XO increases neutrophil adherence to endothelial cells by a dual ICAM-1 and P-selectin-mediated mechanism

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Circulating xanthine oxidase (XO) can modify adhesive interactions between neutrophils and the vascular endothelium, although the mechanisms underlying this effect are not clear. We found that treatment with XO of bovine pulmonary artery endothelial cells (EC), but not neutrophils or plasma, increased adherence, suggesting that XO had its primary effect on EC. The mechanism by which XO increased neutrophil adherence to EC involved binding of XO to EC and production of H2O2. XO also increased platelet-activating factor production by EC by a H2O2-dependent mechanism. Similarly, the platelet-activating factor-receptor antagonist WEB-2086 completely blocked XO-mediated neutrophil EC adherence. In addition, neutrophil adherence was dependent on the β2-integrin Mac-1 (CD11b/CD18), leukocyte functional antigen-1 (CD11a/CD18), and P-selectin ligations, by a mechanism that involves platelet-activating factor and H2O2 as intermediates.

Materials and Methods

Source of reagents. Platelet-activating factor (PAF; from bovine heart lecithin), superoxide dismutase (SOD; bovine erythrocyte, 3,000 U/mg) and XO (grade III from bovine milk, 1.2 U/mg) were obtained from Sigma Chemical (St. Louis, MO). Blocking monoconal antibodies against CD11a and CD54 were from AMAC (Westbrook, ME). Blocking monoclonal antibodies against CD11b were from Dako (Carpinteria, CA). WEB-2086 was a kind gift of Boehringer Ingelheim (Ridgefield, CT). Heparin (porcine intestinal mucosa) was purchased from SoloPak Laboratories (Elk Grove Village, IL). 4-Hydroxy-2,6,6-tetramethylpiperidinyloxyl (Tempol) was obtained from Aldrich (Milwaukee, WI). 3H]acetate (500 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Hypoxanthine (HX) and all other reagents were purchased from Sigma Chemical.

Multicellular Xanthine oxidase; platelet activating factor; CD18; CD11b; Mac-1; CD11a; leukocyte functional antigen-1; heparin; hydrogen peroxide; multiorgan failure; acute respiratory distress syndrome
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37°C. In some experiments, EC were incubated with various agents in 50% pooled normal human plasma. EC were then washed twice with HBSS, and unstimulated polymorphonuclear leukocytes (PMN; 10^6/well) were gently added. In some experiments, WEB-2086 was added to EC and PMN. After a 30-min incubation at 37°C, nonadherent neutrophils were collected and pooled with the first wash. Residual 51Cr in adherent neutrophils was released with NaOH and quantified by liquid scintigraphy. Adherence was defined as the percentage of counts per minute (cpm) in the media and first wash, relative to total cpm in media plus wash plus EC (cpm media + first wash/cpm media + first wash + EC releasate).

Visual inspection under all conditions revealed persistence of EC monolayers before NaOH lysis. In some experiments, monolayers were first pretreated with XO and/or heparin for 60 min at 37°C then triply washed and incubated with neutrophils in the presence of HX. In some experiments, neutrophils were fixed with paraformaldehyde (1%) for 10 min at 25°C after 51Cr loading.

**PAF assay.** EC monolayers in 25-cm² flasks were tripym washed to remove serum, then incubated with 25 µCi [3H]acetate in 1 ml HBSS with 10 mM N-[2-hydroxyethyl]pipera-

**RESULTS**

Effect of XO on neutrophil adherence. Exposure of EC and neutrophils to XO (5–30 mU/ml) and substrate (HX) for 10–30 min increased (P < 0.01) neutrophil adherence to EC monolayers (Figs. 1 and 2). Addition of substrate alone did not affect (P > 0.05) neutrophil adherence. Pretreatment of EC, but not neutrophils, with XO and HX increased (P < 0.001) neutrophil adherence to EC monolayers (Fig. 3). Addition of plasma pretreated with XO and HX had no effect on neutrophil adherence. [3H]acetate incorporation into PAF was calculated (36). Lipids were visualized with iodine vapor, and lanes were scraped off in fractions by using the mobility of authentic PAF as a guide. After quantification of [3H]acetate incorporation, the amount of [3H]acetate incorporated into PAF was calculated (36). VWF release and P-selectin and ICAM-1 surface expression. EC were grown to confluence in 96-well enzyme-linked immunosorbent assay (ELISA) plates and exposed to XO (10 mU/ml) and HX (200 µM) in HBSS for 30 min. Control EC were exposed to IFN-α/β (24,000 U/ml) for 24 h to increase ICAM-1 expression. EC were 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 either anti-P-selectin or anti-ICAM-1 (1:500 in 0.1% BSA) for 30 min at 37°C, washed twice, then incubated with rabbit anti-mouse IgG-peroxidase (1:1,000 in 0.1% BSA) for 20 min at 25°C. After two subsequent washes, the plates were developed with o-phenylenediamine dihydrochloride (OPD). Baseline values (EC treated with OPD in the absence of antibodies) were subtracted from sample values to account for the low levels of endogenous EC peroxidases. VWF released into the media was assessed by ELISA as previously described (13).

**Dynamic neutrophil adherence.** EC were passaged into gelatin-coated glass capillary tubes (1.1-mm ID, Scientific Manufacturing Industries, Emeryville, CA). After attachment of EC, medium was changed once, and EC were allowed to grow overnight to form confluent monolayers on approximately one-half of the internal surface of the tube. After treatment of EC with various agents, the capillary tubes were secured on the stage of an inverted microscope with EC in the dependent position. Neutrophils (10^6/ml) were perfused through the tube with a syringe pump at a constant rate (0.173 ml/min). A second syringe pump infused HBSS + 5% fetal calf serum at a variable rate. From the measured total flow rate and the cylindrical geometry of the tube, the shear at the surface of the tube was calculated by using the Hagen-Poiseuille equation (22). The interaction of neutrophils with EC was recorded on videocassette recorder (Javelin recorder) for later playback analysis. The number of rolling neutrophils crossing a standardized 250-µm bar, neutrophil rolling velocity (average of 6–12 neutrophils), and number of firmly adherent neutrophils (no movement for at least 30 s) per 0.0625 mm² were determined for three random fields per tube.

**Adherence of neutrophils to EC monolayers increased after treatment of endothelial cells (EC) with xanthine oxidase (XO; 5–30 mU/ml) and hypoxanthine (HX; 200 µM) for 30 min (P < 0.01), compared with untreated EC. Values are means ± SE of 6 individual determinations.**

![Fig. 1. Neutrophil adherence increased after treatment of endothelial cells (EC) with xanthine oxidase (XO; 5–30 mU/ml) and hypoxanthine (HX; 200 µM) for 30 min (*P < 0.01), compared with untreated EC. Values are means ± SE of 6 individual determinations.](http://jap.physiology.org/)

![Fig. 2. Adherence of neutrophils to EC monolayers increased after treatment of endothelial cells (EC) with xanthine oxidase (XO; 5–30 mU/ml) and hypoxanthine (HX; 200 µM) for 30 min (*P < 0.01), compared with untreated EC. Values are means ± SE of 6 individual determinations.](http://jap.physiology.org/)

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adherence (not shown). In another experiment, conditioned media from HX/XO-treated EC were added to untreated EC monolayers. Adherence of neutrophils was higher (P < 0.01) to HX/XO-treated EC than to EC exposed to HX/XO-EC conditioned media (control 3.09 ± 0.25%; HX/XO 8.13 ± 0.25%; conditioned media 4.27 ± 0.25%).

Effect of heparin on neutrophil adherence. The ability of EC-bound XO to modify neutrophil adherence was assessed by first preincubating monolayers with XO in the absence of substrate. After removal of unassociated XO by extensive washing, HX was then added and resulted in increased (P < 0.001) neutrophil adherence compared with EC not preincubated with XO (Fig. 4). Heparin, which interferes with binding of XO to cell surfaces (1, 31), decreased (P < 0.001) neutrophil adherence when added with XO to EC in the preincubation phase (Fig. 4). In contrast, heparin did not affect (P > 0.05) neutrophil adherence when added concurrently with both XO and HX in HBSS. Heparin alone had no effect (P > 0.05) on neutrophil adherence, compared with baseline. In addition, heparin had no effect on phorbol myristate acetate-mediated neutrophil adherence (PMA, 46 ± 3%; PMA + heparin, 41 ± 4%, P > 0.05). To investigate the importance of XO binding to EC in a more relevant antioxidant-replete milieu, we exposed EC to HX and XO in 50% human plasma. HX and XO increased neutrophil adherence (P < 0.001) in 50% plasma (Fig. 5). In contrast to the experiment performed in HBSS alone, heparin decreased neutrophil adherence when added concurrently with HX and XO in 50% plasma (P < 0.001). Again, heparin had no effect (P > 0.05) on baseline adherence.

Effect of O₂ metabolites scavengers on neutrophil adherence. Catalase decreased (P < 0.001) XO-mediated adherence to EC monolayers (Fig. 6), whereas SOD or the membrane-permeable SOD mimic Tempol (25, 32) did not affect (P > 0.05) XO-mediated neutrophil adherence. None of these agents altered baseline adherence to non-XO-treated EC (P > 0.05). Conversely, treatment of EC with H₂O₂ (10–100 µM) increased (P < 0.001) neutrophil adherence (Fig. 7).

Effect of XO on PAF production. Treatment of EC with XO and HX for 30 min increased (P < 0.001) [³H]acetate incorporation into PAF (Fig. 8). Catalase (P < 0.01), but not SOD (P > 0.05), decreased [³H]acetate incorporation in XO-treated EC. [³H]acetate incorpora-
tion into PAF also increased after 10 min (4,459 ± 294 cpm) and 20 min (3,873 ± 636 cpm) of exposure to HX and XO, compared with control (1,489 ± 224 cpm) (P < 0.01). In parallel, the PAF-receptor antagonist WEB-2086 was found to decrease (P < 0.001) XO-mediated adherence of neutrophils to EC when present during both XO treatment and incubation with neutrophils (Fig. 9). In separate experiments, we found that treatment of EC with WEB-2086 only during exposure to XO and not during subsequent incubation with neutrophils decreased XO-mediated adherence by only 10 ± 9%. Conversely, treatment of EC with Web 2086 only during incubation with neutrophils diminished XO-mediated adherence by 97 ± 8%. Addition of PAF also increased neutrophil adherence to EC, whether added to neutrophils in the presence of EC (Fig. 9) or to EC alone, before addition of neutrophils (not shown). Web 2086 decreased (P < 0.001) PAF-mediated neutrophil adherence.

Effect of monoclonal antibodies on neutrophil adherence. Antibodies directed against ICAM-1 or CD11b, the α-subunit of the neutrophil β2-integrin Mac-1, decreased (P < 0.001) neutrophil adherence to XO-treated EC (Fig. 10). In contrast, antibodies against CD11a, the α-subunit of the neutrophil integrin leukocyte function-associated antigen-1 (LFA-1), did not affect XO-mediated adherence (P > 0.05). Irrelevant isotype antibodies also had no effect on neutrophil adherence to EC (P > 0.05). Furthermore, fixation of neutrophil proteins with paraformaldehyde also decreased (P < 0.001) adherence to XO-treated EC (Fig. 11). Surface expression of ICAM-1 did not change after treatment of EC with XO (control 0.083 ± 0.007 absorbance units; XO+HX 0.080 ± 0.011, P > 0.05 vs. control; IFN-α/β 0.222 ± 0.033, P < 0.001 vs. control). In addition, abrogation of de novo protein synthesis with cycloheximide did not decrease neutrophil adherence to XO-treated EC (XO+HX 10.1 ± 0.4%; XO+HX+cycloheximide 12.3 ± 0.6%, P > 0.05). Surface expression of P-selectin increased after treatment with XO (control 0.216 ± 0.040 absorbance units; XO+HX 0.445 ± 0.039, P < 0.01), and release of vWF also increased after treatment with XO (control 0.030 ± 0.021 absorbance units; XO+HX 0.108 ± 0.023, P < 0.05).

Effect of XO on neutrophil rolling behavior. Under static conditions, antibodies against P-selectin did not (P > 0.05) affect XO-mediated neutrophil adherence (Fig. 12). Under shear-loaded conditions, more (P < 0.001) neutrophils rolled at all three shear rates after treatment of EC with XO (Fig. 13). Antibodies to
P-selectin decreased rolling behavior ($P < 0.05$), and cotreatment with antibodies to P-selectin and ICAM-1 completely blocked XO-mediated neutrophil rolling ($P < 0.001$). Antibodies to ICAM-1 alone decreased the number of rolling neutrophils at the lowest shear rate ($P < 0.05$). Treatment of EC with XO also decreased neutrophil rolling velocity at a shear rate of $38 \text{s}^{-1}$ ($P < 0.01$) but not at the higher shear rates. Antibodies to P-selectin and/or ICAM-1 did not significantly alter rolling velocity. Finally, XO increased the number of firmly adherent neutrophils to EC at all three shear rates ($P < 0.01$). Antibodies to P-selectin decreased firm adherence at shear rates of 60 and $96 \text{s}^{-1}$ ($P < 0.01$) but not at $38 \text{s}^{-1}$ ($P > 0.05$). Treatment of EC with antibodies to ICAM-1 alone ($P < 0.05$) or cotreatment of EC with antibodies to both P-selectin and ICAM-1 ($P < 0.001$) completely suppressed XO-mediated firm adherence at all three shear rates.

**DISCUSSION**

We recently found that after mesenteric ischemia plasma levels of XO, an efficient enzymatic source of reactive oxygen intermediates, increased and mediated retention of neutrophils in the lung parenchyma (33). The ability of circulating XO to increase neutrophil-EC interactions provides an interesting mechanism by which a primarily injured organ may initiate inflammation in other vascular beds. However, the literature provides conflicting paradigms of the mechanism by which prooxidants may promote retention of neutrophils in inflamed tissues. For instance, high levels (10 mM) of $\text{H}_2\text{O}_2$ were found to rapidly incite PAF production by EC (21), whereas PAF did not appear to be responsible for neutrophil adherence when lower, more physiological doses of $\text{H}_2\text{O}_2$ were employed (27). In addition, conclusions diverge regarding whether $\text{H}_2\text{O}_2$ mediates neutrophil adherence exclusively by P-selectin- (27) or ICAM-1-related mechanisms (23). Similarly, conflicting data exist regarding the mechanism by which XO alters neutrophil adherence (9, 29).

We found evidence to suggest that extracellular XO promotes neutrophil adherence to EC by a mechanism involving the production of PAF by EC and the binding of both ICAM-1 and P-selectin to neutrophil ligands. We found first that XO increased neutrophil adherence}
in concentrations we have previously found in plasma to affect lung neutrophil retention in vivo (33). The effect was rapid, occurring within 10 min, and did not require de novo protein synthesis, precluding involvement of nonconstitutive EC ligands such as E-selectin. Curiously, neutrophil adherence decreased to baseline levels by 40 min. Although the reason for this time-dependent decrease in adherence is not clear, it may relate to a decrease in EC PAF levels, as occurs after stimulation of EC with bradykinin (35) or thrombin (28). Alternatively, β2-integrin expression may also decrease despite continued neutrophil stimulation (24).

The effect of XO-derived oxidants appeared to be primarily on EC, since treatment of EC, but not neutrophils, increased adherence. We did not find evidence for formation of plasma-derived factors to mediate this effect. The direct effect of XO on EC is particularly relevant, since XO binds to anionic EC surface moieties both in vitro and in vivo in a heparin-reversible manner (1, 31). Our data are consistent with these prior studies and further suggest that EC-bound XO can activate neutrophil adherence mechanisms. This close approximation of XO to EC may be particularly germane in vivo, as a means of bypassing the antioxidant-rich milieu of blood. We found evidence for this effect when EC were exposed to XO in 50% plasma. Human plasma is rich in oxidant-scavenging activity (20) and could potentially dampen the effect of XO on EC. In 50% plasma, however, XO continued to mediate an increase in neutrophil adherence, consistent with a site-specific mode of action of XO at the cell surface. In this environment, as opposed to HBSS, heparin decreased neutrophil adherence when added concurrently with XO and HX, providing further evidence that displacement of XO from the surface of EC allows plasma scavengers to intervene and diminish the effects of delocalized XO.

H$_2$O$_2$ was primarily involved in mediating static neutrophil adherence, since catalase, but not SOD or the membrane-permeable SOD mimic Tempol, decreased adherence. It is not clear why some studies of reperfusion implicate XO-derived superoxide anion radical rather than H$_2$O$_2$ in mediating adherence (10, 34), although in such studies endogenous, presumably intra-cellular, XO is likely the source of oxygen metabolites. In the present situation with exogenous XO, the diffusion of superoxide anion radical into EC from external sites through anion channels may limit its participation, or the specific targets may differ.

We also found evidence for PAF as an EC-derived intermediate in neutrophil adherence. First, incorporation of $[^3]$Hacetate into PAF increased after stimulation
with XO, and this effect was also diminished by catalase but not SOD. Both PAF synthesis and neutrophil adherence increased within 10 min. In other systems, oxidative stress such as ischemia-reperfusion (15) or high concentrations of \( \text{H}_2\text{O}_2 \) (21) have been shown to increase PAF levels. Although the mechanism is not clear, it is noteworthy that treatment of some cells with XO increases cytosolic free calcium (7) and phospholipase A\(_2\) activity (37), which may facilitate PAF production. Second, the PAF-receptor antagonist WEB-2086 completely blocked XO-mediated adherence and, as expected, addition of reagent PAF increased neutrophil adherence. It is likely that EC-derived PAF activates neutrophils directly, since WEB-2086 blocked adherence. It is likely that EC-derived PAF activates neutrophils directly, since WEB-2086 blocked adherence only when added with neutrophils and not when used to pre-treat EC; however, the additional role of PAF in transducing EC signals in an autocrine fashion has not been completely excluded. In addition, PAF may increase neutrophil \( \beta_2 \)-integrin expression by undetermined EC or neutrophil intermediates. Our results differ from those of Sellak et al. (29), who found that PAF-receptor antagonism did not diminish XO-stimulated neutrophil adherence to EC. It is unclear what accounts for this discrepancy, although this latter group examined a somewhat shorter treatment interval and employed a different PAF antagonist (BN-52021). PAF levels were not measured in this latter study, so it is unclear whether PAF production by EC increased. Finally, it is likely that PAF remained largely associated with EC, since conditioned media from HX/XO-treated EC increased neutrophil adherence to naive EC monolayers only slightly.

Our data also implicate Mac-1 rather than LFA-1 in mediating XO-dependent adherence to endothelial ICAM-1. This pattern of \( \beta_2 \)-integrin involvement is consistent with the proposed role of PAF in activating neutrophils, since PAF increases the surface expression of Mac-1 in neutrophils (5, 26). It is possible that stimulation of neutrophils with chemotaxins like PAF promotes Mac-1- rather than LFA-1-dependent adherence (2, 30). Importantly, Mac-1 expression on neutrophils is increased in acute respiratory distress syndrome (18), a condition associated with high circulating levels of XO (11). Furthermore, monoclonals directed against the common \( \beta_2 \)-integrin subunit CD18 decrease lung injury after intestinal ischemia-reperfusion (12), another condition associated with elevated levels of circulating XO (33), and also decrease neutrophil adherence after intravascular infusion of purified XO (9).

An important endothelial ligand for Mac-1 in the present investigation appears to be constitutive ICAM-1, since inhibition of protein synthesis did not affect adherence, and surface expression of ICAM-1 did not increase after XO treatment. Although ICAM-1 expression has been reported to increase as early as 30 min after exposure of EC to high levels of \( \text{H}_2\text{O}_2 \) (23), most reports suggest a time frame of 6–24 h after oxidative stress for induction of surface ICAM-1 expression to occur (6, 17).

Besides ICAM-1, an additional endothelial ligand responsible for XO-mediated neutrophil adherence appears to be P-selectin, whose expression increased after exposure to XO. A dynamic system was required to demonstrate an effect of the monoclonal antibody against P-selectin, suggesting that the integrin-mediated adherence dominated the effect seen in the static assay. This may explain the apparent discrepancy between studies that have investigated the role of P-selectin using static (29) vs. dynamic (9) systems. The cooperativity between the selectin and integrin adherence systems was more evident at higher shear rates, where interference with P-selectin markedly suppressed firm adherence. This is consistent with the notion that, under higher levels of shear, rolling is a prerequisite for firm adherence (19). In contrast, XO-mediated firm adherence was not significantly reduced by the antibody to P-selectin at the lowest shear rate tested. Indeed, observations in vivo suggest that ICAM-1-mediated firm adherence can occur at low shear rates even with effective blockade of P-selectin sites (16). A potential limitation of this study is the use of bovine rather than human EC. However, our data suggest that human neutrophils appear capable of interacting with bovine EC via both integrin and selectin ligand pairs.

In summary, extracellular XO can associate closely with EC and stimulate adherence of neutrophils through interactions with endothelial ICAM-1 and P-selectin. We suggest that circulating XO may contribute to the initial recruitment of neutrophils to secondarily affected vascular beds.

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REFERENCES


