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Binding of Xanthine Oxidase to Vascular Endothelium

KINETIC CHARACTERIZATION AND OXIDATIVE IMPAIRMENT OF NITRIC OXIDE-DEPENDENT SIGNALING*

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Concentrations of up to 1.5 milliunits/ml xanthine oxidase (XO) (1.1 μg/ml) are found circulating in plasma during diverse inflammatory events. The saturable, high affinity binding of extracellular XO to vascular endothelium and the effects of cell binding on both XO catalytic activity and differentiated vascular cell function are reported herein. Xanthine oxidase purified from bovine cream bound specifically and with high affinity (K_d = 6 nM) at 4 °C to bovine aortic endothelial cells, increasing cell XO specific activity up to 10-fold. Xanthine oxidase-cell binding was not inhibited by serum or albumin and was partially inhibited by the addition of heparin. Pretreatment of endothelial cells with chondroitinase, but not heparinase or heparitinase, diminished endothelial binding by ~50%, suggesting association with chondroitin sulfate proteoglycans. Analysis of rates of superoxide production by soluble and cell-bound XO revealed that endothelial binding did not alter the percentage of univalent reduction of oxygen to superoxide. Comparison of the extent of CuZn-SOD inhibition of native and succinoylated cytochrome *c* reduction by cell-bound XO indicated that XO-dependent superoxide production was occurring in a cell compartment inaccessible to CuZn-SOD. This was further supported by the observation of a shift of exogenously added XO from extracellular binding sites to intracellular compartments, as indicated by both protease-reversible cell binding and immunocytochemical localization studies. Endothelium-bound XO also inhibited nitric oxide-dependent cGMP production by smooth muscle cell co-cultures in an SOD-resistant manner. This data supports the concept that circulating XO can bind to vascular cells, impairing cell function via oxidative mechanisms, and explains how vascular XO activity diminishes vasodilatory responses to acetylcholine in hypercholesterolemic rabbits and atherosclerotic humans. The ubiquity of cell-XO binding and endocytosis as a fundamental mechanism of oxidative tissue injury is also affirmed by the significant extent of XO binding to human vascular endothelial cells, rat lung type 2 alveolar epthelial cells, and fibroblasts.

Free radicals and their secondary products have been implicated in many types of vascular disorders, including ischemia-reperfusion injury, myocardial infarction, hypertension, and atherosclerosis (1–3). Xanthine oxidoreductase (XOR,[1] EC 1.2.3.2) is a key source of reactive oxygen species in the intravascular compartment, with xanthine oxidase primarily viewed to be an intracellular source of reactive species and (XO) inhibition showing protective effects toward both *in vitro* and *in vivo* model systems. Importantly, salutary actions of XO inhibition also occur at sites remote from the original source of injury. Allopurinol, or pretreatment of animals with the molybdopterin enzyme inhibitor tungsten to inactivate XO, decreases injury to lung vascular barrier function secondary to splanchnic ischemia-reperfusion (4, 5). Also, allopurinol, which exhibits no direct antioxidant properties at pharmacologic concentrations, displays tissue-protective actions in organ systems low in or devoid of detectable endogenous XOR activity (6). For example, both rabbit and clinical myocardial ischemia-reperfusion injury was significantly attenuated by allopurinol, despite several reports revealing that rabbit and human heart have low to undetectable amounts of XO (7–10). It therefore becomes critical to understand the tissue distribution of XO and underlying mechanisms of cell injury that are mediated by a source of reactive species that is widely implicated in various pathological processes.

The ability of circulating XO to bind to vascular cells of remote organs may explain the efficacy of XO inhibitors in protecting organs having low XOR activity. Plasma levels of XO rise following ischemia-reperfusion injury to the splanchnic system or following hypovolemic shock, with further increases in plasma XO observed upon perfusion with high concentrations (1000 units/h) of intravenous heparin (4, 11, 12). Lung-associated XO activity increases following perfusion with XO-rich effluent from reperfused ischemic liver (13). Thus, during diverse pathologic processes, XO released into plasma from tissues replete in XO specific activity (*e.g.* the splanchnic system) can circulate to remote sites and bind to target tissues low in or devoid of XO activity. Cell-bound XO may then be concentrated several thousand-fold at the cell surface or interstitial matrix, where its oxidant products could more readily react with cellular target molecules and disrupt vascular functional and barrier properties. High concentrations of XO associate with endothelium and bind to heparin-Sepharose 6B complexes *in vitro*, but this phenomenon has not been well characterized (11, 14, 15). Herein, we show that endothelial-bound XO retains similar catalytic properties as XO in solution, is initially

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[1] The abbreviations used are: XOR, xanthine oxidoreductase; O_2^-, superoxide, ·NO, nitric oxide; $ONOO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; EC-SOD, extracellular superoxide dismutase; XO, xanthine oxidase; XDH, xanthine dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; M199, medium 199; PBS, phosphate-buffered saline.

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associated with sulfated cell surface proteoglycans, migrates to intracellular compartments via endocytosis, generates reactive species not readily accessible to CuZn-SOD, and finally, impairs vascular cell signal transduction.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-25 PD-10 columns were from Amersham Pharmacia Biotech. Fetal bovine serum and defined iron-supplemented calf serum were from Hyclone Laboratories, Inc. (Logan, UT). All other cell culture media, Hanks' balanced salt solution (HBSS), and trypsin were from Life Technologies, Inc. Xanthine oxidase (bovine cream) and CHAPS were obtained from Calbiochem. CuZn-SOD was from Oxis, Inc. (Portland, OR). Recombinant human EC-SOD was prepared as described previously (16). Polyethylene glycol-derivatized CuZn-SOD was from Sterling Drug, Inc. (Malvern, PA). cGMP EIA kits were from Cayman Chemicals (Ann Arbor, MI). Glycosaminoglycan lyases were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Heparin (porcine intestinal mucosa) was obtained from Polysciences, Inc. (Warrington, PA). All other chemicals were from Sigma. The carboxyl terminus of the human XO sequence (17) was selected for antigen production to ensure that the resultant polyclonal rabbit antiserum would recognize xanthine oxidase generated by proteolytic cleavage of the amino terminus of XDH. Polyclonal antibodies against this recombinant human XOR fragment were developed (18). The carboxyl terminus of human XOR was amplified by polymerase chain reaction corresponding to the 358 carboxyl-terminal codons common to both XDH and XO. The XOR polymerase chain reaction product was cloned into an expression vector, induced for expression of the XOR 42-kDa fusion product. The fusion protein was purified to near homogeneity and injected into rabbits for production of polyclonal antibodies. A unique specific signal corresponding to the recombinant XOR fusion product was observed by Western analysis for all dilutions of anti-XOR sera. Preimmune serum was not immunoreactive for XOR. This antiserum does not cross-react with human, rat, bovine, or rabbit IgG or lactoferrin, previously noted to be a problem for other antisera to XOR (19).

Cell Culture—Bovine aortic and human umbilical vein endothelial cells were isolated as described previously (20). Primary cell culture preparations, subcultures, and cell removal from flasks were conducted in the absence of proteases using scraping techniques. Collagenase (0.1% in HBSS) was used only for preparing primary cell isolates from human umbilical veins. Cells were propagated by subculturing in a 1:4 ratio in medium 199 containing 5% fetal calf serum, 5% iron-supplemented and defined calf serum, and 10 μM thymidine in 75-cm^2 flasks. Cells exhibited typical endothelial cobblestone morphology by phase contrast microscopy and positive immunostaining for factor VIII antigen. For XO-cell binding studies, cells between passages 5 and 8 were seeded at a 1:4 split ratio in 12-well plates (4 cm^2) or 25-cm^2 flasks and used within 30 h of reaching confluency. Primary cultures of fetal rat lung fibroblasts and type 2 alveolar epithelial cells were prepared as before (21).

Xanthine Oxidase Preparation and Assay—Xanthine oxidase was further purified by Sephadex G-25 chromatography to remove ammonium sulfate and possible contaminating proteases. Xanthine oxidase was monitored for protease contamination and, prior to its addition to cell systems, activity was determined spectrophotometrically by the rate of uric acid formation in 0.1 mM xanthine, 50 mM potassium phosphate, pH 7.4, at 295 nm ($\epsilon = 1.1 \times 10^4$ M^{-1}·cm^{-1}), revealing a specific activity of typically ~1.4 units/mg of protein. For some experiments, XO was inactivated by 10 mM KCN for 15 min. The enzyme was then dialyzed extensively against 50 mM potassium phosphate, pH 7.4, and assayed for residual activity. Xanthine oxidase was labeled with ^{125}I using IODO-GEN from Pierce. Briefly, 1 mg of XO in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, 5 mCi of [^{125}I]NaI, and 0.3 μM KI were incubated with 0.1 mg of IODO-GEN for 10 min at 4 °C. The free ^{125}I was separated from the ^{125}I-XO by size exclusion chromatography using a Sephadex G-25 PD10 column equilibrated with 50 mM potassium phosphate, pH 7.4, followed by dialysis at 4 °C with four changes of 1 liter of 50 mM potassium phosphate, 25 μM potassium iodide, pH 7.4, and dialysis with four changes of 1 liter of 50 mM potassium phosphate, pH 7.4. Greater than 95% of the counts were precipitable with 10% trichloroacetic acid. For some enzymatic analyses of XO-dependent reduction of oxygen, succinoylated cytochrome *c* was synthesized as previously described (22).

Xanthine Oxidase-Cell Binding Studies—To assay cell-bound XO activity, cells in 75-cm^2 flasks were rinsed twice with HBSS and incubated at 37 °C with 15 ml of 0–10 milliunits/ml XO for 3 h in M199(−), defined as medium 199 without hypoxanthine or xanthine and supplemented with 5 mM potassium phosphate, pH 7.4. Cells were then washed three times with HBSS and solubilized immediately in 3 ml of 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.1% CHAPS, 0.1 mM EDTA, 10 mM dithiothreitol, and 50 mM potassium phosphate, pH 7.4 (lysis buffer). To measure extents of XO inactivation and/or release, cell monolayers were incubated with XO, washed three times, and further incubated at 37 °C for 0–6 h with 15 ml of M199(−). At intervals, the medium was collected, cells were solubilized with lysis buffer, and both cell-bound and medium-associated XO activity were measured fluorometrically at 37 °C via oxidation of pterin to isoxanthopterin, using methylene blue as an electron acceptor rather than NAD^+ (23). One unit of XO activity was defined as the amount of enzyme required to produce 1 nmol of isoxanthopterin/min, while total XOR activity was defined as the amount of enzyme required to produce 1 nmol of isoxanthopterin/min in the presence of 10 μM methylene blue.

For determining XO binding affinity and specificity, cell-XO incubations were conducted at 4 °C to minimize endocytosis. It was initially determined by treating cells for various times with 1 μg/ml ^{125}I-XO that maximal binding occurred at 5 h. Parallel incubations with additional catalytically inactivated, nonradioactive XO (1 mg/ml) allowed correction for nonspecific binding. Cell binding association constants were analyzed with a nonlinear regression curve fitting program for a single ligand binding model (Enzfitter, Elsevier-BIOSOFT, UK). The influence of BSA, heparin, and pH on XO binding to cells was assessed using cells treated with ^{125}I-XO in M199(−) at 4 °C in 12-well plates. Cells were incubated with 0.5 ml of 10 μg/ml ^{125}I-XO in the absence or presence of 1% BSA or 15 units/ml heparin for 6 h, after which the cells were washed three times in cold HBSS and solubilized in lysis buffer. The pH profile of XO binding to cells at pH 6.8–8.0 was examined by adding 2 μg/ml ^{125}I-XO to cells at 4 °C as above, except PBS was used for all steps to optimize pH control.

To ascertain reversibility of XO binding and time-dependent incorporation of XO into a trypsin-resistant compartment, 1 μg/ml ^{125}I-XO in 0.7 ml of M199(−) was added to cells in 2.2-cm^2 12-well plates for 0–70 min at 37 °C. Cells were washed at intervals with cold Ca^{2+}-, Mg^{2+}-free HBSS prior to treatment with 0.5% trypsin for 15 min at 37 °C. It was previously determined, from analysis of the release of [^{14}C]adenine from prelabeled cells, that trypsin incubations of 15 min or less did not lyse cells. Trypsinized cells were then centrifuged, and the ^{125}I-XO-associated radioactivity of cell pellets and the supernatants collected from trypsinized cell pellets was measured by γ-counting. To ensure that the radioactivity measured was not free ^{125}I dissociated from XO, both cell lysates and media were made 1% with BSA, and total protein was precipitated with 10% trichloroacetic acid (w/v) to determine proportions of acid-soluble and insoluble ^{125}I. To ascertain the latency of cell-bound XO release or degradation, cells were incubated with 5 milliunits/ml XO for 1.5 h at 37 °C or 1 μg/ml ^{125}I-XO at 4 °C in M199. Cells were then washed and postincubated with M199 at 37 °C for up to 6 h, and both cells and media were obtained at intervals for XO activity assay or ^{125}I-XO protein content determination.

The ability of various compounds to displace cell-bound XO was determined by incubating cells grown in 12-well plates with 1 μg/ml ^{125}I-XO for 3 h at 4 °C, after which cells were washed twice with 4 °C HBSS and postincubated for 1 h at 4 °C with gentle shaking in 0.7 ml of M199(−) containing no additions (control cells), 50 units/ml EC-SOD, 1 or 100 μg/ml poly-D-lysine, 15–100 units/ml heparin (176 units/mg), 2.5% BSA, or 2.5% bovine calf serum. Cells were then washed with cold HBSS and solubilized with lysis buffer, and cell-associated radioactivity was determined. Cells were also pretreated for 3 h at 37 °C with 4 milliunits/ml heparinase, heparitinase, or chondroitinase AC in M199 prior to the addition of ^{125}I-XO. The activity of endoglycosidases under these conditions was determined spectrophotometrically (ϵ_{232} = 3800 M^{-1} cm^{-1}) using 100 μg/ml heparin, heparan sulfate, or chondroitin sulfate as substrate (24). After endoglycosidase treatment, cells were washed with HBSS and incubated with 1 μg/ml ^{125}I-XO in M199(−) for 3 h at 4 °C. Cells were then washed three times with cold HBSS and solubilized with lysis buffer. Cell-associated ^{125}I-XO was then compared with that of parallel control cells not treated with endoglycosidases. Potential protease contamination of chondroitinase AC was ruled out using azocasein and fluorescamine-casein as substrates, with the assay having a lower limit of detection of 10^{-5}% contamination.

XO Immunolocalization—Polyclonal rabbit anti-XOR was used for immunolocalization studies. Bovine aortic endothelial cells were grown to confluency on two-well chamber slides (Nalge-Nunc International, Milwaukee, WI) and incubated for 1 or 3 h with 5 milliunits/ml XO in M199 at 37 °C. Primaquine (10 mM) was added in some cases to inhibit endocytosis. Cells were washed with 0.15 M NaCl, 10 mM potassium phosphate, pH 7.4 (PBS) and fixed with 4% paraformaldehyde in PBS

for 20 min at 25 °C. Fixed cells were blocked for 15 min with 50 mM lysine in PBS followed by the addition of 10% goat serum in PBS for 1 h at 25 °C. Permeabilized cell groups had 0.1% Triton X-100 added to both lysine and goat serum-containing blocking buffers. Cells were then incubated 12–15 h at 4 °C with polyclonal rabbit anti-XOR antibody in PBS containing 10% goat serum, followed by 30-min incubation at 22 °C with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove PA). Incubation with the secondary antibody was terminated by extensive washing and 5-min postfixation with 4% paraformaldehyde in PBS. Nuclei were counterstained by incubation for 15 min with 1 μg/ml 4,6-diamino-2-phenylindole in deionized H_2O. Stained cells were mounted with Prolong mounting medium (Molecular Probes, Inc., Eugene, OR). Immunolocalization of added XO and endogenous XOR was performed using an Olympus IX-70 inverted microscope with an Olympix digital cooled camera, and images were analyzed using Esprit software (Life Sciences Resources, Cambridge, UK). The same exposure times and sensitivity settings were used in the capture and processing of all digital images. Antibody specificity was determined by incubating the primary antiserum with 3.6 μg/ml bovine XO overnight at 4 °C before addition to fixed cell monolayers.

Extracellular Superoxide Release and Xanthine Oxidase Kinetic Constants—Superoxide production by cell monolayers was measured spectroscopically in HBSS supplemented with 10 mM potassium phosphate, 0.1 mM horse heart ferricytochrome *c*, 100 μM xanthine, pH 7.4 (HBSS-cyt *c*) at 550 nm (ϵ = 21 mM^{-1} cm^{-1}). Xanthine oxidase was bound to cells in 25-cm^2 flasks by incubation with 5 ml of 0–10 milliunits/ml XO in M199(−) for 3 h at 37 °C, followed by extensive washing with HBSS. Cell monolayers were then incubated at 37 °C for 3 h in 3 ml of HBSS-cyt *c* with or without other additions. A 1-ml aliquot of medium was removed every 10 min for absorbance measurement and then returned to flasks. The rate of xanthine or pterin consumption by bound XO was similarly determined by measuring production of uric acid or isoxanthopterin. Xanthine or pterin-containing HBSS was added to cells, and a 1-ml aliquot of medium was removed every 10 min for spectrophotometric determination of xanthine oxidation to urate (ϵ_{295} = 1.1×10^4 M^{-1} cm^{-1}) or fluorometric analysis of rates of isoxanthopterin oxidation to pterin (isoxanthopterin; λ_{ex} = 340 nm, λ_{em} = 395 nm).

[^{14}C]Adenine Release—Cells grown in 12-well plates were incubated with 4 μM (0.2 μCi/ml) [^{14}C]adenine for 3 h in HBSS at 37 °C. During the last 2 h of [^{14}C]adenine labeling, XO was added, followed by extensive washing with HBSS. Following an 8-h postincubation of washed and radiolabeled cells in HBSS supplemented with 0.1 mM xanthine, aliquots of media were removed, and cell lysis was determined by [^{14}C]adenine and [^{14}C]adenine-labeled metabolite release into the medium. The extent of [^{14}C]adenine release from cells solubilized with 1% CHAPS indicated 100% lysis.

Smooth Muscle Cell cGMP Measurement—Endothelial cells grown to confluence on 2.2-cm^2 Transwell micropore filters were incubated with 10 milliunits/ml XO in M199(−) for 3 h at 37 °C, followed by extensive washing with HBSS. Endothelial cell-containing filters were then placed in six-well plates containing confluent rat aortic smooth muscle cells and equilibrated for 10 min with Dulbecco's PBS. Dulbecco's PBS supplemented with 100 μM xanthine, 50 units/ml CuZn-SOD, and 0.5 mM isobutylmethylxanthine with or without 100 μM allopurinol was then added to smooth muscle cells, while Dulbecco's PBS with 100 μM xanthine, 50 units/ml SOD, 0.5 mM isobutylmethylxanthine, 6.7 μM ionomycin with or without 100 μM allopurinol was added to overlaid endothelial cell-containing Transwell filter wells for 15 min. A low concentration of CuZn-SOD (5 units/ml) was added to all groups to scavenge variable rates of light-induced O_2^- production in the medium induced by intermittent exposure to dimmed laboratory lighting conditions and autoxidation of medium constituents. The basal generation of O_2^- by medium under these conditions was confirmed via chemiluminescent assay of accelerated rates of ·NO decay when medium was exposed to laboratory lighting conditions, as opposed to slower rates of ·NO decay in darkness or in the presence of 5 units/ml SOD. Sodium nitroprusside (10 μM) was added to preparations of smooth muscle cells not exposed to endothelium-derived mediators to determine maximal smooth muscle cell cGMP production. In some cases, heparin-binding EC-SOD (50 units/ml) or higher concentrations of CuZn-SOD (1000 units/ml) was added to the media of both the effector (endothelial) and target (smooth muscle) cells during the the 15-min exposure period. Endothelial cell-containing Transwell inserts were then removed from smooth muscle cell-containing wells, and 0.5 ml of cold 50 mM sodium acetate, pH 4, was added to smooth muscle cells for 1 h at 4 °C. Cells were then removed by scraping and centrifuged at 2000 × *g* for 5 min, and the supernatant was assayed for cGMP by EIA. Smooth muscle cells were not morphologically affected by any of the above manipulations. For all experiments, protein concentrations were measured at 550 nm by a modified Bradford assay using Coomassie Plus reagent (Pierce) with bovine serum albumin as a standard (25). Samples were diluted with water to 5–20 μg/ml to which an equal volume of Coomassie Plus reagent was added.

Statistical Analysis—Data were analyzed with a nonlinear regression curve-fitting program (Enzfitter, Elsevier-Biosoft, UK) using a Michaelis-Menten equation to determine kinetic constants. Unless otherwise noted, data represent mean ± S.D. for experiments repeated at least two times, all giving similar results. Statistical significance of $p < 0.05$ was determined by one-way analysis of variance with Duncan's post hoc analysis.

RESULTS

Xanthine Oxidase Binding to Endothelial Cells—Initial endothelial cell ^{125}I-XO binding studies were conducted at 4 °C to minimize possible endocytosis and proteolysis. Maximum binding occurred by 5 h (mean k_{on} = 0.39 ± 0.21 μg h^{-1}) and was resistant to removal by extensive washing. Xanthine oxidase binding was saturable, exhibiting an average K_d of 0.9 μg/ml (6 nM XO, Fig. 1), with experiment to experiment K_d values ranging from 0.3 to 2.2 μg/ml (2–15 nM XO) for different bovine endothelial cell lines. Saturable binding was 80–90% inhibitable by the addition of a 100-fold excess of unlabeled XO. The addition of 15 units/ml (85 μg/ml) heparin during XO-cell incubation (2 μg/ml XO) inhibited binding 43%, while BSA (100 μg/ml) had no effect (not shown). Pretreatment of cells with heparitinase or heparinase did not affect subsequent XO binding, while pretreatment with chondroitinase AC (determined to have no detectable contaminating protease activity) inhibited XO binding 47% (Table I). Alkaline pH enhanced binding of XO to cells 4.3-fold at pH 8, compared with pH 7.4 (Table II). Poly-D-lysine and heparin, added for 1 h at 4 °C, following binding of XO to cells, displaced XO 45 and 26%, respectively, compared with buffer-treated controls (Table III). Heparin-binding EC-SOD, BSA, and fetal bovine serum did not displace cell-bound XO.

XO Binding at 37 °C—Endothelial cells bound XO at 37 °C in a time- and concentration-dependent manner. The cell-associated XO remained active, with exposure to 2.5 milliunits/ml XO or greater resulting in significant increases in cell XO catalytic activity (Fig. 2). At 37 °C, maximum cellular XO catalytic activity and ^{125}I-XO binding occurred within 1 h of XO addition (k_{on} = 5.5 ± 1.2 h^{-1}). Endothelial XDH, as well as XO, activity increased after XO incubation with cells, such that the proportion of XO activity compared with total xanthine oxidoreductase activity remained virtually unchanged (34 ± 4% for control cells, 39 ± 3% for cells incubated with 5 milliunits/ml XO; data not shown). The possible endocytosis of cell-bound XO during cell exposure to XO at 37 °C was probed using 0.5% trypsin to remove cell surface-associated proteins. Cells were exposed to trypsin for the maximal time possible before disruption of cell membrane integrity became apparent. There was an initial rapid increase, followed by a slower increase, in the pool of trypsin-resistant cell-associated XO, implying ongoing endocytosis of XO while binding to cells (Fig. 3).

Further evidence of bound XO endocytosis by endothelial cells was observed immunocytochemically with anti-XO antibodies (Fig. 4). Endothelial cells exposed to 5 milliunits/ml XO at 37 °C demonstrated increasing amounts of XO on the cell surface, while unexposed cells had no XO on the cell surface. When cells were permeabilized to immunolocalize both intra- and extracellular XO, unexposed cells displayed punctate staining, suggesting that intracellular XO was contained in organelles, possibly endosomes. Cells exposed to XO and then permeabilized had a greater degree of intracellular staining than unexposed cells. The addition of the endocytosis inhibitor primaquine to cells slightly increased extracellular XO

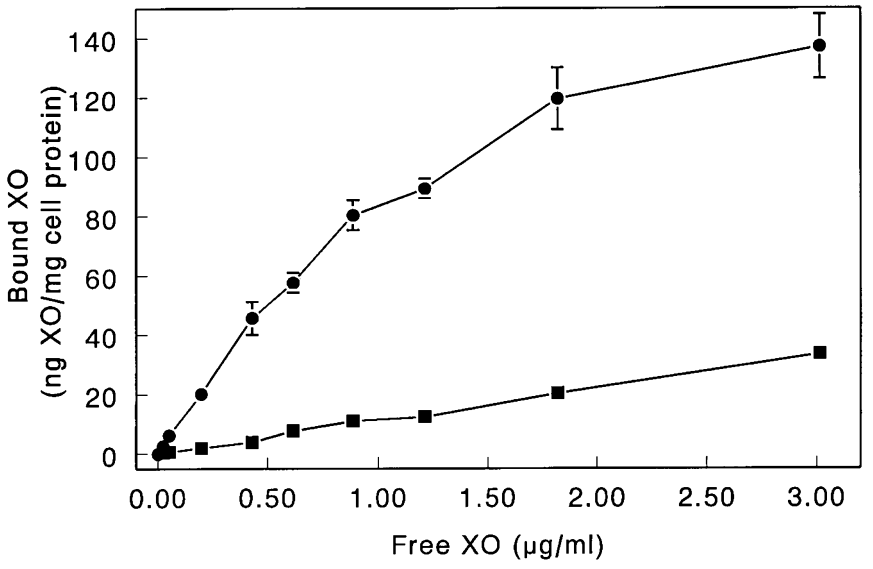


FIG. 1. **Specific and nonspecific binding of xanthine oxidase to endothelial cells.** Cells were incubated with increasing concentrations of ^{125}I-XO at 4 °C for 6 h in the absence (●) or presence (■) of 1 mg/ml native XO. Data represent mean ± S.D. of a representative experiment (n = 6).

TABLE I
The role of endoglycosidases in endothelial cell-xanthine oxidase binding

Cells were treated with glycosaminoglycan lyases at 37 °C for 3 h prior to incubation with 1 μg/ml ^{125}I-XO at 4 °C for 3 h. Data represent mean ± S.D. of three experiments (n = 4).

Treatment	Control
	%
4 milliunits/ml heparinase	102 ± 5
4 milliunits/ml heparitinase	103 ± 3
4 milliunits/ml chondroitinase AC	72 ± 3[a]
40 milliunits/ml chondroitinase AC	53 ± 13[a]

[a] $p < 0.05$ from control.

TABLE II
The influence of pH on endothelial cell-xanthine oxidase binding

Cells were incubated with 2 μg/ml ^{125}I-XO in PBS at various pH values for 6 h at 4 °C. Cells were washed with PBS (adjusted to the same pH) three times and solubilized, and cell-bound ^{125}I-XO was determined. Data represent mean ± S.D. of three separate experiments, n = 12 for each.

pH	Xanthine oxidase binding
	ng/mg cell protein
6.8	58 ± 70
7.1	45 ± 50
7.4	128 ± 40
7.7	419 ± 90[a]
8.0	822 ± 160[a]

[a] $p < 0.05$ from pH 7.4 values.

immunostaining and inhibited the increase in intracellular staining of XO in cells exposed to XO. No fluorescence was detected when using primary antiserum preadsorbed with 5 milliunits/ml purified bovine XO.

After XO was bound to cells at 37 °C, changes in cell-associated XO activity (Fig. 5*A*) and trypsin-dissociable ^{125}I-XO (Fig. 5*B*) were examined over time. Augmented cell XO activity and cell-associated ^{125}I-XO declined with half-lives of 1.8 and 2.8 h, respectively. All of the ^{125}I-XO released from cells was detected in the media as trichloroacetic acid-precipitable ^{125}I-labeled protein. Part of the XO specific activity lost by cells was recovered as intact active enzyme in the media within the first hour, after which there was no significant increase in medium XO activity, despite a continued loss of cell-associated XO activity. All of the activity detected in the medium was XO in the oxidase rather than dehydrogenase form.

TABLE III
Displacement of endothelial cell-bound xanthine oxidase

Cells were incubated at 4 °C with 1 μg/ml ^{125}I-XO in M199 without hypoxanthine or xanthine for 3 h. Cells were washed and incubated with M199 without hypoxanthine or xanthine in the absence (control cells) or presence of the noted additions for 1 h at 4 °C. The concentration of ^{125}I-XO associated with cells was then measured. Data represent mean ± S.D. of two experiments, n = 6.

Addition	Control
	%
2.5% BSA	98 ± 10
5% fetal bovine serum	104 ± 15
1 μg/ml poly-D-lysine	101 ± 3
100 μg/ml poly-D-lysine	55 ± 19[a]
15 units/ml heparin	81 ± 5[a]
100 units/ml heparin	74 ± 6[a]
300 units/ml EC-SOD	98 ± 7

[a] $p < 0.05$ from control.

Superoxide Production by Cell-associated XO—Increased cell-bound XO activity, following incubation with 0–10 milliunits/ml XO for 3 h, was paralleled by increased rates of cytochrome *c* reduction in the presence of xanthine (Fig. 2). The rate of cytochrome *c* reduction by cells having bound XO was inhibited 74% by allopurinol, 17–29% by 300 units/ml CuZn-SOD, polyethylene glycol-conjugated SOD, or Mn-SOD and 41% by 50 units/ml EC-SOD (Table IV). When succinoylated cytochrome *c* was used to detect O_2^- rather than native cytochrome *c*, CuZn-SOD completely inhibited cytochrome *c* reduction (Table IV). There was no detectable cytochrome *c* reduction induced by the media during analysis of extracellular O_2^- production by cells with bound XO, nor was there significant SOD-inhibitable cytochrome *c* reduction by cells prior to XO binding.

The K_m for oxidation of xanthine and pterin by cell-associated XO was determined in intact cell monolayers. The K_m for xanthine oxidation, indicated by rates of uric acid production, was 3.4 ± 0.8 μM for cell-bound XO and separately, 3.6 ± 0.5 μM for soluble XO. The K_m for pterin oxidation by cell-bound XO, measured fluorometrically by isoxanthopterin formation, was 1.5 ± 0.3 μM for cell-bound XO and, separately, 1.6 ± 0.8 μM for soluble XO (Table V). Minimal xanthine and pterin oxidation was due to cell-associated XO being released into the media during analysis. Both xanthine and pterin oxidation was fully allopurinol-inhibitable. Comparison of rates of xanthine oxidation and cytochrome *c* reduction at similar xanthine concentra-

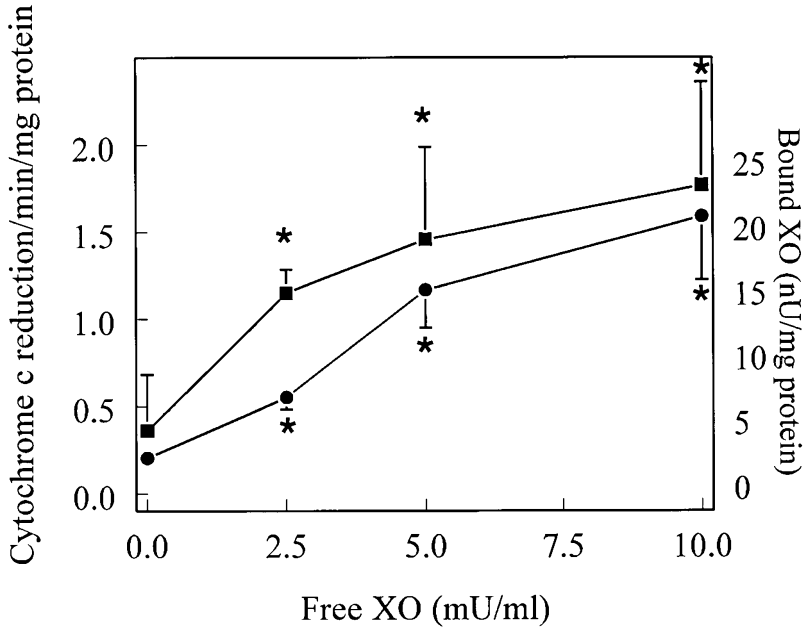


FIG. 2. **Xanthine oxidase binding increases endothelial cell xanthine oxidase specific activity and rates of superoxide production.** Xanthine oxidase (0–10 milliunits/ml) was incubated with the cells for 3 h at 37 °C in M199 without hypoxanthine or xanthine, after which the cells were washed and either assayed for XO activity via fluorometric analysis of pterin oxidation (■) or incubated with HBSS supplemented with 0.1 mM cytochrome *c* and 0.1 mM xanthine for determining rates of O_2^- production (●). Reduction of cytochrome *c* was monitored at 550 nm for 2–3 h. Data represent mean ± S.D., $n = 3$ (cytochrome *c* reduction) and $n = 4$ (XO activity) of a representative experiment. *, $p < 0.05$ compared with no addition of XO.

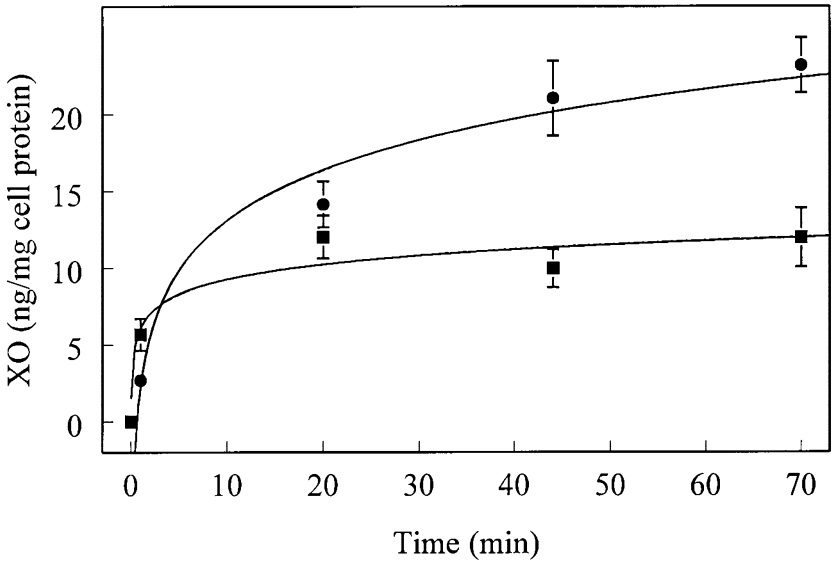


FIG. 3. **Time-dependent endocytosis of cell-bound xanthine oxidase.** Cells were incubated with 1 μg/ml ^{125}I-XO in M199 at 37 °C in the absence of added substrate for 0–70 min, washed, and treated with 0.5% trypsin. Trypsinized cells were centrifuged, and ^{125}I-XO was measured in both trypsin-treated cell pellets (●) and supernatants of trypsinized and centrifuged cells (■). Data represent the mean ± S.D. of a representative experiment ($n = 4$).

tions indicated that 22 ± 6% of the electron flux to oxygen by bound XO was univalent, yielding O_2^-.

Binding of XO to Other Cell Types—The binding of 5 milliunits/ml XO to cultured human umbilical vein endothelium, fetal rat lung fibroblasts, and fetal rat lung type 2 epithelial cells also resulted in significant increases in cell XO specific activity. This yielded now-detectable levels of XO in human vascular endothelium, fibroblasts, and type 2 cells as well as a 7-fold increase in bovine aortic endothelial cell XO specific activity (Table VI). Cell-XO binding was dose- and time-dependent, being saturable for epithelial cells but not fibroblasts (not shown).

Influence of Bound XO on Endothelial Function—To determine whether cell-bound XO influenced endothelial function, endothelial cells were incubated with XO at 37 °C, washed, and then further incubated with 100 μM xanthine. After exposure to xanthine for up to 8 h, it was observed that cellular membrane integrity was not significantly impaired, as indicated by release of [^{14}C]adenine and labeled cell metabolites into the media (>80% trichloroacetic acid-soluble; data not shown). Cell-bound XO, in the presence of xanthine, interrupted ionomycin-stimulated endothelial ·NO-dependent signaling to smooth muscle cells, as revealed by a significant reduction in endothelial-stimulated cGMP production by smooth muscle cells treated with the phosphodiesterase inhibitor isobutylmethylxanthine (Fig. 6*A*). Inactivation of XO by pretreatment with KCN or the addition of allopurinol to incubations abrogated the inhibitory action of XO toward ·NO-dependent signaling. The addition of 1000 units/ml CuZn-SOD or 50 units/ml EC-SOD to ionomycin-stimulated endothelial cells having bound XO did not restore smooth muscle cell guanylate cyclase activation (Fig. 6*B*).

DISCUSSION

The observation that XO binds to endothelial cells (11, 14) has been extended to define the affinity and nature of cell binding, retention of catalytic activity by cell-associated XO, the endocytosis of cell-bound XO, and finally, the cellular structural and functional consequences of a concentrated production of reactive species at sites of XO-cell association. The specific and reversible binding of XO by endothelial cells was demonstrated by saturable binding at 4 °C (Fig. 1) and 80–90% inhibition of ^{125}I-XO binding by a 100-fold excess of unlabeled XO. The latter also confirmed that iodination did not significantly

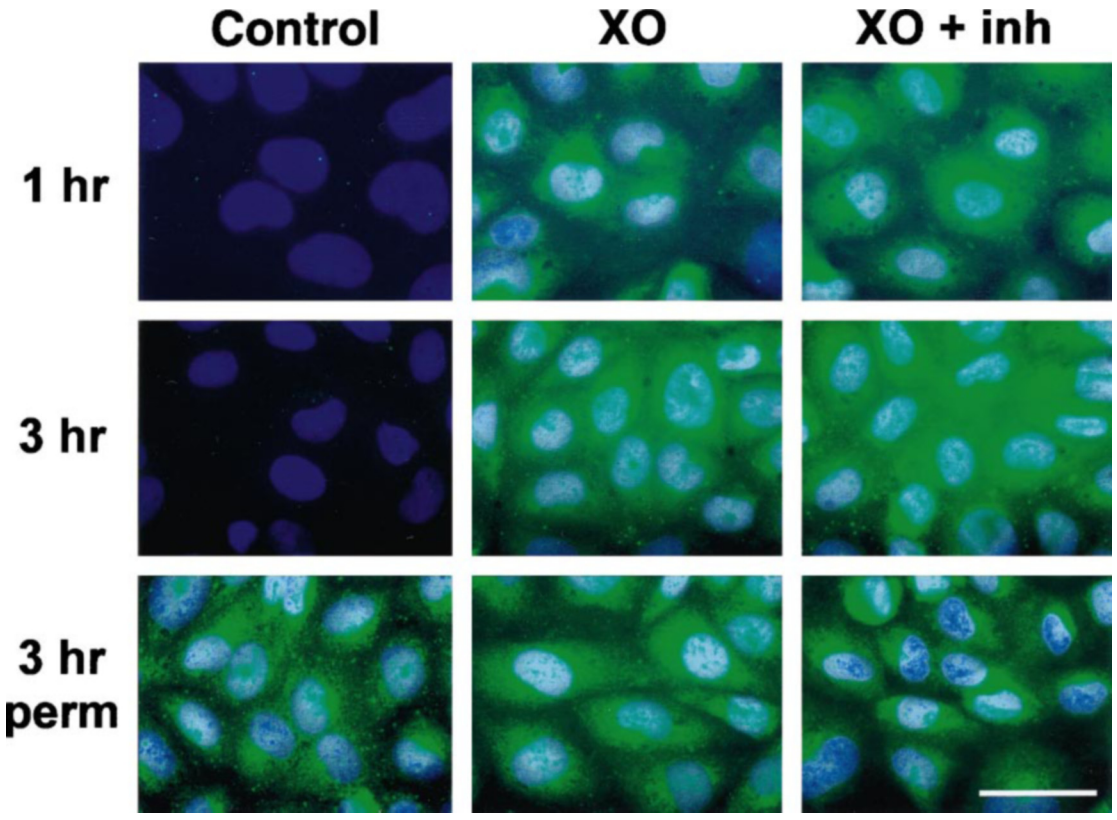


FIG. 4. **Immunolocalization of the distribution of endogenous and added exogenous xanthine oxidase in vascular endothelial cells.** Cells were incubated with 5 milliunits/ml XO in M199 at 37 °C for 1 or 3 h and washed extensively. Primaquine was added to the XO + inh group during XO incubation to limit endocytosis. Control and treated cells were then fixed with paraformaldehyde and blocked in the presence or absence of 0.1% Triton X-100 to permeabilize the cell surface. The presence of XO was then probed at 4 °C using rabbit polyclonal anti-XDH/XO antibody and fluorescein isothiocyanate-conjugated secondary antibody. The nuclei were counterstained with 4,6-diamino-2-phenylindole. *Scale bar*, 50 μm.

modify XO-cell interactions. Binding was not inhibited by albumin and serum, indicating the potential for *in vivo* occurrence as well as the absence of nonspecific XO binding to cell surface or culture dish sites (Table III). The K_d of XO binding (6 nM) to endothelial cells is comparable with other macromolecules having specific endothelial binding sites, including lipoprotein lipase, diamine oxidase, and EC-SOD (26–28). Early evidence that exogenous XO could bind to and damage endothelial cells came from the observations that the addition of heparin to XO-treated cells partially limited oxidative injury by mechanisms that did not include the direct scavenging of reactive species by heparin (29, 30). Previous studies supported the concept that the endogenous XOR content of non-rodent vascular cells is too low in specific activity to contribute significantly to oxidative signal transduction events or cell injury (31, 32). The capacity of the splanchnic system and both normoxic and hypoxic vascular endothelial cells to express and release XOR into the circulation affirm that extracellular XO has the potential to contribute to tissue pathogenesis (13, 33–35).

There are undetectable or only trace levels of XO activity in normal human plasma (36, 37), with up to 3% of total IgM being anti-human XOR, existing predominantly as immune complexes with endogenous XOR (38). During pathological conditions such as reperfusion injury, hepatitis, adult respiratory distress syndrome, or atherosclerosis, human plasma XO concentrations increase to as much as 1.5 milliunits/ml, an amount of XO that would occupy a significant fraction of cellular binding sites, assuming a specific XO activity of 1.4 units/mg of protein (12, 36, 37). These elevated XO levels can persist for at least 120 min following reperfusion of ischemic tissue, a time frame permitting maximal target cell binding to occur (Fig. 3; Refs. 11, 12, and 40). Normal levels of plasma purine substrates for XO (*e.g.* hypoxanthine) are 1–2 μM, increasing up to 15 μM during anaerobic exercise and in the plasma of patients having chronic impaired lung function (*e.g.* neonatal and adult respiratory distress syndromes). Also, immediately after reperfusion of ischemic tissues, plasma purine concentrations can rise to over 100 μM (41–44). Thus, circulating XO can bind in the vascular compartment of humans, utilize endogenous purine substrates to generate reactive species, and impair vascular function. This concept is supported by recent observations in both animal models and clinical studies of ischemia-reperfusion, hypertension, and atherosclerosis (45–48). For example, the addition of heparin to the vascular compartment of normal humans and rat hemorrhagic shock models causes an immediate increase in plasma XO content in the absence of additional acute cell injury, suggesting reversible binding of XO to the vascular lumen (11, 14). Also, isolated lungs perfused with XO-rich effluent from ischemic rat livers exhibit a 1.4-fold increase in whole lung XO activity (13). Rabbit aortic rings exposed to XO displayed heparin and allopurinol-reversible impairment of endothelial-dependent relaxation, underscoring the adverse functional consequences of cell-XO association (45). It is noteworthy that the calf serum utilized in tissue culture systems is rich in XOR. Thus, cell binding of this frequent culture medium component can contribute to net cell XO specific activity, changes in cell XO distribution, and an elevation in endogenous rates of cell oxidant production (49).

Although the endothelial XO binding site has not been identified at a molecular level, its functional characteristics are revealed herein. The nonlinearity of Scatchard binding analysis and the variability in XO binding constants for different

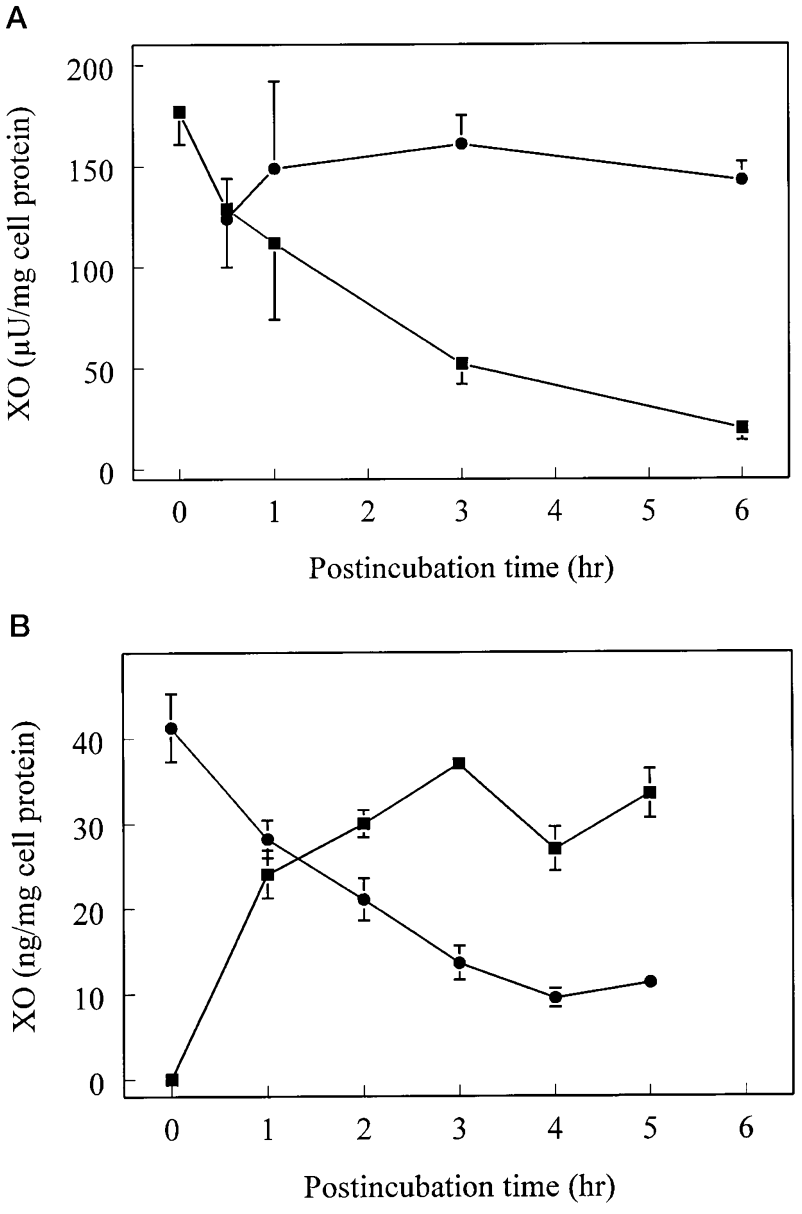


FIG. 5. **Latency of endothelial cell-bound xanthine oxidase.** Cells were incubated with 5 milliunits/ml XO at 37 °C (*A*) or 1 μg/ml ^{125}I-XO at 4 °C (*B*) in M199. Cells were then washed and further incubated with M199 for various times before assessing the XO activity or ^{125}I-XO protein associated with cells (●) and media (■). Data represent the mean ± S.D. of a representative experiment (n = 4).

passages and lines of cells suggests heterogeneity in XO binding sites. Some heparin-binding molecules (*e.g.* thrombospondin, EC-SOD, and basic fibroblast growth factor) are >80–90% inhibited from cell binding by low concentrations (1–10 μg/ml) of heparin or after cellular treatment with heparin lyases (50–52). Derivatization of lysine and arginine residues of XO limited binding to heparin-Sepharose conjugates (53). Herein, the addition of high heparin concentrations and hydrolysis of heparin and chondroitin sulfate-containing proteoglycans prior to or after endothelial cell incubation with XO only partially inhibited XO binding, suggesting heterogeneous binding sites that include chondroitin sulfate-containing proteoglycans (Tables I and III). The partial displacement of XO binding by heparin may be due to the polyanionic character of heparin and its ability to bind to cationic motifs of XO, in turn competing for XO binding to chondroitin sulfate or other glycosaminoglycan-containing cellular proteoglycans. Evidence that XO does not exclusively bind to heparin-containing cell proteoglycans comes from comparing XO kinetic properties following binding to heparin-Sepharose and to endothelial cells. Xanthine oxidase immobilized by heparin-Sepharose had an increased K_m for xanthine and an increased proportion of univalent electron flux (15). In contrast, cell-bound XO manifested the same K_m for xanthine and percentage of univalent flux as XO in solution. In addition, XO binding to heparin-Sepharose increased with acidic pH, while XO binding to cells increased with alkaline pH (Table II; Ref. 15).

The association of XO with cells led to increased endothelial XO activity and content of ^{125}I-labeled XO (Figs. 2 and 3). Cell-bound XO became incorporated by endocytosis, as indicated by the temporally increasing proportion of cell-associated XO resistant to trypsin dissociation (Fig. 3) and the diminished intracellular XO immunoreactivity of XO-treated cells when coincubated with the endocytosis inhibitor primaquine (Fig. 4). The decline of cell-associated XO specific activity, coupled with changes in cell *versus* medium distribution of both catalytically active XO and ^{125}I-XO, revealed that cell-bound XO was also being both inactivated and rereleased from cells (Fig. 5), similar to the fates of EC-SOD and lipoprotein lipase (26–28). The reversibly bound XO found in media that retained activity did not reveal the modest (~20%) increased rates of substrate oxidation upon the addition of methylene blue observed for control preparations of XO (not shown). Methylene blue directly oxidizes the FeS centers of XO, unlike oxygen, which

TABLE IV
Inhibition of endothelial cell monolayer cytochrome c reduction by superoxide dismutases and allopurinol

Cells were incubated with or without XO for 3 h at 37 °C. Monolayers were then washed three times and incubated with 0.1 mM native or succinoylated ferricytochrome *c*, 0.1 mM xanthine, and bovine CuZn-SOD, polyethyleneglycol-conjugated bovine CuZn-SOD (PEG-SOD), human Mn-SOD, EC-SOD, or allopurinol. Data represent the mean ± S.D. of two separate experiments ($n = 3$).

Condition	Cytochrome *c* preparation	Cytochrome *c* reduction	O_2^- production
		$pmol \cdot min^{-1} \cdot cm^{-2}$	% inhibition
Untreated	Native	2.0 ± 0.9[a]	
Bound XO		5.8 ± 1.3	
+CuZn-SOD (300 units/ml)		4.1 ± 1.5	29
+PEG-SOD (300 units/ml)		4.3 ± 1.0	27
+Mn-SOD (300 units/ml)		4.8 ± 0.4	17
+EC-SOD (50 units/ml)		3.4 ± 0.5[a]	41
+Allopurinol (100 μM)		1.5 ± 0.7[a]	74
Untreated	Succinoylated	2.0 ± 1.7[a]	
Bound XO		4.9 ± 1.8	
+Cu ZnSOD (300 units/ml)		−0.3 ± 1.7[a]	107
+Allopurinol (100 μM)		2.0 ± 1.4[a]	58

[a] $p < 0.05$ from the corresponding cell-bound XO-mediated basal rates of native or succinoylated cytochrome *c* reduction.

TABLE V
Kinetic constants of xanthine oxidase in solution and following endothelial cell binding

Cells were incubated with 5 milliunits/ml XO for 3 h at 37 °C. Monolayers were washed, and rates of substrate oxidation were determined for 0.02–20 μM xanthine or pterin. Similar analyses were conducted for native bovine XO free in solution.

	K_m (xanthine oxidation)	K_m (pterin oxidation)
	μM	μM
Soluble XO	3.6 ± 0.5	1.6 ± 0.8
Cell-bound XO	3.4 ± 0.8	1.5 ± 0.3

TABLE VI
Xanthine oxidase binding to vascular endothelial and pulmonary cells

Cells were incubated with 5 milliunits/ml XO in HBSS at 37 °C for 3 h prior to washing and assay of cell-associated XO activity by pterin oxidation to isoxanthopterin. Data represent mean ± S.D. of a representative experiment ($n = 3$–5).

Cell type	Xanthine oxidase activity	
	Control	With xanthine oxidase
	microunits/mg cell protein	
Bovine thoracic aortic endothelial cells	35 ± 15	233 ± 70
Human umbilical vein endothelial cells	7 ± 14	740 ± 210
Fetal rat lung type II epithelial cells	14 ± 17	577 ± 154
Fetal rat lung fibroblasts	4 ± 6	372 ± 66

oxidizes the FAD cofactor (54). The lack of an influence of methylene blue on rate indicates that cell-released XO utilized oxygen as an electron acceptor, while the original preparation of XO contained partial dehydrogenase activity. This, coupled with the observation that the addition of XOR to fresh plasma immediately converts from the dehydrogenase to oxidase form (11, 55), implies that both release of XOR from tissues into the circulation and binding of XOR to cells will favor the production of reactive oxygen species. Since XOR also displays significant NADH oxidase activity, the cell-bound form may be one of multiple loci for the extracellular NAD(P)H-dependent production of O_2^- by vascular tissues (45, 56–58).

Superoxide generated by cell-bound XO was resistant to scavenging by CuZn-SOD, a phenomenon also observed for heparin-Sepharose 4B-immobilized XO (Table IV; Ref. 15). Cytochrome *c* reduction by XO in solution was completely inhibited by 50 milliunits/ml CuZn-SOD, while cytochrome *c* reduction by cell-associated XO was only inhibited 28–40% by 300 milliunits/ml CuZn-SOD, Mn-SOD, PEG-derivatized CuZn-SOD, and 50 units/ml EC-SOD. There was, however, a significant difference in the extent of inhibition of native cytochrome *c* reduction by CuZn-SOD (28% of control), compared with the heparin-binding EC-SOD (40% of control). This suggested that SOD-resistant, XO-dependent cytochrome *c* reduction arises from partitioning of XO-derived O_2^- production into an anionic compartment. Alternatively, a component of SOD-resistant XO-dependent cytochrome *c* reduction could be the result of direct cytochrome *c* reduction, since proteolyzed XO can directly reduce cytochrome *c* (28). To address this issue, it is noted that native cytochrome *c* at pH 7.4 bears a +9.5 charge, thus avidly associating with the glycocalyx, while CuZn-SOD is electrostatically repelled by cells (59). To reveal whether cell-bound XO reduced O_2 to O_2^- or directly reduced cytochrome *c*, cytochrome *c* was succinoylated to lend a net negative charge at pH 7.4 and limit association with the glycocalyx and cell-bound XO. The net negative charge on succinoylated cytochrome *c* also decreases the rate constant for reduction by O_2^-, making it necessary to use greater concentrations of succinoylated cytochrome *c* to achieve the same rate of reduction as native cytochrome *c* (22). The complete inhibition of cell-associated XO-dependent succinoylated cytochrome *c* reduction by CuZn-SOD confirmed the production of O_2^- by cell-bound XO rather than direct electron transfer to cytochrome *c* (Table IV). The inability of native CuZn-SOD to inhibit O_2^- production by cell-bound XO, due to formation of O_2^- in a sequestered microenvironment, has important implications for devising antioxidant strategies for treatment of diseases including a pathogenic role for XO-derived reactive oxygen species and other cellular sources of O_2^-. The data herein reveal that XO inactivation, inhibition of cell-XO binding, and the administration of proteoglycan-binding forms of SOD or more cell-avid SOD mimetics will have greater pharmacologic impact on this reaction pathway than native CuZn-SOD (60, 61).

Evidence that cell-bound XO can impair vascular cell function and produces O_2^- in a sequestered microenvironment comes from the inhibition of ·NO-dependent signal transduction by endothelial cells having bound XO (Fig. 6) Stimulation of control endothelial cell ·NO production by ionomycin increased smooth muscle cell cGMP production in co-cultures of endothelial and smooth muscle cells as before (62, 63). Superoxide produced by cell-bound XO can then react with ·NO at rates 10-fold faster than for SOD scavenging of O_2^-, yielding $ONOO^-$ and attenuating ·NO-dependent production of cGMP by smooth muscle cells (58, 64–66). Peroxynitrite generated from the reaction of XO-derived O_2^- with ·NO was not expected to directly inhibit endothelial nitric-oxide synthase (67). The prevention of XO-mediated inhibition of endothelial-dependent smooth muscle cell cGMP production by allopurinol indicated that XO did not irreversibly inhibit nitric-oxide synthase activity or deplete cellular substrates required for nitric-oxide synthase activity (Fig. 6). The lack of an effect of CuZn-SOD or EC-SOD on XO-mediated inhibition of smooth muscle cell cGMP production also reinforced the concept that glycosaminoglycan-bound XO partitions into an SOD-resistant compartment (15).

Multiple lines of evidence reveal that circulating and cell-associated XO contribute to the pathogenesis of vascular disease. Patients with atherosclerosis that display impaired vascular function also exhibit increased circulating XO levels, a phenomenon that may predispose to impaired ·NO-dependent

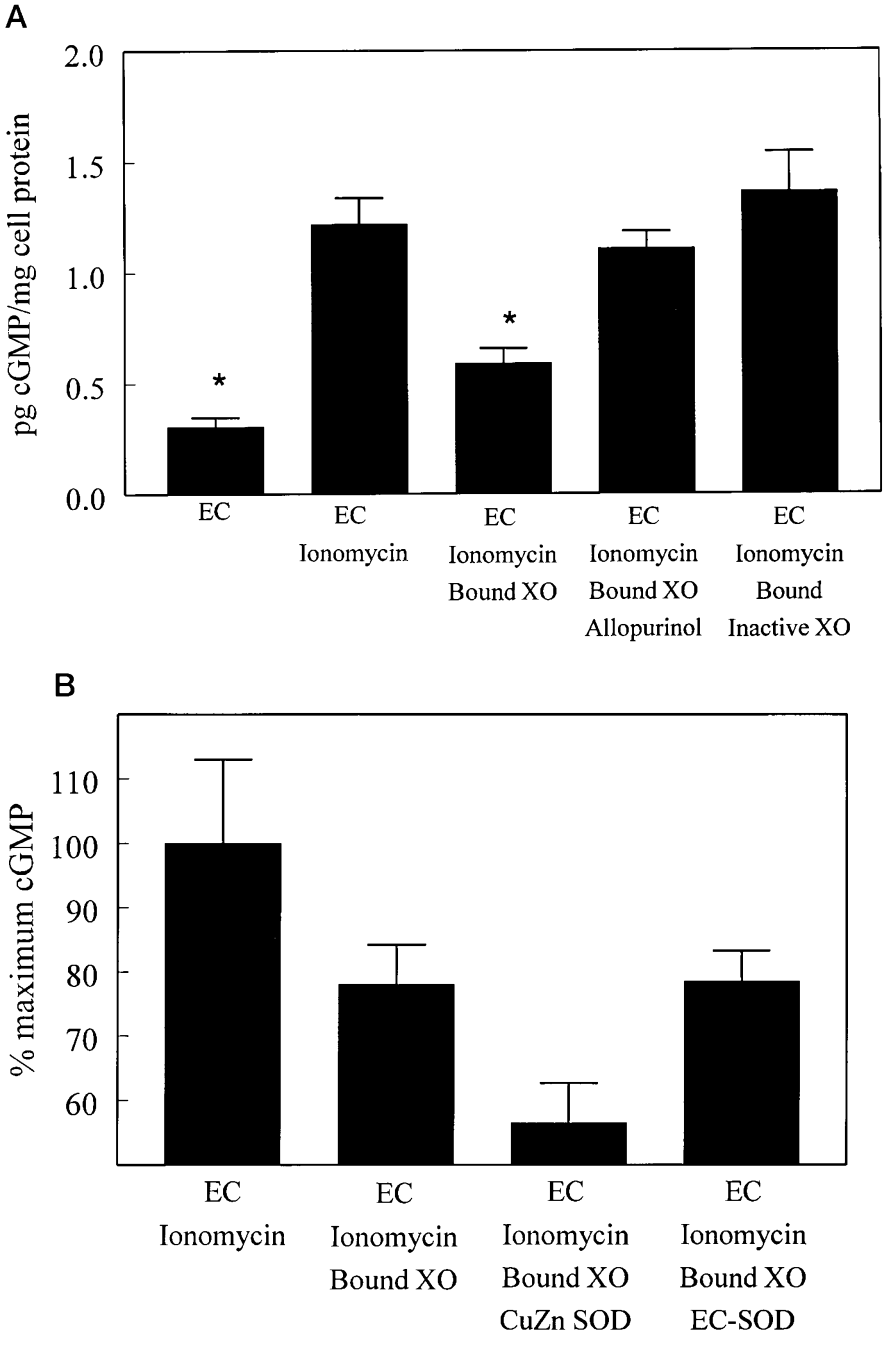


FIG. 6. *A*, inhibition of nitric oxide-dependent signal transduction by cell-bound xanthine oxidase. Vascular endothelial cells (*EC*) cultured on Transwell filters were incubated with 10 milliunits/ml XO for 3 h and washed extensively. Endothelial cell-containing filters were then transferred to dishes containing smooth muscle cells and incubated with xanthine (100 μM), SOD (50 units/ml), isobutylmethylxanthine (0.5 mM), and ionomycin (6.7 μM) with or without allopurinol for 15 min. Smooth muscle cell cGMP concentration was then determined by EIA. Sodium nitroprusside (10 μM) was added directly to smooth muscle cells to reflect maximum attainable cGMP concentrations. Data represent mean ± S.E. of six experiments ($n = 3$). *, $p < 0.05$ compared with ionomycin-treated endothelial cells. *B*, inability of EC-SOD or CuZn-SOD to inhibit cell-bound xanthine oxidase-mediated impairment of nitric oxide-dependent signaling. Vascular endothelial cells and smooth muscle cells were treated as described above with the further addition of 50 units/ml EC-SOD or 950 units/ml CuZn-SOD to the media of both cell types during the 15-min coincubation period. Data represent mean ± S.E. of a representative experiment ($n = 3$).

signal transduction and further development of arterial lesions via oxidative modification of low density lipoproteins and depletion of plasma antioxidants (39). While XO can be detected in a heparin-reversible vascular compartment in healthy adults (14), increased levels of XO are also evident in the vessel wall and plaque of individuals with atherosclerosis (68). This increase in vessel wall XO may come from enhanced expression of XO by local stimuli (69–71) as well as from binding of plasma XO to the endothelium and intima. Then vessel wall XO can initiate a cascade of oxidant-mediated events that culminate in a chronic inflammatory response, promotion of cellular destruction, and plaque formation (58, 72–75). Importantly, endothelial-bound XO inhibits $^{\cdot}$NO-dependent signal transduction events that mediate vessel relaxation (Fig. 6). This phenomenon, a hallmark of vascular pathology in both animal models of atherosclerosis and atherosclerotic humans, is amenable to normalization by allopurinol (45, 46, 76).

In summary, diverse cell types possess glycosaminoglycan-containing binding sites for XO. Upon cell binding, XO retains catalytic activity and becomes incorporated via endocytosis. The reactive oxygen species derived from cell-bound XO are generated in an SOD-resistant compartment to inhibit $^{\cdot}$NO-dependent signaling and initiate proinflammatory events.

Acknowledgments—We acknowledge the skillful technical assistance of Gina Park and Daniel S. Gelman.

Note Added in Proof—It was recently reported that xanthine oxidoreductase is asymmetrically localized on the exofacial surface of human endothelial and epithelial cells, as well as in the cytosol (Rouquette, M., Page, S., Bryant, R., Benboubetra, M., Stevens, C. R., Blake, D. R., Whish, W. D., Harrison, R., and Tosh, D. (1998) *FEBS Lett.* **426,** 397–401). This infers that mechanisms exist for the translocation of intracellular enzyme and supports the concept that reactive species derived from xanthine oxidase participate in cell signaling.

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