Endogenous nitric oxide decreases xanthine oxidase-mediated neutrophil adherence: role of P-selectin

Circulating xanthine oxidase (XO), which is increased in patients with acute respiratory distress syndrome (6), may participate in the systemic activation of inflammatory cells, which is the signature event defining multiple organ failure. For instance, hepatic ischemia-reperfusion causes massive release of XO into the circulation and consequent lung injury (23). In addition, after intestinal ischemia-reperfusion, circulating XO levels increase and promote lung neutrophil retention (17). However, endogenous protective mechanisms that may be activated during such secondary pulmonary injury have not been well studied. Recently, we found that endogenous nitric oxide (NO) diminished lung injury and neutrophil recruitment after intestinal ischemia-reperfusion (20). We therefore hypothesized that exposure of endothelial cells (EC) to XO would stimulate the production of NO by EC, and NO, in turn, would diminish adhesive interactions between neutrophils and EC. In the present study, we demonstrate that EC stimulated with XO produce NO in an apparent negative-feedback cycle with respect to neutrophil adherence. The opposing effects of XO and NO on neutrophil adherence appear to be mediated through their disparate effects on P-selectin expression.

MATERIALS AND METHODS

Source of reagents. Sodium nitrite was obtained from Mallinckrodt (St. Louis, MO), blocking monodonal antibody CLB-throm/6 against P-selectin from Monosan, and unconjugated and peroxidase-conjugated rabbit polyclonal antibodies to von Willebrand factor (vWF) from Dako (Carpinteria, CA). XO (grade II from bovine milk, 1.2 U/mg), superoxide dismutase (SOD; bovine erythrocyte, 3,000 U/mg), N-nitro-arginine (L-NNA), sodium nitroprusside (SNP), and all other reagents were obtained from Sigma Chemical (St. Louis, MO).

Endothelial cell culture. Bovine pulmonary artery EC were harvested by using collagenase digestion, passed twice in d-valine minimum essential medium (MEM) to minimize smooth muscle contamination, and cultured in Eagle's MEM with 10% fetal calf serum (18). EC were studied after 2–3 passages.

Nitrite release. Production of NO by EC was assessed by release of nitrite (5). EC were plated in 96-well enzyme-linked immunosorbent assay (ELISA) plates and grown to confluence. EC were washed twice with Hanks' balanced salt solution (HBSS) and exposed to 10 µM XO, 200 µM hypoxanthine (HX), and 30 U/mL SOD and/or 200 µM L-NNA for 30 min at 37°C in a total volume of 200 µL HBSS/well. Media (50 µL) were removed and added to 50 µL Griess reagent in a separate ELISA plate and incubated for 10 min at room temperature with shaking. The optical density at 540 nm was measured with a microplate reader (Bio-Tek EL 340, Winooski, VT) and compared with a standard curve of sodium nitrite in HBSS.

Neutrophil-EC dynamic interactions. Neutrophils were isolated from healthy human donors by Percoll gradient separation (18). EC were passaged into gelatin-coated glass capillary tubes 1.1 mm in internal diameter (Scientific Manufacturing Industries, Emeryville, CA). Fresh medium was flushed once through the tubes 4–5 h after seeding, and EC were grown overnight into confluent monolayers occupying approximately one-half of the internal surface of the tube. After treatment with 10 µM XO, 200 µM HX, 200 µM L-NNA, 25 µM SNP, and/or 4 µg/mL anti-P-selectin antibodies for 30 min at 37°C, capillary tubes were used to stage the section of an inverted phase-contrast microscope (Nikon) with EC in the dependent position and kept at 37°C. Neutrophils (2 × 10^6/mL in HBSS with 5% fetal calf serum) were infused by syringe pump at a constant flow rate. With the use of the Hagan-Poiseille equation (14), the shear rate was calculated to be 96 s^-1. With the assumption of a viscosity of 0.0084 Pa s at 37°C (13), this corresponds to an approximate shear stress of 0.81 dyn/cm². Neutrophil-EC interactions were recorded on VCR (Javelin, CA) for later playback analysis. The number of rolling neutrophils passing a standardized 500-µm bar and the number of firmly adherent (no movement for 30 s) neutrophils per 0.25 mm² were determined for at least three random fields per tube.

P-selectin surface expression and vWF release. EC were grown to confluence in 96-well ELISA plates and then exposed to 10 µM XO, 200 µM HX, and/or 200 µM L-NNA for 30 min at 37°C. EC were fixed with 1% paraformaldehyde and then washed twice with HBSS and blocked with 2% bovine
serum albumin (BSA) in HBSS at 25°C for 30 min. After two subsequent washes, EC were incubated with anti-P-selectin (1:500 in 0.1% BSA) for 30 min at 37°C, washed twice, incubated with rabbit anti-mouse immunoglobulin G-peroxidase (1:1,000 in 0.1% BSA) for 20 min at 25°C, washed twice, and developed with o-phenylenediamine dihydrochloride. VWF release was measured by sandwich ELISA as previously described (7) with the use of unconjugated and peroxidase-conjugated antibodies at a 1:500 dilution and expressed as absorbance units.

Statistical analysis. Group means were analyzed by one-way analysis of variance with Student-Newman-Keuls multiple-group comparisons.

RESULTS

Effect of XO and L-NNA on nitrite release. Treatment of EC with HX and XO (HX + XO) increased (P < 0.01) release of nitrite into the media, compared with control EC (Fig. 1). Addition of SOD to prevent superoxide anion radical-mediated oxidation of NO to nitrate further increased nitrite release from HX + XO-treated EC (P < 0.001) compared with SOD-treated controls or HX + XO-treated EC not exposed to SOD. Addition of the NO synthase inhibitor L-NNA decreased nitrite levels (P < 0.001) released from HX + XO-treated EC exposed to SOD.

Effect of XO and L-NNA on neutrophil-EC interactions. Treatment of EC with either HX + XO or L-NNA increased the numbers of rolling (Fig. 2, P < 0.01) and adherent (Fig. 3, P < 0.05) neutrophils at a shear rate of 96 s⁻¹ compared with untreated controls. In addition, treatment of EC with both HX + XO and L-NNA increased rolling (P < 0.001) and adherence (P < 0.05) compared with EC treated with either HX + XO or L-NNA alone. Cotreatment with antibodies against P-selectin decreased both rolling (P < 0.05) and adherence (P < 0.05) of neutrophils to baseline values in EC treated with HX + XO and/or L-NNA but did not alter rolling or firm adherence of neutrophils to untreated control EC (P > 0.05). Neutrophil rolling increased (P < 0.01) as early as 15 min into L-NNA exposure, although only at a lower shear rate of 38 s⁻¹ (data not shown).

Effect of XO and L-NNA on P-selectin surface expression and VWF release. Treatment of EC with HX + XO or L-NNA increased P-selectin surface expression (P < 0.001) compared with control EC (Fig. 4). Moreover, treatment of EC with both HX + XO and L-NNA further

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**Fig. 1.** Treatment of endothelial cells (EC) with xanthine oxidase (XO; 10 µM/ml) plus hypoxanthine (HX; HX + XO; 200 µM) increased release of nitrite into the media compared with control EC (*P < 0.01). Values are means ± SE; n = 12 individual determinations. Treatment of EC with superoxide dismutase (SOD; 30 U/ml), XO, and HX had increased release of nitrite compared with EC treated with SOD alone or HX + XO alone (**P < 0.001). N⁴-nitro-L-arginine (L-NNA) (200 µM) decreased nitrite release from EC treated with SOD, XO, and HX (***P < 0.001). HBSS, Hanks' balanced salt solution.

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**Fig. 2.** Treatment of EC with either XO (10 µM/ml) plus HX (200 µM) or L-NNA (200 µM) increased the number of rolling neutrophils compared with untreated controls (open bars, filled bar; *P < 0.01). Values are means ± SE; n = 5–11 individual capillary tubes. Treatment of EC with both HX + XO and L-NNA increased neutrophil rolling compared with EC treated with either HX + XO or L-NNA alone (**P < 0.001). Cotreatment with antibodies against P-selectin (hatched bars) decreased neutrophil rolling in EC treated with HX + XO and/or L-NNA (**P < 0.05) but did not alter rolling in untreated control EC (P > 0.05). Neutrophil rolling in all groups treated with anti-P-selectin were not different from untreated controls (P > 0.05).

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**Fig. 3.** Treatment of EC with either XO (10 µM/ml) plus HX (200 µM) or L-NNA (200 µM) increased the number of firmly adherent neutrophils compared with untreated controls (*P < 0.05). Values are means ± SE; n = 5 individual capillary tubes. Treatment of EC with both HX + XO and L-NNA increased neutrophil adherence compared with EC treated with either HX + XO or L-NNA alone (**P < 0.001). Cotreatment with antibodies against P-selectin (hatched bars) decreased adherence to EC treated with HX + XO and/or L-NNA (**P < 0.05) but did not alter adherence to untreated control EC (P > 0.05). Neutrophil adherence in all groups treated with anti-P-selectin were not different from untreated controls (P > 0.05).
increased P-selectin surface expression relative to EC treated with either HX+XO or L-NNA alone (P < 0.001). Treatment of EC with HX+XO and/or L-NNA also increased release of vWF into media (P < 0.05) compared with control EC (Fig. 5). vWF levels after treatment of EC with both HX+XO and L-NNA were not significantly different (P > 0.05) from vWF levels observed after treatment of EC with either HX+XO or L-NNA alone.

Effect of SNP on neutrophil rolling. Treatment of EC with HX+XO increased the number of rolling (P < 0.001) and firmly adherent (P < 0.01) neutrophils compared with untreated controls (Fig. 6). EC co-treated with both HX+XO and SNP supported less neutrophil rolling (P < 0.001) and firm neutrophil adherence (P < 0.05) compared with EC treated with HX+XO alone.

**DISCUSSION**

After injury to one organ, XO is released into the circulation and increases neutrophil sequestration in other organs (17). However, endogenous mechanisms that may be activated to limit the secondary spread of inflammation have not been studied. Recently, we observed that endogenous production of NO by the lung prevented pulmonary inflammation following mesenteric ischemia-reperfusion (20), suggesting a possible protective endothelial response against circulating XO. In the present study, we found that XO stimulated NO production by EC, and this enhanced NO generation caused a decrease in P-selectin-dependent neutrophil adhesion to EC.

We found first that XO increased L-NNA-inhibitable nitrite release from EC, consistent with an increased production of NO. The effect was relatively rapid, occurring within 30 min, consistent with activation of constitutive endothelial NO synthase (eNOS). Although the mechanism by which XO may activate eNOS is unclear, it is notable that XO increases cytosolic free calcium in EC (2) and induces vWF secretion from EC, a process that is also Ca^{2+} dependent (22).

The XO-stimulated release of NO appeared to partially counteract the proadhesive effects of XO because cotreatment of EC with L-NNA further increased neutrophil rolling behavior and adherence to XO-treated EC. In support of this interpretation, addition of the
NO donor SNP decreased neutrophil rolling and adherence to XO-treated EC. These data are consistent with observations that exogenous administration of NO by NO donors decreases leukocyte adherence to XO-treated mesenteric venules (3). The situation may be similar to ischemic-reperfused tissues, although conflicting studies report that exogenous NO donors either decrease (4) or do not affect (9) leukocyte rolling in reperfused vessels. In the study of Gauthier et al. (4), however, the NO donor was given intravenously 10 min before reperfusion, whereas it was superfused just before reperfusion in the study of Kubes et al. (9). Notwithstanding, the effect of endogenously produced NO on oxidant-induced neutrophil adhesion has not previously been recognized, and this mechanism needs to be considered for its ability to alter neutrophil adhesion.

Treatment of EC with l-NNA alone also increased both neutrophil rolling and adherence, suggesting that basal production of NO by EC prevents excessive adhesion of neutrophils to uninjured endothelium. This correlates well with observations that N\textsuperscript{G}-nitro-l-arginine methyl ester (l-NNAME) increases leukocyte rolling (1) and adherence (10, 12) in mesenteric vessels within 30–60 min in vivo. In one study, lack of an effect of l-NNAME on static neutrophil adherence in vitro in a similar time frame (16) may relate to the lack of shear stress, which rapidly induces NO release by EC (11).

NO decreased neutrophil-EC interactions, at least in part, by decreasing P-selectin expression. First, blocking monoclonal antibodies against P-selectin eliminated XO- and l-NNA-induced neutrophil rolling and adherence. The effect of anti-P-selectin on adherence may be explained by the requirement of rolling for firm adherence to occur at the shear rate studied in this system (19). Second, surface expression of P-selectin increased after treatment of EC with l-NNA and/or XO. P-selectin is a preformed glycoprotein that is stored in endothelial Weibel-Palade bodies. Accordingly, it is significant that treatment of EC with l-NNA and/or XO also caused release of vWF, the other principal protein associated with these organelles (15). Therefore, XO and endogenous NO appear to have opposing effects on the activation of Weibel-Palade bodies. This Yin-Yang relationship may also explain why the selective antagonist fucoidan abolishes lung injury in l-NNAME-treated rats subjected to intestinal ischemia-reperfusion (20), a condition that causes increased circulating levels of XO (17).

Suppression of endogenous NO also increases P-selectin expression in vivo (1), and this in part has been attributed to activation of mast cells with consequent release of histamine, a potent stimulus for P-selectin expression (21). For example, treatment of rats with l-NNAME causes intestinal mast cell degranulation (8). The present study, in which mast cells are not present, suggests that NO can have a direct effect on EC, acting in an autocrine fashion to stabilize Weibel-Palade bodies and decrease P-selectin-mediated neutrophil tethering.

In summary, treatment of EC with XO initiates both pro- and antiadhesive pathways, the latter being mediated by endogenous NO. Such stimulation of NO production by oxidant-stressed endothelium may be an important protective response that diminishes indiscriminate dissemination of inflammation during systemic illnesses such as sepsis, shock, and multiorgan failure.

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