ORIGINAL ARTICLE

Nitric Oxide and Thromboxane A_2–Mediated Pulmonary Microvascular Dysfunction

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Objectives: To examine whether the lung releases nitric oxide (NO) in response to thromboxane A_2 and to examine the local release of NO as a protective compensatory mechanism by which the lung responds to the proinflammatory and vasoactive effects of thromboxane A_2.

Design: The lungs of anesthetized Sprague-Dawley rats were perfused in vitro with Krebs-Henseleit buffer that contained an inhibitor of NO synthase (nitroglycerine-nitro-L-arginine methyl ester [L-NAME]) (10^{-4} mol/L), an NO donor (sodium nitroprusside) (10^{-8} mol/L), or perfusate alone. Following equilibration, the thromboxane A_2 receptor agonist 9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46 619) (7.1×10^{-8} mol/L) was added to the perfusate. Fifteen minutes later, the capillary filtration coefficient, pulmonary arterial pressure, and vascular resistance were measured. Pulmonary NO release was assessed by quantitating the release of cyclic guanosine monophosphate into the perfusate.

Results: The capillary filtration coefficient of lungs exposed to U-46 619 was 3.5 times greater than that of lungs perfused with buffer alone ($P<.05$). The addition of sodium nitroprusside reduced the increase in capillary filtration coefficient associated with U-46 619 by 50% ($P<.05$) whereas L-NAME had no effect. The addition of U-46 619 to the perfused lung caused a 3.0 ± 0.4 mm Hg increase in pulmonary artery pressure ($P<.01$) with a corresponding rise in total vascular resistance ($P<.05$). This effect was exacerbated by L-NAME ($P<.05$) and inhibited by sodium nitroprusside ($P<.05$). Exposure of the isolated lungs to U-46 619 caused a 4-fold increase in cyclic guanosine monophosphate levels within the perfusate.

Conclusion: These data are consistent with the hypothesis that NO release may be an important protective mechanism by which the lung responds to thromboxane A_2.

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THROMBOXANE A_2 (TXA_2) has important proinflammatory effects on the pulmonary microvasculature that include vasoconstriction, enhanced permeability, and neutrophil and platelet activation. It has been incriminated in the pathogenesis of acute lung injury following remote organ reperfusion,[1] pulmonary ischemia-reperfusion injury,[2] and endotoxemia.[3] Clinically, TXA_2 is thought to be involved in the pathogenesis of adult respiratory distress syndrome and primary and secondary pulmonary hypertension.[4]

In each of these inflammatory states, the lungs release substances possessing anti-inflammatory and vasodilatory effects. These agents may serve to limit or abrogate the potentially injurious effects of TXA_2 and other proinflammatory mediators. The constitutive release of nitric oxide (NO) by the vascular endothelium likely serves a particularly important function. The release of picomolar quantities of NO by the vascular endothelium has been shown to maintain normal microvascular permeability,[5] inhibit neutrophil-endothelial cell adherence,[6] and maintain normal tissue perfusion through its role as a potent and important vasodilator.[7] Our series of experiments examine the role of NO in counteracting the proinflammatory and vasoactive effects of TXA_2. In particular, we examine whether the lungs generate NO in response to TXA_2 exposure and whether this is one of the protective mechanisms by which the lungs compensate for the vasoactive and proinflammatory effects of TXA_2.

RESULTS

EFFECT OF TXA_2 RECEPTOR ACTIVATION ON PULMONARY cGMP RELEASE

The addition of U-46 619 to the perfusate of the isolated, perfused lung caused a 4-fold increase in cGMP release. This increase was totally prevented by the presence of L-NAME within the perfusate. The concentration of cGMP within the perfusate of lungs exposed to U-46 619 was 0.43 ± 0.03 pmol/mL per gram of lung

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MATERIALS AND METHODS

ISOLATED, PERFUSED LUNG MODEL

Pathogen-free Sprague-Dawley rats (weight, 250-350 g) were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally). The pulmonary arterial trunk and left atrium were cannulated via the right and left ventricles, respectively, and the heart and lungs excised en bloc. The lungs were suspended by a ligature from a force transducer (TSD 125C; Biopac Systems Inc, Santa Barbara, Calif) for continuous measurement of lung weight. The lungs were perfused with Krebs-Henseleit buffer that contained 3% bovine serum albumin at 0.04 mL · gram body weight^{-1} · minute^{-1} and ventilated with room air at a rate of 60 strokes per minute. Pulmonary arterial (PA) and left venous (PV) pressures were continuously measured with pressure transducers (TSD 104A; Biopac Systems Inc) with 0 reference at the level of the apex of the lung. These measurements were continuously recorded by a data acquisition unit (MP100 Manager version 3.2.3; hardware version 1.1f, Biopac Systems Inc) interfaced with a personal computer (Dell Computer Corp, Austin, Tex). The perfusate was maintained in a 37°C water bath. The first 75 mL of perfusate was discarded to remove blood elements from the vascular space; the perfusate was recirculated after that. Pulmonary venous pressure was maintained at 2 to 3 mm Hg and all lungs were perfused under zone 3 conditions with PA > PV > PAIRWAY. The lungs were isogravimetric throughout the experiment.

EXPERIMENTAL PROTOCOL

The experimental protocol is shown in **Figure 1**. The lungs were perfused with Krebs-Henseleit buffer either alone (10^{-8} mol/L) or containing the NO donor sodium nitroprusside (SNP) (10^{-10} mol/L) or the NO synthase inhibitor nitroglycerine-nitro-L-arginine-methyl ester (L-NAME) (Sigma-Aldrich Co, St Louis, Mo) (10^{-4} mol/L and 10^{-2} mol/L). These doses of SNP have been previously demonstrated to induce vasodilatation in a similar experimental protocol using the isolated, perfused lung model.[8] The dose of L-NAME has been shown to inhibit NO formation by nearly 100% in cultured endothelial cells[9] and has been previously used in similar experiments with the isolated, perfused lung model.[10,11] Following a 15-minute period of equilibration, baseline measurements of PA and PV were obtained, from which pulmonary vascular resistance (R_t) was calculated. The TXA_2 receptor agonist 9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46 619) (7.1×10^{-8} mol/L) was then added to the perfusate. Fifteen minutes later, PA and PV pressures were recorded and the capillary filtration coefficient (K_f) and R_t determined. This dose of U-46 619 is similar to that used by other investigators in this experimental system[12] and induces an increase in pulmonary microvascular permeability similar to that of several in vivo injury models, including intestinal reperfusion injury.[1] In preliminary experiments, a dose-response relationship was demonstrated between increases in U-46 619 concentrations (10^{-11} to 10^{-7} mol/L) and increases in K_f. Furthermore, this increase in K_f was totally prevented by the TXA_2 receptor antagonist SQ-29 548 (data not shown). Perfusate was collected for the measurement of cyclic guanosine monophosphate (cGMP) concentration.

MEASUREMENT OF PULMONARY MICROVASCULAR DYSFUNCTION

Capillary Filtration Coefficient

Pulmonary microvascular permeability was quantitated by determining the K_f as described by Drake et al.[13] Fifteen minutes after the addition of U-46 619, the capillary pressure was measured using the double occlusion technique.[14] Pulmonary venous pressure was then elevated from

weight. This was significantly greater than that of lungs perfused with Krebs-Henseleit buffer alone (0.10 ± 0.01 pmol/mL per gram of lung weight, $P<.001$). The concentration of cGMP within the perfusate of lungs exposed to U-46 619 and L-NAME was 85% less than that of lungs exposed to U-46 619 alone ($P<.001$).

EFFECT OF TXA_2 RECEPTOR ACTIVATION ON PULMONARY MICROVASCULAR FUNCTION

Capillary Filtration Coefficient

The addition of U-46 619 to the isolated, perfused lung model resulted in a 3-fold increase in K_f when compared with lungs perfused with buffer alone ($P<.01$). This is illustrated in **Figure 2**. The K_f of lungs perfused with U-46 619 (7.1×10^{-8} mol/L) and SQ-29 548 (2 μmol/L) was 0.006 ± 0.002 g · minute^{-1} · millimeters of mercury^{-1} · 100 g body weight^{-1}. This was not different than that of lungs exposed to Krebs-Henseleit buffer alone (0.007 ± 0.001 g · minute^{-1} · millimeters of mercury^{-1} · 100 g of body weight^{-1}). These data suggest that the effects of U-46 619 are mediated through activation of the TXA_2 receptor and not a toxic or nonspecific effect.

Pulmonary Vascular Resistance

The addition of U-46 619 to the isolated, perfused lung model resulted in prompt vasoconstriction, with an average increase in PA of 3.0 ± 0.3 mm Hg over baseline measurements ($P<.01$). These data are shown in **Figure 3**. As expected, U-46 619 altered R_t in a manner similar to PA. These data are shown in **Figure 4**.

EFFECT OF NO ON TXA_2-MEDIATED CHANGES IN PULMONARY MICROVASCULAR FUNCTION

Capillary Filtration Coefficient

Inhibition of NO release with L-NAME (10^{-4} mol/L) had no effect on U-46 619–induced changes in K_f. These data are shown in Figure 2. Even a 100-fold increase in the dose of L-NAME (10^{-2} mol/L) did not significantly alter the effect of U-46 619 on K_f (0.019 ± 0.003 g · minute^{-1} · millimeters of mercury^{-1} · 100 g of body weight^{-1}). The addition of SNP (10^{-8} mol/L) to the perfusate reduced U-46 619–induced changes in K_f by 50% ($P<.01$). A 100-fold decrease in the concentration of SNP (10^{-10} mol/L) had a similar

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8 to 10 mm Hg by raising the height of the venous reservoir. This resulted in a 2-component weight gain consisting of an initial rapid increase related primarily to recruitment and distention of the vascular bed (minutes 0-1) and a second slow, constant weight increase due to fluid filtration across the microvasculature (minutes 1-5). After 5 minutes of partial venous occlusion, capillary pressure was again measured (prior to releasing the partial outflow occlusion). The K_f was calculated:

$$K_f = (\Delta W/\Delta T)/\Delta P,$$

where ΔW is the change in lung weight between minutes 1 and 5 of partial venous outflow occlusion; ΔT is the duration of partial outflow occlusion during which ΔW is measured; and ΔP is the difference between the partial outflow occlusion and the capillary pressure. The K_f is normalized to body weight and expressed as gram · minute^{-1} · millimeters of mercury^{-1} · 100 g body weight^{-1}.

Pulmonary Vascular Resistance

Immediately prior to the addition of U-46 619, PA and PV pressures were recorded and compared with measurements obtained 15 minutes after the addition of U-46 619. The total R_t was calculated as the total pressure drop across the lung:

$$R_t = (PA - PV)/Q,$$

where Q is the flow through the isolated perfused lung. The calculation of pulmonary vascular resistance was normalized for body weight and expressed as the difference before and after treatment with U-46 619 (millimeters of mercury · milliliters^{-1} · minute^{-1} · 100 g body weight^{-1}).

REAGENTS

Krebs-Henseleit buffer was made fresh daily from a stock solution. The pH of the solution was 7.4 to 7.5 and remained stable throughout the perfusion. Bovine serum albumin (3 g/100 mL; Sigma-Aldrich Co) was added to the perfusate immediately prior to use. Sodium nitroprusside (Gensia Pharmaceuticals Inc, Irvine, Calif) was suspended in sterile water and then diluted in the perfusate daily for a working solution. Nitroglycerine-nitro-L-arginine-methyl ester (Sigma-Aldrich Co) was suspended in perfusate daily prior to use. The U-46 619 (Cayman Chemical Co, Ann Arbor, Mich) was supplied as a stock solution in methyl acetate and diluted in perfusate daily for a final concentration of 7.1×10^{-8} mol/L. In one experiment, the specificity of U-46 619 was examined by adding the TXA_2 antagonist SQ-29 548 (2 µmol/L) (Cayman Chemical Co) to the perfusate of the isolated, perfused lung apparatus. This agent was suspended in ethanol and then diluted in perfusate for a concentration of 200 mol/L prior to use.

MEASUREMENT OF NO RELEASE

A 1-mL aliquot of perfusate was obtained from the venous port of the isolated, perfused lung at the conclusion of the experiment. This was frozen at –20°C until assayed for cGMP by a commercially available enzyme immunoassay (Cayman Chemical Co). Nitric oxide activates soluble guanylyl cyclase, converting GMP to cGMP. Increased concentrations of cGMP are thought to reflect endogenous NO generation.[12]

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. Analysis of unpaired data from 2 groups was performed using a 2-tailed, unpaired Student *t* test. Data from multiple groups were compared by analysis of variance with a Fisher post hoc test (StatView R 4.0; Abacus Concepts, Berkeley, Calif). Statistical significance was considered for a type I error of less than 5%. All experiments have been approved by the Committee on the Care and Use of Animals at the University of Texas Southwestern Medical School and Dallas Veterans Affairs Medical Center.

effect on U-46 619–induced changes in K_f (0.006 ± 0.001 g · minute^{-1} · millimeters of mercury^{-1} · 100 g body weight^{-1}). In the absence of U-46 619, the addition of SNP or L-NAME had no effect on the K_f of Krebs-Henseleit buffer–perfused lungs.

Pulmonary Vascular Resistance

The effect of L-NAME and SNP on U-46 619–induced vasoconstriction is shown in Figures 3 and 4. In contrast to its effect on K_f, the presence of L-NAME in the perfusate of the isolated, perfused lung notably enhanced the vasoconstrictive effects of U-46 619. The change in PA induced by U-46 619 in the presence of L-NAME was twice that induced by U-46 619 alone ($P<.01$). The change in R_t of lungs exposed to L-NAME and U-46 619 was about 60% greater than that of lungs treated with U-46 619 alone ($P = .06$). The addition of the 10-mmol/L concentration of L-NAME to the isolated, perfused lung did not result in greater vasoconstriction than that induced by the 100-µmol/L concentration. The addition of SNP (10^{-8} and 10^{-10} mol/L) to the perfusate completely abolished the vasoconstrictive effects of U-46 619 with or without L-NAME. The addition of L-NAME or SNP to the perfusate of the isolated, perfused lung in the absence of U-46619 had no effect on PA or R_t.

COMMENT

These experiments are consistent with the hypothesis that the lungs release NO as a protective, compensatory mechanism in response to TXA_2. Evidence is provided by the observations that (1) cGMP is released by the lungs in response to TXA_2 receptor activation; (2) inhibition of the constitutive release of NO by the lungs exacerbates the vasoconstrictive effects of U-46 619; and (3) the provision of exogenous NO attenuates the vasoconstriction and increased capillary permeability that characterizes TXA_2-mediated microvascular dysfunction.

Thromboxane A_2 has been incriminated in the pathogenesis of pulmonary microvascular dysfunction in adult respiratory distress syndrome[4] and in a plethora of animal models of acute lung injury.[1-3] The U-46 619 binds and activates TXA_2 receptor and is a useful tool for studying its effects on microvascular function.[15] The principal effects of the activation of TXA_2 receptor on the mi-

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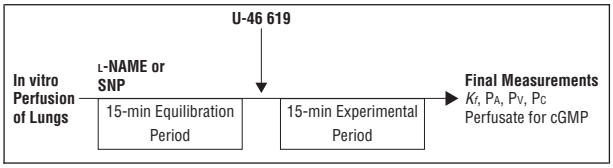

Figure 1. *In the experimental protocol, normal rat lungs were perfused with Krebs-Henseleit buffer (10^{-4} mol/L) with or without the nitric oxide synthase inhibitor nitroglycerine-nitro-L-arginine methyl ester (L-NAME) (10^{-10} mol/L) or the nitric oxide donor sodium nitroprusside (SNP) (10^{-4} and 10^{-2} mol/L). The thromboxane A_2 receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46 619) (7.1×10^{-8} mol/L) was added to the perfusate. Pulmonary microvascular dysfunction was then quantitated by measuring the capillary filtration coefficient (K_f) and pulmonary artery (PA), venous (PV), and capillary (PC) pressures. Perfusate was also sampled for measurement of cyclic guanosine monophosphate (cGMP) concentrations as an index of nitric oxide release.*

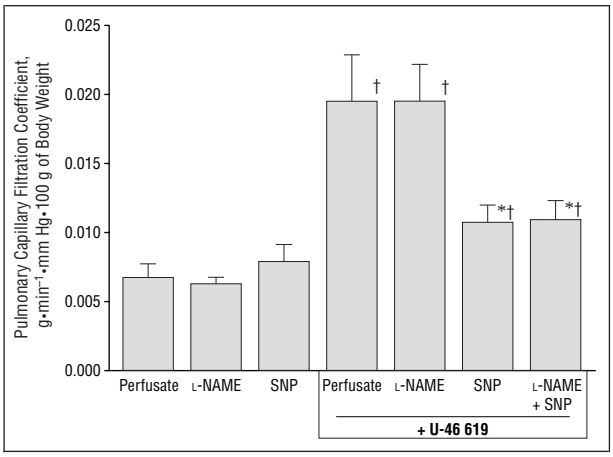

Figure 2. *The effect of the thromboxane A_2 receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostoglandin $F_{2\alpha}$ (U-46 619) (7.1×10^{-8} mol/L) on the pulmonary capillary filtration coefficient (K_f) of isolated, perfused lungs exposed to nitroglycerine-nitro-L-arginine methyl ester (L-NAME) (10^{-4} mol/L) and sodium nitroprusside (SNP) (10^{-8} mol/L). The K_f is expressed as grams · minutes^{-1} · millimeters of mercury^{-1} · 100 g body weight^{-1} (asterisk indicates P<.05 vs perfusate alone; dagger, P<.05 vs perfusate + U-46 619).*

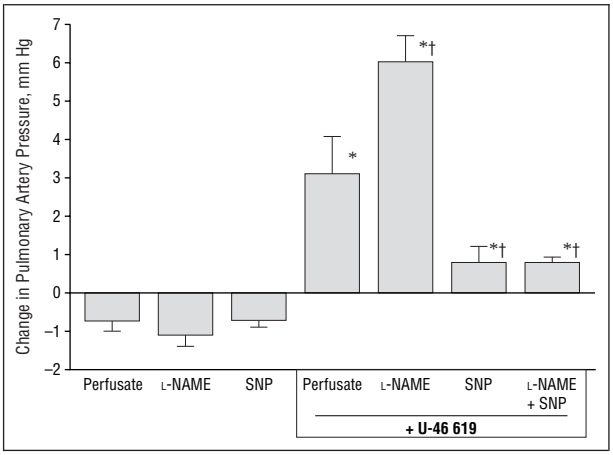

Figure 3. *The effect of thromboxane A_2 receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46 619) (7.1×10^{-8} mol/L) on the pulmonary artery pressure of Krebs-Henseleit buffer–perfused lungs exposed to nitroglycerine-nitro-L-arginine methyl ester (L-NAME) (10^{-4} mol/L) and sodium nitroprusside (SNP) (10^{-8} mol/L). These data are expressed as the difference in pulmonary artery pressure before and 15 minutes after the addition of U-46 619 to the perfusate (asterisk indicates P<.01 vs perfusate alone; dagger, P<.01 vs U-46 619 alone).*

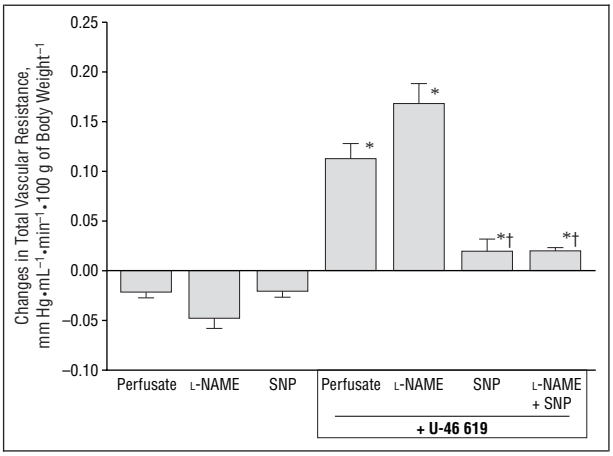

Figure 4. *The effect of thromboxane A_2 receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46 619) (7.1×10^{-8} mol/L) on the total vascular resistance of Krebs-Henseleit buffer–perfused lungs exposed to nitroglycerine-nitro-L-arginine methyl ester (L-NAME) (10^{-4} mol/L) and sodium nitroprusside (SNP) (10^{-8} mol/L). These data are expressed as the difference in vascular resistance before and 15 minutes after the addition of U-46 619 to the perfusate and as millimeters of mercury · milliliters^{-1} · minutes^{-1} · 100 g of body weight^{-1} (asterisk indicates P<.01 vs perfusate alone; dagger, P<.01 vs U-46 619 alone).*

crovasculature include vasoconstriction that results in increased capillary hydrostatic pressure and enhanced permeability to fluid and solutes. Together, these phenomena contribute to the development of pulmonary edema during systemic inflammatory states.

Our study demonstrates that within 15 minutes of TXA_2 receptor activation there is a 4-fold increase in pulmonary NO release. These findings are similar to those of Wilson et al[12,16] using a similar experimental model. Many investigators have implicated increased vascular shear stress as an important signal for endothelial NO release.[12,16-18] Vascular shear stress is proportional to the flow rate and viscosity of the perfusate and inversely proportional to the diameter of the vessel. Hence, vasoconstrictors such as U-46 619 should increase the shear forces in vessels perfused at a constant flow rate such as in our study. Wilson et al[12] demonstrated that the addition of U-46 619 to lungs perfused at a constant rate increased NO release; this effect was not observed with those perfused at a constant pressure. Other investigators have related NO release in isolated lung models to increasing perfusate viscosity[16] and flow rates.[19]

Our study also suggests that the constitutive release of NO by the pulmonary vascular endothelium attenuates the vasoconstrictive effects of TXA_2. Evidence is provided by the observations that L-NAME enhances and SNP prevents U-46 619–induced pulmonary vasoconstriction. Similar results have been reported by other investigators using the isolated, perfused lung model in which U-46 619–induced vasoconstriction was found to be augmented by the NO synthase inhibitor N^{ω}-methyl-L-arginine and the soluble, guanylyl cyclase inhibitor 6-anilino-5,8-quinolinodione (LY 83583).[12] In other in vivo and in vitro models of acute lung injury, NO administered by inhalation or intravascular NO donors attenuates the vasoconstrictive effects of oxidants,[10,20] remote organ injury,[21,22] and endotoxemia[23]; agents that inhibit NO release enhance the vasoconstrictive effects of these

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injuries.[10,20,21,23] The therapeutic potential of these findings has been realized with the use of inhaled NO in the management of patients with adult respiratory distress syndrome and other disease states in which pulmonary hypertension is a important component.[24,25]

It is less clear whether endothelial-derived NO protects the lung from TXA_2-mediated changes in microvascular permeability. This conclusion is based principally on the observation that inhibiting NO release with L-NAME had no effect on U-46 619–induced changes in K_f; however, the NO donor SNP attenuated U-46 619–induced changes in permeability. One would have anticipated that if endogenous NO release protects the pulmonary microvasculature from TXA_2-mediated changes in permeability, inhibition of NO synthesis would exaggerate the changes in K_f associated with U-46 619.

These results may have been related to the neutrophil-depleted nature of this experimental model. Investigators using similar buffer-perfused lung models have demonstrated that inhibition of NO synthase does not affect microvascular permeability in either normal or acutely injured lungs.[10,11] In contrast, the provision of even small quantities of NO has been shown to protect the lung from TXA_2-mediated microvascular dysfunction and dysfunction associated with oxidant exposure,[10] pulmonary ischemia-reperfusion injury,[11] and remote tissue reperfusion injury.[21,22,26] Interestingly, in vivo models of acute lung injury show that inhibition of NO synthase with L-NAME notably increased the extravasation of plasma proteins into the lung.[21,26] In each of these models, L-NAME–induced increases in pulmonary microvascular permeability have been associated with increases in neutrophil sequestration within the lung, a phenomenon prevented by NO donors. These data are consistent with the findings of Kurose et al,[27] who attributed, at least in part, L-NAME–induced increases in intestinal microvascular permeability to neutrophil recruitment and to neutrophil-mediated tissue injury. In these and subsequent studies, inhibition of NO synthesis with L-NAME has been shown to up-regulate the expression of many endothelial adhesion glycoproteins, including P-selectin and intracellular adhesion molecule–1, suggesting an important functional relationship between endothelial-derived NO production and the expression of various endothelial adhesion molecules.[27,28]

There are several potential mechanisms by which exogenously administered NO may have attenuated TXA_2-mediated changes in microvascular permeability. One of these mechanisms is a direct effect on the endothelial cytoskeleton along with stress fiber disassembly and expansion of intercellular junctions.[29-31] Nitric oxide has been postulated to maintain normal microvascular permeability by preserving endothelial cytoskeletal integrity and normal intercellular junctions,[27,32] although the mechanisms by which this might occur are poorly defined.

Given the neutrophil-depleted nature of the isolated, perfused lung system used in our study, it would seem unlikely that the primary effector of TXA_2-mediated changes in microvascular function is the neutrophil. Nevertheless, Siebert et al[33] have suggested that neutrophils sequestered within the pulmonary microvasculature during harvesting of the lungs may contribute to the microvascular dysfunction in this experimental model. This may be particularly relevant given the previously described relationship between the constitutive release of NO and neutrophil-mediated tissue injury.[21,26-28]

Last, the release of NO by the vascular endothelium may also serve to limit oxygen-free, radical-mediated endothelial injury. Nitric oxide inhibits neutrophil superoxide anion production by a direct effect on nicotinamide adenine dinucleotide phosphate oxidase.[34] Furthermore, many investigators have presented evidence that NO released constitutively by the endothelium scavenges superoxide radical and prevents oxidant-mediated tissue injury.[35,36] In addition, NO may also induce the synthesis and activity of other antioxidants such as heme oxygenase.[37]

Many laboratory studies and a handful of clinical studies have sparked tremendous enthusiasm for the development of therapeutic regimens that either augment NO delivery to the microvasculature or inhibit its exaggerated production. Dysfunction of NO formation by the vascular endothelium has been implicated in the pathogenesis of diseases as diverse as hypertension, hypercholesterolemia, diabetes mellitus, ischemia reperfusion injury, angina pectoris, and various forms of circulatory shock.[38-43] Several investigators have suggested that the administration of NO by inhalation may be associated with notable improvement in pulmonary function in patients with adult respiratory distress syndrome.[24,25] Our study suggests that the beneficial effect of exogenous administration of NO may be due to the restoration of normal pulmonary microvascular permeability and reduction of pulmonary capillary hydrostatic pressure. Other studies have related the hemodynamic "collapse" associated with septic shock to the overproduction of NO. These investigators postulated that inhibition of this exaggerated NO release will prevent or attenuate the hypotension associated with septic shock.[44-46] The observations in our study are consistent with those of other investigators,[47,48] suggesting that the pharmacologic inhibition of endothelially derived NO (eg, that which occurs following treatment with nonselective NO synthase inhibitors such as L-NAME or nitroglycerine-monomethyl-L-arginine) may in fact be detrimental. In patients with acute lung injury, the nonselective inhibition of NO release may actually worsen pulmonary hypertension, ventilation-perfusion mismatch, and oxygen delivery in these already critically ill patients.

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