Kubes et al., 1991; Gaboury et al., 1993) that, in turn, are thought to contribute to increased vascular permeability (Kubes et al., 1991; Kurose et al., 1993). Thus, in ALI, it is possible that there is a pathophysiological amplification of injury to the barrier function of the microcirculation mediated by the primary endothelial cell dysfunction as well as by the associated adherence of activated PMNs to the injured endothelium. The finding that the vascular endothelium produces NO that, in addition to relaxing the

ABBREVIATIONS: PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; ALI, acute lung injury; Ppa, mean pulmonary arterial pressure; Psw, mean systemic arterial pressure; Pia, mean left atrial pressure; Pmv, microvascular pressure; EVLW, extravascular lung water; L-NAME, N^G-nitro-L-arginine methyl ester; TPP, transpulmonary pressure; Kfc, pulmonary capillary filtration coefficient; ECV, ethchlorvynol; DMSO, dimethyl sulfoxide; NO, nitric oxide; ECV, ethchlorvynol; ARDS, adult respiratory distress syndrome.
underlying vascular smooth muscle (Furchgott and Zawadzki, 1980), is capable of regulating the interaction of the endothelium with inflammatory cells (Kubes et al., 1991; Gaboury et al., 1993) suggests a pivotal role for NO the pathophysiology of ALI.

Although a great deal of evidence has accumulated in support of the hypothesis that NO participates in the hypotension associated with sepsis in experimental animals (Thiemermann and Vane, 1990; Julou-Schaeffer et al., 1991; Klemm et al., 1995) and in humans (Evans et al., 1993), inhibition of NO synthesis in this setting produced conflicting results (Minnard et al., 1994; Robertson et al., 1994; Petros et al., 1994; Mitaka et al., 1995). Thus, administration of inhibitors of NO synthesis in endotoxin-induced shock resulted in improved hemodynamics but did not diminish mortality in rats (Klemm et al., 1995) and was associated with pulmonary hypertension in swine (Robertson et al., 1994). Moreover, in a limited study in human subjects with severe sepsis-associated hypotension, although the administration of an inhibitor of NO synthesis resulted in improvement in several hemodynamic parameters, survival was not improved (Petros et al., 1994). The results of studies such as these suggest that endogenous NO is not simply a pathophysiological mediator of arterial hypotension, but, more importantly, may subserve a role that is beneficial for survival in sepsis. We investigate the hypothesis that endogenous NO opposes increases in pulmonary vascular permeability and microvascular pressure that occur in an animal model of neutrophil-mediated ALI.

Materials and Methods

Intact dog preparation. Adult heart worm- and microfilaria-free, male, mongrel dogs (20-30 kg) were anesthetized with pentobarbital sodium (30 mg/kg, i.v.; followed by infusion of 0.05 mg/kg/min). Animals were ventilated (Harvard ventilator) with room air with a tidal volume of 15 ml/kg at 12 to 15 breaths/min. A positive end-expiratory pressure of 5 cm H2O was maintained and the lungs were inflated to 20 cm H2O every 10 min to avoid atelectasis. After 10 min, animals were exsanguinated. Via left lateral thoracotomy, the heart and lungs were removed en block and ventilation was maintained. Blood-filled catheters were placed into the pulmonary artery and vein of the left lower lobe. The remaining lobes were ligated and removed. The isolated lobe was ventilated with a gas mixture containing 15% O2, 6% CO2, balance N2 with tidal volume adjusted such that peak airway pressure was the same as that recorded during whole lung ventilation. A positive end-expiratory pressure of 5 cm H2O was maintained. The isolated lobe was suspended from a force transducer (Grass, Quincy, MA, FT03C) and perfused with autologous blood at constant flow (5-7 ml/g of lung weight) with a recirculating volume of 700 ml to achieve an initial inflow pressure of 13 to 15 mm Hg. Blood exiting the lobe was returned to a venous reservoir where it was gassed with 15% O2, 6% CO2, balance N2. The blood was warmed (37-38°C) by passage through a heat exchanger and circulated with a Masterflex pump (Cole-Parmer Instrument Co., Barrington, IL, model 7523-00). Ppa (inflow) and Pla (outflow) pressure were measured continuously. The isolated lobe, venous reservoir and connecting tubing were enclosed in an insulated cabinet with temperature held constant at 37°C. A screw clamp on the venous outflow line was adjusted to maintain Ppa at 2.0 to 2.5 mm Hg. Lungs were perfused under Zone III conditions (Ppa–Pla=airway pressure). TTP was calculated as Ppa-Pla.

Measurement of Kfc and Pmv in isolated perfused lungs.

The Kfc of isolated perfused lungs was determined by the measurement of weight gain over time in response to increased Pla. Thus, Pla was increased rapidly to 20 cm H2O while lung perfusion rate was maintained constant. The increase in Pla was sustained for 3 min and lung weight was monitored continuously. In response to the increase in Pla, the initial rapid increase in lung weight reflects the increase in lobar blood volume due to recruitment and distension of the vasculature. The subsequent slower rate of weight gain represents the filtration of fluid out of the microvasculature (Drake et al., 1978). The slower component of the weight gain was used to calculate the Kfc. A semilogarithmic plot of the slow weight gain was extrapolated to obtain the initial rate of weight gain at time zero. Because Kfc is a representation of the change in fluid movement in response to a change in microvascular pressure, the determination of Kfc demands that Pmv be determined immediately before and during the period of increased venous pressure. Pmv was determined by the double occlusion method (Hakim et al., 1979). Kfc was calculated by dividing the initial change in rate of weight gain by the change in Pmv and is reported as ml/min/cm H2O per 100 g lung tissue.

Statistical analysis. Statistical significance between means was determined by an analysis of variance. In the event that the F ratio indicated that differences were present, Tukey’s least significant difference test was used to identify individual differences among means. P ≤ 0.05 was considered statistically significant. Values are expressed as means ± S.E.

Experimental protocols. In intact dogs, after hemodynamic and blood gas stability were achieved, measurements of cardiac output and EVLW were made. The animals then received either L-NAME (10 mg/kg, i.v. followed by 5 mg/kg/hr, n = 5) or its vehicle (saline, n = 6) and determination of cardiac output and EVLW were repeated after 30 min. PMA (10 μg/kg, i.v.) was then administered and, after 60 min, the final determinations of cardiac output and EVLW were made. PMA was administered i.v. over 5 min and was dissolved in DMSO (2 mg/ml) and diluted with 15 ml of saline resulting in a

Isolated perfused dog lung preparation.

Dogs were anesthetized with pentobarbital sodium (30 mg/kg i.v.) and intubated and ventilated (Harvard ventilator) with room air. A catheter was placed into a femoral artery and heparin (10,000 U i.v.) was administered. A Swan-Ganz catheter was advanced into the main pulmonary artery via the femoral artery. Body temperature was maintained via a peripheral vein for continuous measurement of Ppa. For measurements of Psa and blood gas tensions, a catheter was placed into the superior vena cava. The dye concentration curve was determined by withdrawing blood (Sage pump, model 351) via the femoral artery. EVLW was quantified by a microprocessor-based system (9310 computer, Edwards Laboratories). The dye concentration curve and the thermal curve were determined simultaneously. The lung water computer estimates EVLW based on the difference between the mean transit times for each indicator. EVLW, expressed in ml/kg body weight, is reported as the mean of two to three determinations. The cardiac output was calculated as the integral of the area under the thermal curve divided by the duration of the curve.

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Measurement of extravascular lung water and cardiac output in intact dogs. Estimates of EVLW were made by the measurement of extravascular thermal volume. A 5-French thermodilution catheter (model D402-A, Waters, Rochester, MN). The dye concentration
solution containing no more than 1% DMSO. This dose of PMA was chosen because it had been previously reported that, in intact anesthetized dogs, 10 μg/kg of PMA were associated with increased pulmonary vascular resistance and decreased cardiac output, but EVLW was unaltered, i.e., this dose of PMA was not associated with the development of pulmonary edema (Sprague et al., 1990).

In isolated blood-perfused dog lungs, baseline determinations of Pmv and Kfc were made after stability of hemodynamic parameters and blood gasses was achieved (minimum of 30 min). Either L-NAME (100 μM) or its vehicle (saline) was then added to the perfusate and, after 30 min, Pmv and Kfc were again determined. Finally, 30 min after the addition of PMA (4.2 × 10^-8 M) or its vehicle, final determinations of Kfc and Pmv were made. In five additional experiments, L-arginine (1 mM) was added to the perfusate 30 min before the administration of L-NAME. In the latter group, Pmv and Kfc were determined 30 min after L-NAME. A 2 mg/ml stock solution of PMA dissolved in DMSO was diluted in 10 ml of saline immediately before use (DMSO concentration < 1%). The final concentration of PMA in the perfusate was achieved by addition of 18 μl of the diluted solution to the perfusate reservoir over a 3-min period. Four experimental protocols were followed; 1) the vehicle for L-NAME followed by PMA (n = 6), 2) L-NAME followed by PMA (n = 5), 2) L-arginine followed by L-NAME followed by PMA (n = 5) and 4) the vehicle for L-NAME followed by the vehicle for PMA (n = 4). Finally, in two experiments, L-NAME was administered in the absence of PMA or L-arginine to demonstrate that L-NAME itself was without effect on Pmv and Kfc.

Results

Effect of L-NAME administration in intact anesthetized dogs. The administration of L-NAME to anesthetized dogs resulted in a decrease in cardiac output with no change in either Psa (table 1) or Ppa (fig. 1A). These results suggest that L-NAME produced increases in both systemic and pulmonary vascular resistance. Importantly, L-NAME administration was not associated with any increase in EVLW (fig. 1B). The administration of the vehicle for L-NAME was without effect on hemodynamic parameters, arterial oxygen tension or EVLW (table 1; fig. 1, A and B).

Effect of PMA administration in the absence and presence of L-NAME in intact anesthetized dogs. In the absence of L-NAME, PMA was not associated with a change in Ppa (fig. 1B). There was a numerical increase in Ppa over control values in this group, however, the values did not differ statistically (fig. 1A). In contrast, in animals that were pretreated with L-NAME, PMA administration resulted in 3-fold increases in both Ppa and EVLW (fig. 1, A and B). Although the administration of PMA in the presence of L-NAME resulted in the development of increased EVLW, the accompanying increase in Ppa was so great that the mechanism responsible for the edema formation could not be determined. Thus, it is possible that the edema formation could have resulted from increased vascular permeability, increased microvascular hydrostatic pressure or a combination of the two. To resolve this important issue, experiments were performed in isolated blood-perfused dog lungs in which the effects of PMA on Pmv and microvascular Kfc in the absence and presence of L-NAME could be determined.

Effect of L-NAME or L-arginine followed by L-NAME in isolated blood-perfused dog lungs. The administration of either L-NAME, L-arginine followed by L-NAME or their vehicle (saline) was without effect on TPP, Pmv or Kfc (figs 2, A and B and 3). In four experiments, isolated lungs were prepared in an identical manner but did not receive any active drugs. The latter studies were performed to demonstrate that any effects on Pmv and/or Kfc were related to the action of the agents and not due to the passage of time. In

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<th>Group</th>
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<tr>
<td>Vehicle</td>
<td>Psa (mm Hg)</td>
<td>130 ± 5</td>
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<tr>
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<td>152 ± 14</td>
<td>153 ± 11</td>
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<tr>
<td>Vehicle</td>
<td>Cardiac output (liter/min)</td>
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<td>L-NAME</td>
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<td>3.0 ± 0.2</td>
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Values are means ± S.E.

^a Different from value before L-NAME.

^b Different from vehicle group.

Fig. 1. Effect of PMA (10 μg/kg, i.v.) on Ppa (A) and EVLW (B) in the absence (n = 5) of L-NAME (10 mg/kg) i.v. followed by 5 mg/kg/hr) or its vehicle (saline, n = 6) in anesthetized dogs. *different from respective pre-PMA value. †different from the group that did not receive L-NAME. Values (means ± S.E.) represent those obtained at baseline, 30 min after administration of either L-NAME or its vehicle (saline) and 60 min after PMA.
these studies there were no changes in any measured parameter over time and final values for Pmv and Kfc were 9.4 ± 0.9 mm Hg and 0.26 ± 0.09 ml/min/cm H2O/100 g lung weight, respectively. It was also possible that the administration of L-NAME itself might result in an increase in Pmv of Kfc in the absence of PMA. To address this issue, in two experiments, L-NAME administration was followed by the addition of the vehicle for PMA to the perfusate. In the latter studies, L-NAME was without effect on Pmv or Kfc with final values of 9.5 mm Hg and 0.22 ml/min/cm H2O/100 g lung weight, respectively.

**Effect of the administration of PMA in the absence and presence of L-NAME in isolated blood-perfused dog lungs.** In the presence of either the vehicle for L-NAME or L-NAME, the addition of PMA to the perfusate resulted in an increase in TPP and Pmv (fig. 2, A and B). However, the increases in both TPP and Pmv were larger in the lungs that were pretreated with L-NAME (fig. 2, A and B). Thirty min after PMA administration, Kfc was increased solely in lungs pretreated with L-NAME (fig. 3).

**Effect of the administration of PMA in the presence of L-arginine and L-NAME in isolated blood-perfused dog lungs.** It was reported that L-NAME and other arginine analogues may have effects in addition to the inhibition of endogenous NO synthesis (Peterson et al., 1992; Buxton et al., 1993). However, if an effect of these agents is prevented by the administration of an excess of L-arginine, then that action can be attributed to inhibition of NO synthesis (Buxton et al., 1993). To confirm that the effects of L-NAME on the response to PMA administration were due to inhibition of NO synthesis, in 5 additional experiments L-arginine (1 mM) was added to the perfusate of isolated dog lungs 30 min before L-NAME. Pretreatment with L-arginine prevented the L-NAME-associated increases in TPP, Pmv (fig. 2, A and B) and Kfc (fig. 3) in response to PMA administration.

**Discussion**

There is significant controversy regarding the contribution of endogenous NO to the edema formation of ALI. This controversy is illustrated by the divergent results of studies investigating the effect of inhibitors of NO synthesis in different models of ALI. For example, it was reported that in isolated rat lungs perfused with a physiological salt solution, ALI produced by i.v. administration of paraquat was attenuated by an inhibitor of endogenous NO synthesis (Berishia et al., 1994) leading to the conclusion that endogenous NO is a significant contributor to the edema formation in this model of ALI. In contrast, it was reported that inhibition of endogenous NO synthesis resulted in increased pulmonary edema formation after ischemia-reperfusion (Abdih et al., 1994). The latter data support the hypothesis that endogenous NO opposed the development of pulmonary edema in the latter model of ALI.

One problem inherent in the study of edema formation in ALI is that pulmonary edema often occurs concomitant with increases in pulmonary vascular pressures. Indeed, in most studies in isolated lungs, the end point for determination of an effect of NO on edema formation is the wet-to-dry weight ratio of the lungs. The latter technique cannot be used to determine if edema formation is the result of increases in hydrostatic pressure, increases in microvascular permeability or a combination of the two. Thus, this technique is of limited value in assessing the mechanism by which NO influences the development of pulmonary edema. In our work, we examined the effect of an inhibitor of endogenous NO synthesis on both the accumulation of extravascular lung water in intact dogs as well as microvascular pressure and microvascular permeability in isolated perfused dog lungs. A strength of the isolated perfused lung preparation used in this study is that it permits the independent assessment of the effects of agents on both Pmv and Kfc, the latter an estimate of microvascular permeability.

The efforts of this laboratory have, for some time, been directed toward the understanding of those mechanisms that are responsible for the pulmonary edema associated with
ARDS. To that end, we have developed two distinct models of ARDS in intact anesthetized dogs as well as in isolated perfused dog lungs. The first model is the lung injury resulting from the administration of the sedative-hypnotic agent, ECV. When administered i.v. to intact anesthetized dogs, ECV causes a lung injury characterized by the development of nonhydrostatic pulmonary edema and hypoxemia (Stephenson et al., 1984, Sprague et al., 1986). The injury that occurs after ECV administration does not require the presence of neutrophils and the pulmonary edema formation is due to increased microvascular permeability, presumably via a direct effect of ECV on the endothelial cell (Millen et al., 1978; Wysolmerski et al., 1984). The second model of ARDS is the lung injury that occurs after the i.v. administration of PMA. In intact anesthetized dogs, the administration of PMA at a dose of 20 to 30 μg/kg was associated with systemic hypotension, pulmonary hypertension, reduced cardiac output, reduced circulating white blood cell counts, arterial hypoxemia and pulmonary edema (Sprague et al., 1990). Importantly, we found that a smaller dose of PMA (10-15 μg/kg) was associated with pulmonary hypertension, but not with edema formation (Sprague et al., 1990). In addition, in contradistinction to the lung injury that occurs after ECV administration, PMA-induced ALI is dependent on the presence of circulating neutrophils (Shasby et al., 1982). The involvement of neutrophils in one model of ALI (PMA) and not in the other (ECV) permits the investigation of the contribution of endogenous NO to the interaction of these inflammatory cells with the endothelium in ALI.

In studies in which ALI was induced in intact anesthetized dogs by the administration of ECV (neutrophil-independent ALI), we found that pretreatment with L-NAME resulted in a small, but significant, reduction in pulmonary edema formation (Sprague et al., 1995). We report that, in intact anesthetized dogs, inhibition of endogenous NO synthesis results in increased pulmonary vascular pressures (figs 1A and 2) as well as the development of pulmonary edema (fig 1B) after the administration of a dose of PMA that was without effect on pressures or edema formation in the absence of L-NAME. Moreover, the studies in the isolated perfused dog lung demonstrate that the edema formation observed when this dose of PMA is administered in the presence of L-NAME is the result of increases in both Pmv (fig. 2) and Kfc (fig. 3). These data demonstrate that only in the presence of an inhibitor of endogenous NO does the dose of PMA used in this study result in the development of pulmonary edema in intact dogs and increases in both Kfc and Pmv in isolated perfused lungs. Although the development of increased microvascular permeability alone would be expected to result in pulmonary edema formation, a concomitant increase in microvascular pressure, as observed in this model, would augment the movement of water and solutes out of the vascular space and into the interstitium of the lung.

One interpretation of the findings of this work is that endogenous NO present in the lung acts to oppose the effects of PMA on microvascular pressure and permeability and that when this NO is eliminated by the application of L-NAME, the effect of PMA is unopposed. The finding that L-NAME failed to increase the accumulation of lung water after ECV, but increased edema formation in PMA-induced ALI may be due to the fact that ECV-induced lung injury is the result of a direct effect of ECV on the endothelium (Wysolmerski et al., 1984), whereas PMA-induced lung injury requires neutrophils (Shasby et al., 1982). An extension of this interpretation is that the mechanism by which endogenous NO attenuates PMA-induced increases in microvascular permeability is via some effect of NO on the interaction between neutrophils and the endothelium. This interpretation is supported by the fact that the administration of L-NAME in the absence of PMA or ECV had no effect on EVLW suggesting that under “basal” conditions, i.e. when neutrophil activation would not be expected, inhibition of endogenous NO synthesis neither promoted edema formation in intact anesthetized dogs nor resulted in increases in Pmv or Kfc in isolated perfused dog lungs.

Our results support the hypothesis that endogenous NO acts to oppose increases in both Pmv and Kfc after PMA administration, however, determination of the cellular origin of the NO is beyond the scope of the present work. In addition to the endothelium, epithelial cells as well as cells present in the interstitium of the lung and inflammatory cells are capable of producing NO. Although the source of endogenous NO was not identified in our study, the endothelium is a likely candidate. The integrity of the endothelium is critical for the maintenance of normal microvascular permeability. Moreover, the endothelial cell is a rich source of endogenous NO and is in direct contact with circulating neutrophils.

In summary, the results of these experiments support the hypothesis that, under conditions that mimic those present in patients with ARDS, i.e., in a lung injury that requires the presence of activated/adherent neutrophils, endogenous NO opposes the development of pulmonary edema. Moreover, the data suggest that the protection against edema formation afforded by endogenous NO in neutrophil-dependent lung injury is via its ability to oppose increases in both pulmonary microvascular permeability and microvascular pressure.
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References

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