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Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits

(superoxide/nitric oxide/peroxynitrite/glycosaminoglycan/vascular relaxation)

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ABSTRACT **Reactive oxygen species play a central role in vascular inflammation and atherogenesis, with enhanced superoxide ($O_2^{\cdot-}$) production contributing significantly to impairment of nitric oxide ($^{\cdot}NO$)-dependent relaxation of vessels from cholesterol-fed rabbits. We investigated potential sources of $O_2^{\cdot-}$ production, which contribute to this loss of endothelium-dependent vascular responses. The vasorelaxation elicited by acetylcholine (ACh) in phenylephrine-contracted, aortic ring segments was impaired by cholesterol feeding. Pretreatment of aortic vessels with either heparin, which competes with xanthine oxidase (XO) for binding to sulfated glycosaminoglycans, or the XO inhibitor allopurinol resulted in a partial restoration (36–40% at 1 μM ACh) of ACh-dependent relaxation. Furthermore, $O_2^{\cdot-}$-dependent lucigenin chemiluminescence, measured in intact ring segments from hypercholesterolemic rabbits, was decreased by addition of heparin, allopurinol or a chimeric, heparin-binding superoxide dismutase. XO activity was elevated more than two-fold in plasma of hypercholesterolemic rabbits. Incubation of vascular rings from rabbits on a normal diet with purified XO (10 milliunits/ml) also impaired $^{\cdot}NO$-dependent relaxation but only in the presence of purine substrate. As with vessels from hypercholesterolemic rabbits, this effect was prevented by heparin and allopurinol treatment. We hypothesize that increases in plasma cholesterol induce the release of XO into the circulation, where it binds to endothelial cell glycosaminoglycans. Only in hypercholesterolemic vessels is sufficient substrate available to sustain the production of $O_2^{\cdot-}$ and impair NO-dependent vasorelaxation. Chronically, the continued production of peroxynitrite, ($ONOO^-$) which the simultaneous generation of NO and $O_2^{\cdot-}$ implies, may irreversibly impair vessel function.**

In recent years, numerous studies have shown that vascular function is compromised in a number of pathological conditions including atherosclerosis and hypertension (1–3). In atherosclerosis, blood vessels undergo marked changes in both structure and function that may predispose for angina and myocardial infarction. Defects in lipoprotein metabolism and vascular reactivity are fundamental pathological responses to hypercholesterolemia, with reactive oxygen species playing an important role in the initiation and progression of these lesions (4, 5). Isolated blood vessels from atherosclerotic patients (6, 7) and hypercholesterolemic animals (8–11) exhibit impaired, endothelium-dependent vascular relaxation. In these models, superoxide ($O_2^{\cdot-}$) is generated at greater rates in both intracellular and extracellular compartments, reacting with $^{\cdot}NO$ to inhibit endothelium-dependent relaxation and yielding peroxynitrite ($ONOO^-$) (12). Since $ONOO^-$ is capable of oxidizing lipids and proteins, it may further contribute to foam cell formation in the atherosclerotic lesion (13–15).

While oxidative injury plays a pivotal role in these processes, the cellular mechanisms of $O_2^{\cdot-}$ production and sites of reaction remain to be clearly defined. Sources of $O_2^{\cdot-}$ that have been identified include a plasma membrane NAD(P)H oxidase (16, 17) and the enzyme NO synthase when deprived of its substrate L-arginine (18). Recently, it has been shown that vascular endothelial cells possess glycosaminoglycan (GAG)-rich receptors, which reversibly bind xanthine oxidase (XO) via saturable, high-affinity binding sites (19, 20). This serves to concentrate an enzyme at the cell surface, which is capable of generating $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) when supplied with its substrates xanthine or hypoxanthine. This association may be relevant to atherosclerosis, since it has been argued that an early response to hypercholesterolemia is a localized hypoxia in the vessel wall, which would lead to ATP catabolism and the formation of XO substrate (21).

GAGs are synthesized by all vascular cells and are located in intracellular secretory granules, at the cell surface, and in the extracellular matrix (22). These macromolecules serve diverse cellular functions including cell adhesion, proliferation, and differentiation as well as mechanical support. Numerous factors influence GAG synthesis and function in both normal and pathological states. Lipid emulsions promote an increase in the relative proportion of heparin-sulfate GAGs in endothelial cells (23), and GAG biosynthesis and content of aortic neointima is enhanced in cholesterol-fed rabbits (24). Reactive species derived from XO may also stimulate the synthesis of cellular GAGs (25), at the same time inhibiting the expression of extracellular superoxide dismutase type C (26). Thus, vascular concentration of XO can be enhanced at the same time that defense mechanisms against $O_2^{\cdot-}$ are being impaired. We report herein that the binding of XO to vessel wall GAGs contributes to the impaired $^{\cdot}NO$-dependent relaxation associated with hypercholesterolemia.

MATERIALS AND METHODS

Vascular Relaxation Studies. New Zealand white rabbits (2.5–3.0 kg; Myrtle's Rabbitry, Thompson Station, TN) were assigned at random to one of two experimental groups. The first group was placed on a modified laboratory chow (Purina) containing 1% cholesterol for 4–6 weeks. A second group of age- and weight-matched rabbits were fed a standard laboratory chow and served as controls. Endothelium-dependent

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Abbreviations: GAG, glycosaminoglycan; XO, xanthine oxidase; K-H, Krebs-Henseleit; ACh, acetylcholine; SOD, Cu,Zn superoxide dismutase; HB-SOD, heparin-binding SOD; XDH, xanthine dehydrogenase.

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relaxation was measured in isolated aortic ring segments of control and experimental rabbits as described (12). Upon sacrifice, the aorta was excised and cleansed of fat and adhering tissue. Individual ring segments (3–4 mm wide) were cut from the aorta and suspended from a force-displacement transducer in a tissue bath. Ring segments were bathed in bicarbonate-buffered, Krebs-Henseleit (K-H) solution of the following composition: NaCl, 118 mM; KCl, 4.6 mM; $NaHCO_3$, 27.2 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.75 mM; Na_2EDTA, 0.03 mM; and glucose, 11.1 mM. A passive load of 3 g was applied to all ring segments and maintained at this level throughout the experiment. At the beginning of each experiment, KCl (70 mM)-induced contractions were elicited in indomethacin (5 μM)-treated ring segments to determine the maximal contractile capacity of each vessel. Rings were then thoroughly washed and allowed to equilibrate for 1 hr. In subsequent experiments, vessels were submaximally contracted to $\approx$40% of the KCl response with phenylephrine (3×10^{-8}–10^{-7} M). When tension development reached a plateau, acetylcholine (ACh) (10^{-9}–3×10^{-6} M) was added cumulatively to the bath to invoke EC-dependent relaxation. At the end of each dose response protocol, sodium nitroprusside (5 μM) was added to elicit residual endothelium-independent relaxation. Real-time data was collected for all experiments and downloaded to an IBM PC for later analysis using commercially available software (EXPERIMENTER'S WORKBENCH). In one series of experiments, rings were incubated with heparin (1000 units/ml) for 10 min and then thoroughly washed with K-H solution before phenylephrine/ACh dose–response protocols. In related studies, ring segments were treated with allopurinol (100 μM) for 10 min.

Superoxide-Dependent Lucigenin Chemiluminescence. Lucigenin (0.25 mM) was used to detect $O_2^{\cdot-}$ production by vascular ring segments placed in scintillation vials containing 2 ml of phosphate-buffered saline. Chemiluminescence was measured using an LKB Rackbeta scintillation counter set to operate in the out-of-coincidence mode, with sampling at 30-sec intervals over a 10-min time period. The effects of heparin (1000 units/ml), allopurinol (50–100 μM), native Cu,Zn superoxide dismutase (SOD; 100 units/ml), heparin-binding SOD (HB-SOD; 40 units/ml), and xanthine (50 μM) were tested.

***In Vitro* Incubation of Control Ring Segments with Purified XO.** XO (Calbiochem) was desalted by elution on a Sephadex PD-10 gel filtration column. Collected fractions were assayed for enzymatic activity by the rate of formation of uric acid at 295 nm ($\varepsilon_M = 1.1 \times 10^4\ M^{-1}cm^{-1}$). Rings were incubated with purified XO (10 milliunits/ml) for 1 hr in aerated K-H buffer before the study. At the end of this incubation period, tissues were washed four times with 15 ml of K-H solution to remove unbound enzyme. The response to ACh was then determined in vessels submaximally contracted with phenylephrine. Where stated, exogenous xanthine (100 μM) was added to the bath 10 min before ACh. In some experiments, rings that had been preincubated with XO as described above were then incubated with heparin (1000 units/ml) or allopurinol (100 μM) for 10 min. The tissue was then thoroughly washed, and the response to ACh was again determined.

Plasma Xanthine Oxidoreductase Activity. XO and dehydrogenase (XDH) activity of plasma from control and cholesterol-fed rabbits was measured using HPLC (27). At sacrifice, plasma samples were obtained from test animals and immediately frozen at −80°C. Before measuring enzymatic activity, endogenous urate was removed by eluting the sample on a Sephadex G-25 column. Samples were then treated with oxonic acid (2 mM) to inhibit plasma uricase activity. Xanthine (75 μM) was then added, and XO/XDH activity assessed by monitoring the production of urate. These reactions were performed in the absence and presence of NAD^+ (0.5 mM) and pyruvic acid (5 mM) to assess XO and total oxidoreductase (XO plus XDH) activity, respectively. The specificity of this detection method for urate production by XO/XDH was verified by inhibition of urate formation following allopurinol addition in some samples.

RESULTS

Cumulative administration of ACh in aortic ring segments from cholesterol-fed rabbits showed severe impairment of endothelium-dependent relaxation (Fig. 1). Incubation of rings from cholesterol-fed rabbits with heparin (1000 units/ml) or allopurinol (100 μM) for 10 min before ACh exposure resulted in a significant recovery of $^{\cdot}NO$-dependent relaxation (Fig. 1). Interestingly, heparin treatment did not show a protective effect on ACh-induced relaxation in ring segments excised from rabbits maintained on the cholesterol-enriched diet for periods of 3 and 6 months (data not shown).

Superoxide generation of isolated ring segments from cholesterol-fed rabbits was assessed using lucigenin-dependent chemiluminescence. Fig. 2 depicts the composite chemiluminescence profile of ring segments from cholesterol-fed rabbits. We observed that chemiluminescence yields in these vessels were elevated above those of control vessels and gradually increased for several minutes before reaching a stable maximum value at between 5 and 10 min (Fig. 2*a*). The effect of HB-SOD is to decrease the steady increase in vessel wall $O_2^{\cdot-}$ generation while leaving the baseline chemiluminescence unchanged. We conclude, therefore, that the initial signal is not due to $O_2^{\cdot-}$ generation and has been subtracted in subsequent analyses. To assess the effects of treatments that partially restored $^{\cdot}NO$-dependent relaxation on vessel wall $O_2^{\cdot-}$ formation, tissues were exposed to heparin or allopurinol. Heparin incubations were performed in the tissue bath and included a washing procedure before $O_2^{\cdot-}$ measurement. Using the maximum chemiluminescence signal obtained at 10 min, the results obtained are reported in Fig. 2*b*. The composite signal was inhibited 49% by heparin, 97% by allopurinol, and 68% by HB-SOD. The addition of either exogenous xanthine (50 μM) or SOD (100 units/ml) had no significant effect on the chemiluminescence of rings from cholesterol-fed rabbits. Basal

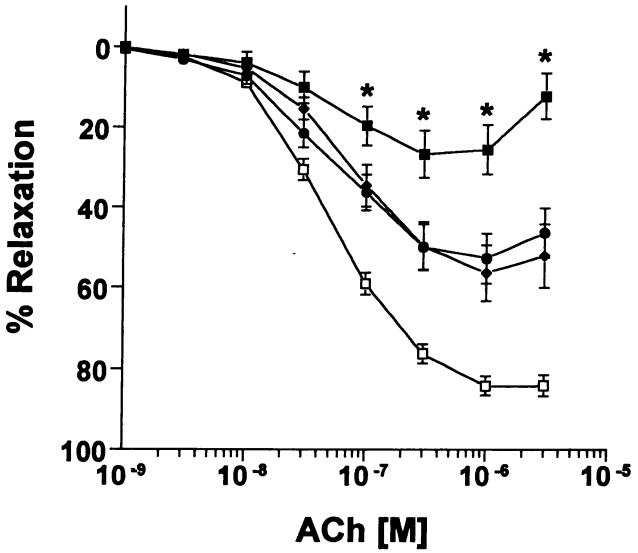

FIG. 1. Endothelium-dependent relaxation of rabbit aortic ring segments. Aortic rings were isolated from rabbits that had been maintained on a 1% cholesterol diet for 6 weeks. Cumulative dose–response profiles to ACh were obtained in phenylephrine-contracted rings of control (□, n = 31) and cholesterol-fed rabbits (■, n = 20). Pretreatment of ring segments with 1000 units/ml heparin (●, n = 17) or 100 μM allopurinol (◆, n = 13). Data are means ± SEM. *, $p <$ 0.05 from the control and heparin- and allopurinol-treated groups.

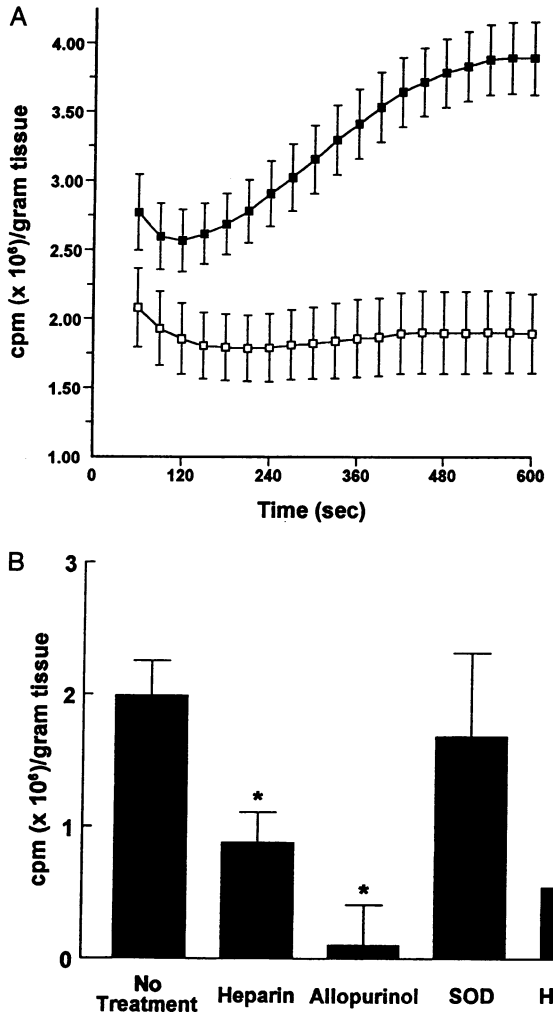

FIG. 2. Superoxide-dependent, lucigenin chemiluminescence of intact aortic ring segments. Tissues were placed in vials containing 2 ml of phosphate-buffered saline with lucigenin (0.25 mM) and xanthine (50 μM). (*A*) Chemiluminescence profiles of ring segments from control (□, $n = 6$) and cholesterol-fed (■, $n = 12$) rabbits monitored over a 10-min time period. (*B*) Effects of heparin ($n = 8$), allopurinol ($n = 6$), SOD ($n = 8$), and HB-SOD ($n = 3$) on chemiluminescence of ring segments of cholesterol-fed rabbits. Peak chemiluminescence yields are reported for the 10-min time point. Data represent mean ± SEM. *, $p < 0.05$ from the cholesterol-fed group receiving no additional treatment.

chemiluminescence of rings from control rabbits fed a normal diet was 2.04 ± 0.22 (x 10^6 cpm), which was not significantly different from the allopurinol-treated or HB-SOD values of vessels from cholesterol-fed animals. This data supports a contributory role of XO to $O_2^{\cdot-}$ generation in isolated vessels.

Measurement of XO/XDH activity in plasma from cholesterol-fed rabbits was measured *ex vivo* by the stimulation of urate production in the presence of xanthine substrate (Fig. 3). Hypercholesterolemia results in a greater than two-fold increase in the concentration of circulating XO, which was inhibited ≈90% by allopurinol (100 μM). To measure total xanthine oxidoreductase (XO plus XDH) activity, 0.5 mM NAD^+ and 5.0 mM pyruvate were included in the sample. The rates of urate production were not significantly different from samples without addition of these reagents, inferring that the plasma enzyme is predominantly in the oxidase form.

Experiments were designed to test whether XO would bind to vessel segments from control rabbits and whether this treatment induced changes in endothelium-dependent relaxation. Incubation of isolated vessels with purified XO did not

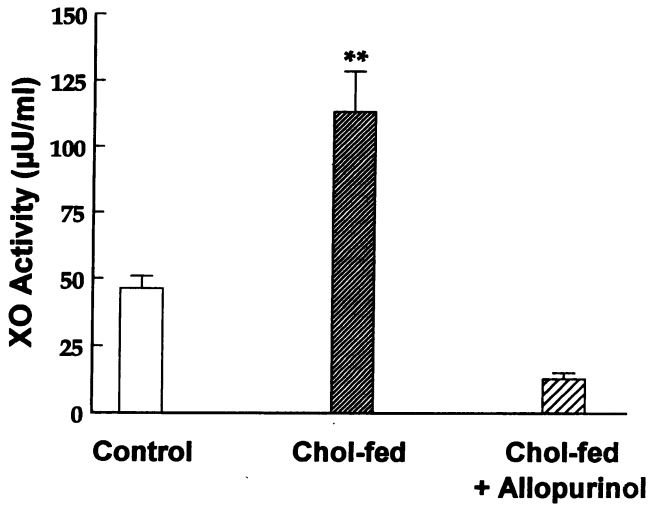

FIG. 3. Plasma XO activity in normal and hypercholesterolic rabbits. Uric acid production in plasma samples from control ($n = 11$) and cholesterol-fed ($n = 16$) rabbits was initiated by the addition of 75 μM xanthine. After incubation at 37°C for 60 min, samples were extracted for measurement of urate by HPLC. In seven plasma samples, allopurinol was included at a concentration of 100 μM to determine the specificity of these reaction conditions for the measurement of XO activity. Data are means ± SEM. *, $p < 0.05$ from control and allopurinol-treated groups.

change ACh-induced relaxation of aortic ring segments from control rabbits. However, following the addition of xanthine, the response to ACh was significantly impaired (Fig. 4*a*). The addition of xanthine alone did not affect ACh-dependent relaxation (data not shown). Similar to results with hypercholesterolemic vessels, heparin pretreatment (10 min) prevented the observed loss in responsiveness to ACh in xanthine/XO-treated vessels (Fig. 4*b*). Similarly, allopurinol prevented the shift in ACh responses of ring segments treated with XO plus xanthine, suggesting that the impaired relaxation response seen in XO-treated vessels was $O_2^{\cdot-}$-dependent (Fig. 4*b*). Incubation of control vessels with heparin alone did not alter sensitivity to ACh.

DISCUSSION

The targeted delivery of the $O_2^{\cdot-}$ scavenger SOD to the vessel wall in hypercholesterolemic rabbits partially reverses impaired $^{\cdot}NO$-dependent relaxation, consistent with a major role of $O_2^{\cdot-}$ in this defect (12, 28–30). The reaction of $O_2^{\cdot-}$ with $^{\cdot}NO$ results in diminished activation of smooth muscle cell guanylate cyclase, preventing the synthesis of cGMP and thus relaxation. Although mechanisms of $^{\cdot}NO$ production in the vessel wall have been well documented, the biochemical pathways leading to $O_2^{\cdot-}$ production are only now beginning to be defined. Potential sources include an endothelial cell plasma membrane NAD(P)H oxidase or the uncoupling of $^{\cdot}NO$ synthase (16–18). Superoxide may also be derived from the enzyme XO. In the present studies, we found that catalytically active, circulating XO is elevated more than two-fold in the plasma of cholesterol-fed rabbits compared with controls. This increase in plasma XO could be due to remote hepatic injury induced by hypercholesterolemia and subsequent association of XO with GAGs at the vascular lumen (27) and interstitial matrix in a manner reminiscent of extracellular SOD type C vascular distribution (31). *In vitro* studies show that XO and extracellular SOD type C exhibit high-affinity, saturable binding to sulfated GAGs, which is reversible by heparin treatment or inhibited by enzymatically cleaving cellular GAGs with heparinase and heparitinase (19, 20, 31–33). GAG association

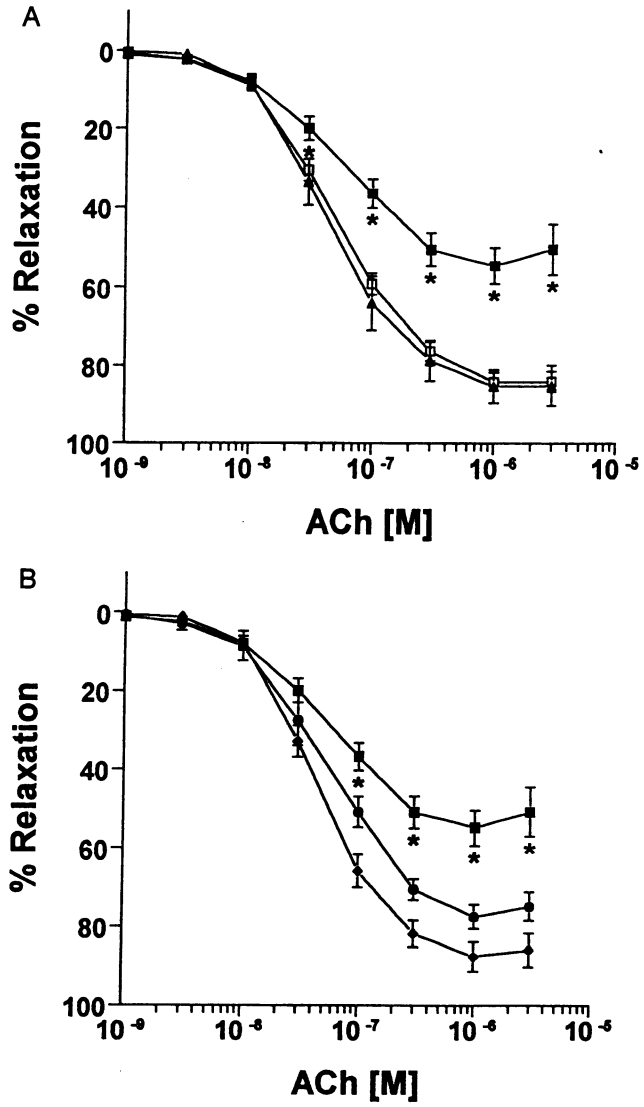

FIG. 4. Endothelium-dependent relaxation of control rabbit aortic ring segments in the presence of bound XO and xanthine. Cumulative dose–response profiles to ACh were obtained in phenylephrine-contracted rings of control rabbits. (*A*) Vessels were incubated with XO (10 milliunits/ml) for 1 hr and then thoroughly washed with K-H buffer to remove unbound enzyme. Acetylcholine dose–responses were performed in XO-incubated ring segments in the presence (■, $n = 16$) and absence (▲, $n = 6$) of 100 μM xanthine. The ACh dose–response curve for control (□, $n = 31$) ring segments is included for comparison of the effects of these treatments. (*B*) XO-incubated rings were incubated with 1000 units/ml heparin (●, $n = 7$) for 10 min before the addition of 100 μM xanthine substrate. Heparin pretreatment prevented the impairment of $^{\cdot}$NO-mediated relaxation seen upon addition of xanthine. Similarly, rings pretreated with 100 μM allopurinol (◆, $n = 6$), an inhibitor of XO, prevented the shift in the ACh dose–response profile seen in XO-treated vessels upon addition of 100 μM xanthine. Data are means ± SEM. *, $p < 0.05$ from all treatment groups.

can also promote the endocytic incorporation of membrane-bound molecules (34, 35). Thus, XO may also be internalized by endothelial cells to react with intracellular purines and produce reactive species.

In the present studies, heparin partially reversed the impaired $^{\cdot}$NO-dependent relaxation of aortic ring segments from rabbits fed a cholesterol diet for 4–6 weeks. Both *in vitro* and *in vivo* studies (19, 20, 32) demonstrate that heparin competitively binds to and displaces GAG-associated molecules, inferring that the effect of heparin on restoring vascular relaxation was due to displacement of GAG-bound XO, thus attenuating the reaction of $O_2^{\cdot-}$ with endothelium-derived $^{\cdot}$NO. Allopurinol, an inhibitor of XO, also shifted ACh dose–response profiles to an extent similar to heparin, suggesting that $O_2^{\cdot-}$ derived from XO was involved in the production of altered vessel reactivity. Another XO inhibitor, 4-amino-6-hydroxypyrazolo[3,4-D]pyrimidine, similarly potentiates vasorelaxation in rabbit and rat vessels (36). Concurrent administration of allopurinol and heparin did not demonstrate an additive effect on vessel relaxation (results not shown).

Heparin may stimulate both cGMP concentration and nitrate/nitrite levels in human umbilical vein endothelial cells, indicating enhanced rates of $^{\cdot}$NO production (37). It is possible that the observed protective effect of heparin in ring segments of hypercholesterolemic rabbits may be due to an effect of heparin on $^{\cdot}$NO production. However, our results show that the partial restoration of $^{\cdot}$NO-mediated relaxation by heparin is due to limitation of vessel wall $O_2^{\cdot-}$ production via displacement of GAG-bound XO. This is supported by the observation that both heparin and allopurinol reduce $O_2^{\cdot-}$-dependent, lucigenin chemiluminescence in ring segments of cholesterol-fed rabbits. Additionally, a chimeric heparin-binding SOD (HB-SOD) was a potent scavenger of $O_2^{\cdot-}$ and caused reduced chemiluminescence yields. Native SOD did not afford this protection, presumably due to its electrostatic repulsion from cell membranes and inability to bind in the same GAG-containing environment as HB-SOD. These treatments had little or no effect on the chemiluminescence of ring segments from control rabbits, suggesting that XO-dependent $O_2^{\cdot-}$ production in these tissues was negligible.

Addition of purified XO to control rabbit vessel segments also showed reversible XO binding to GAGs and resulted in inhibition of ACh-induced vessel relaxation in the presence of xanthine. Heparin and allopurinol similarly prevented the observed shift in sensitivity to ACh in xanthine/XO-treated vessels. The lack of an effect of XO on relaxation in the absence of xanthine indicates that insufficient endogeneous substrate was available in normal vessels. In the lesioned vessels of atherosclerotic animals, however, a localized hypoxia can be induced in regions adjacent to plaque deposits. These conditions would favor the catabolism of adenosine to xanthine or hypoxanthine, both substrates for XO. The allopurinol-inhibitable chemiluminescence of blood vessels from cholesterol-fed rabbits in the presence or absence of supplemental xanthine was similar, suggesting that adequate endogenous substrate was available in these tissues to support XO-dependent, $O_2^{\cdot-}$ production.

XO-derived $O_2^{\cdot-}$ has been linked to oxidative injury in ischemia/reperfusion models (38). In an isolated rat hindlimb preparation subjected to ischemia-reperfusion injury, circulating plasma XO activity was increased, and ACh-mediated relaxation was severely compromised in the reperfused vascular bed (39). In this and other studies, heparin has been reported to be cytoprotective toward XO-induced endothelial cell injury and recovery of endothelium-dependent relaxation in the absence of direct oxidant scavenging mechanisms (39, 40). In a hemorrhagic shock model of ischemia-reperfusion, XO activity is released in high concentrations to the rat vasculature after reperfusion (20). Furthermore, XO was bound to the endothelium in a heparin-reversible fashion, suggesting that in ischemia-reperfusion injury, XO can become concentrated on the vessel wall at sites remote from the primary locus of injury (20).

Results of the current studies provide new information regarding basic mechanisms associated with the development of atherosclerotic disease. It is apparent that impaired blood vessel function in atherosclerosis is due in part to the reaction of $^{\cdot}$NO with $O_2^{\cdot-}$, concomitantly leading to both the production of the potent oxidant $ONOO^-$ and a loss of the physiological actions of $^{\cdot}$NO. Free radical injury to low density lipoprotein

and atherosclerotic blood vessels may be linked to the binding and concentration of XO at GAG sites on endothelial cells and the subsequent production of reactive species such as O_2^-. Furthermore, GAG function or expression may be altered by hyperlipidemia, thereby facilitating incorporation of lipoproteins in the vessel wall. This microenvironment will then serve as a site for XO binding and incorporation serving as an important locus in the pathological reactions associated with the development and progression of atherosclerotic disease.

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