

# L-Arginine inhibits xanthine oxidase-dependent endothelial dysfunction in hypercholesterolemia

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**Abstract** Xanthine oxidase (XO)-derived superoxide contributes to endothelial dysfunction in humans and animal models of hypercholesterolemia (HC). Since L-arginine supplementation prevents defects in NO signaling, we tested the hypothesis that L-arginine blunts the inhibitory effect of XO on vascular function. Acetylcholine-mediated relaxation was significantly impaired in ring segments of HC rabbits, a response that was associated with an increase in plasma XO activity. L-Arginine treatment of HC rabbits reduced plasma XO and improved endothelial function. L-Arginine also modestly prolonged the lag time for oxidation in isolated lipoprotein samples. These results reveal that the principal action of L-arginine is to protect against the XO-dependent inactivation of NO in arteries of HC rabbits.

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**Key words:** Xanthine oxidase; Hypercholesterolemia; Superoxide; Nitric oxide; Peroxynitrite; Vascular reactivity

## 1. Introduction

Vascular function is compromised under conditions of inflammation and atherogenesis [1,2]. Defects in lipoprotein metabolism and vascular reactivity are fundamental pathological responses to hypercholesterolemia, with extensive evidence suggesting that reactive oxygen and nitrogen species play an important role in the initiation and progression of these lesions [3,4]. Blood vessels from atherosclerotic patients and hypercholesterolemic (HC) animal models exhibit impaired, endothelium-dependent relaxation [5,6]. Nitric oxide (NO) becomes modified in a hyperlipidemic environment via its reaction with superoxide ( $O_2^{\cdot-}$ ), resulting in diminished NO bioactivity and yielding the potent oxidant peroxynitrite ( $ONOO^-$ ) [7,8]. Peroxynitrite may promote atherogenesis by reducing the beneficial physiological actions of NO and oxidizing lipoproteins [7,9].

Potential sources of  $O_2^{\cdot-}$  in the vascular compartment include a plasma membrane NADPH oxidase [10], the enzyme nitric oxide synthase (NOS) when deprived of its substrate

L-arginine [11] and xanthine oxidoreductase (XOR) [12]. Xanthine oxidase (XO) is produced from XOR via intramolecular thiol oxidation or limited proteolysis [13]. In the presence of  $O_2$  and purine substrate, XO yields uric acid and the oxidants  $O_2^{\cdot-}$  and hydrogen peroxide ( $H_2O_2$ ). The identification of XO in vascular lesions of HC humans suggests that the enzyme may be a clinically relevant target for the therapeutic treatment of atherosclerosis [14]. This is underscored by findings that infusion of the XO inhibitor oxypurinol in humans increases forearm blood flow in HC, but not hypertensive, patients [15].

Circulating plasma XO activity is increased in HC rabbits, with XO capable of specific binding to glycosaminoglycan (GAG)-rich proteoglycan receptors on vascular cells, resulting in the concentration of XO at the cell surface [12]. GAG association also promotes the endocytic incorporation of bound enzyme, facilitating XO reaction with intracellular purines and production of reactive oxygen species [16]. Superoxide derived from XO also appears to play a role in the modification of NO function, since treatment of atherosclerotic blood vessels with the XO inhibitor allopurinol improves endothelium-dependent relaxation [12,17].

Anti-atherogenic effects of L-arginine have been reported in humans and animal models [18,19]. Oral treatment with L-arginine enhances endothelial NO bioactivity in HC humans [18]. Results of laboratory studies also suggest that chronic administration of L-arginine to HC animals prevents structural and functional changes in the vasculature [19,20]. The mechanism underlying the vascular protective effects of L-arginine has been attributed to an increased availability of substrate for the endothelial NOS isoform. In the current study, it is revealed that an additional effect of chronic L-arginine administration is to limit hepatocellular injury induced by HC and increases in both plasma XO activity and the production of  $O_2^{\cdot-}$  in the vessel wall of HC rabbits.

## 2. Materials and methods

### 2.1. Animals

New Zealand white rabbits (2.5–3.0 kg) were maintained on a modified rabbit chow containing 1% cholesterol for 6 weeks prior to study (HC group). A subgroup of HC rabbits received L-arginine (2.25%) administered in the drinking water. Age- and weight-matched rabbits were fed a standard laboratory diet and served as controls in these studies. All protocols were approved by the Institutional Animal

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Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

## 2.2. Plasma XO activity

XO activity was detected in plasma samples using high performance liquid chromatography (HPLC) with electrochemical detection. Prior to 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  $\mu$ M) was then added at 37°C for 60 min, and XOR activity assessed by monitoring the production of urate. These reactions were performed in the absence and presence of NAD<sup>+</sup> (0.5 mM) and pyruvic acid (5 mM) in order to assess XO and total XOR activity, respectively. The specificity of this detection method for urate production by XOR was verified by inhibition of urate formation following allopurinol addition.

## 2.3. Plasma cholesterol and alanine aminotransferase measurements

Plasma cholesterol concentration was determined as described by Allain et al. [21]. Plasma samples were first treated with cholesterol esterase to cleave esterified cholesterol. The free cholesterol in samples was then oxidized by cholesterol oxidase with the concomitant formation of hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol to produce the chromophore quinoneimine. The absorbance of quinoneimine at 500 nm was monitored photometrically and was directly proportional to cholesterol content in the sample.

The alanine aminotransferase (ALT) activity of plasma samples was determined using a modification of the procedure described by Henry et al. [22]. ALT catalyzes the transfer of an amino group from L-alanine to  $\alpha$ -ketoglutarate, resulting in the formation of pyruvate and L-glutamate. Pyruvate may then be reduced to lactate in the presence of lactate dehydrogenase and NADH. ALT activity of plasma samples is determined indirectly by monitoring the oxidation of NADH at 340 nm. Since all reactants were present in excess, the rate-limiting step in this reaction is catalyzed by ALT.

## 2.4. Vessel reactivity studies

Endothelium-dependent and -independent relaxation was monitored in aortic ring segments as described previously [12]. Ring segments were passively stretched to a tension of 3 g, corresponding to the optimum range of their length–tension curve. In order to assess endothelium-dependent, NO-mediated relaxation, aortic rings were submaximally contracted with phenylephrine (PE) followed by cumulative addition of acetylcholine (ACh). In other experiments, ring segments were exposed to the endothelium-independent vasodilator sodium nitroprusside (SNP). Relaxation responses are reported as a decrease in vessel tension as a percentage of the pre-existing tone generated by PE.

## 2.5. Lipoprotein oxidation studies

$\beta$ -very low density lipoprotein ( $\beta$ -VLDL) was isolated from plasma by differential centrifugation. After dialysis against Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline containing NaCl (140 mM), KCl (2.7 mM), Na<sub>2</sub>HPO<sub>4</sub> (8.13 mM), KH<sub>2</sub>PO<sub>4</sub> (1.47 mM) and EDTA (10  $\mu$ M), the lipoprotein was sterilized by filtration through a 0.22  $\mu$ m filter. The oxidizability of purified  $\beta$ -VLDL samples was monitored to determine whether L-arginine treatment increased the resistance of  $\beta$ -VLDL to oxidative modification. Oxidation of  $\beta$ -VLDL (25  $\mu$ g/ml) was initiated by exposure to 10  $\mu$ M CuSO<sub>4</sub>. Lipid peroxidation results in conjugated double bond formation in polyunsaturated fatty acids which was monitored spectrophotometrically at 234 nm.

## 3. Results

The effects of chronic L-arginine treatment on plasma XO activity, endothelium-dependent relaxation and the oxidizability of plasma lipoproteins in HC rabbits were examined. Total cholesterol was similar in plasma samples obtained from HC (1682  $\pm$  165 mg/dl) and L-arginine-treated (1353  $\pm$  230 mg/dl) HC rabbits, and was elevated in both groups compared to controls (31  $\pm$  4 mg/dl). Vascular reactivity studies, performed

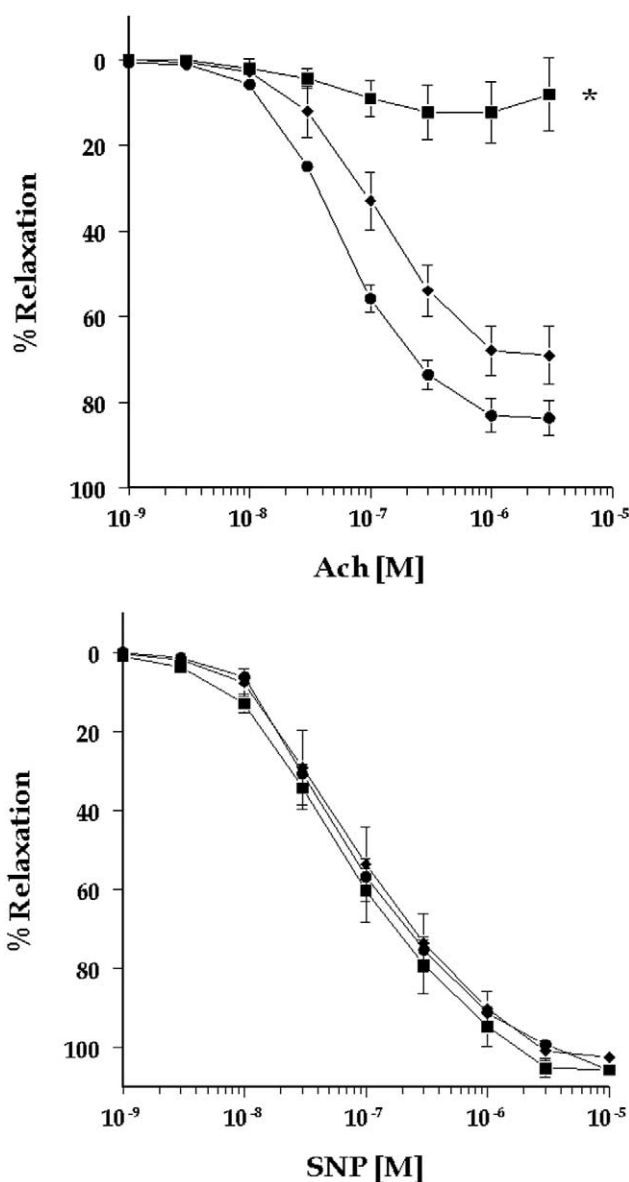


Fig. 1. Endothelium-dependent and -independent relaxation of rabbit aortic ring segments. The vasodilator response of aortic ring segments from control ( $\bullet$ ,  $n=10$ ), HC ( $\blacksquare$ ,  $n=9$ ), and HC rabbits receiving L-arginine ( $\blacklozenge$ ,  $n=7$ ) was tested by cumulative addition of ACh or SNP. Top panel: ACh-induced relaxation was significantly impaired in vessels of HC rabbits. Chronic L-arginine treatment prevented the development of endothelial dysfunction in cholesterol-fed rabbits. Bottom panel: Endothelium-independent relaxation was monitored by addition of SNP. No difference in the response to SNP was noted between groups. Data are means  $\pm$  S.E.M. \* $P < 0.05$ , significant difference compared to control and L-arginine-treated HC rabbits.

on aortic ring segments of HC rabbits, demonstrated a severe impairment of ACh-stimulated relaxation (Fig. 1). Maximum relaxation ( $R_{\max}$ ) of ring segments occurred at 1  $\mu$ M ACh in HC rabbits and was  $8 \pm 8\%$  compared to  $84 \pm 4\%$  in controls. While cumulative administration of ACh induced minimal relaxation in HC rabbits, L-arginine treatment of HC rabbits blunted the impairment of NO function as demonstrated by a downward shift in the ACh dose–response profile ( $R_{\max} = 69 \pm 7\%$ ; see Fig. 2). Endothelium-independent relaxation induced by cumulative addition of SNP was not different be-

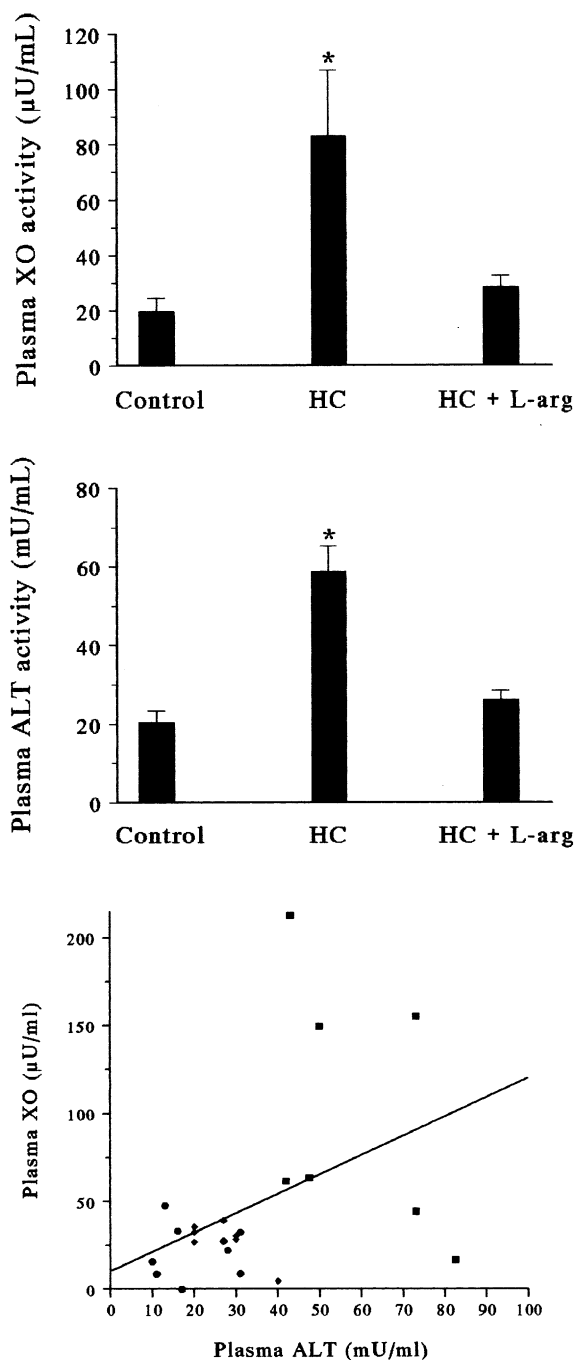


Fig. 2. Plasma measurements of XO and ALT activity. Top panel: XO activity was assessed by monitoring urate formation using HPLC with electrochemical detection. XO activity was elevated approximately two-fold in plasma from HC rabbits ( $n=9$ ). L-Arginine treatment of HC rabbits ( $n=7$ ) reduced plasma XO activity to levels comparable to those of control rabbits ( $n=10$ ). Middle panel: Since ALT is a marker for hepatic injury and is associated with the hepatic release of XO into the circulation, plasma enzyme activity was monitored. L-Arginine treatment caused a significant reduction in ALT activity in HC rabbits suggesting a hepatoprotective effect of L-arginine. Bottom panel: There was a positive correlation ( $r=0.419$ ,  $P<0.05$ ,  $n=23$ ) between plasma XO and ALT activities. Data are means  $\pm$  S.E.M. \* $P<0.05$ , significant difference compared to control and L-arginine-treated HC rabbits.

tween groups, supporting that the endothelium is a critical target of injury in the HC rabbit (Fig. 1).

XO activity was increased approximately four-fold in plasma of HC rabbits ( $83 \pm 23$   $\mu$ U/ml) compared to normolipidemic, control rabbits ( $20 \pm 5$   $\mu$ U/ml) (Fig. 2). Plasma XO and XOR activities were not statistically different, suggesting that the enzyme is present predominantly in the oxidase form. L-Arginine supplementation of HC rabbits reduced plasma XO activity ( $28 \pm 4$   $\mu$ U/ml) to a level comparable to that observed in control rabbits (Fig. 2). There was a significant inverse correlation between plasma XO and  $R_{\max}$  ( $r=-0.784$ ,  $P<0.00005$ ,  $n=22$ ) (Fig. 3). These data establish a relationship between increased plasma levels of XO and the impairment of endothelium-dependent relaxation. Since increases in plasma ALT activity are associated with XO release from the liver [23], we monitored ALT levels in plasma obtained from the three treatment groups (Fig. 2). ALT activity was distributed in a similar manner as XO with a significant increase in plasma of HC rabbits ( $59 \pm 6$  mU/ml) compared to controls ( $20 \pm 3$  mU/ml). L-Arginine supplementation of HC rabbits significantly reduced circulating levels of ALT ( $26 \pm 3$  mU/ml). Plasma levels of ALT and XO were positively correlated ( $r=0.419$ ,  $P<0.05$ ,  $n=23$ ).

To further test the effects of L-arginine on vascular redox activity, we monitored the oxidizability of isolated lipoproteins.  $\beta$ -VLDL was isolated from plasma of HC and L-arginine-treated HC rabbits. Oxidation profiles were obtained by exposing the lipid to 10  $\mu$ M  $\text{CuSO}_4$  and monitoring the formation of conjugated dienes spectrophotometrically.  $\beta$ -VLDL, composed of apolipoprotein and diverse endogenous antioxidants such as  $\alpha$ -tocopherol and  $\beta$ -carotene, displays a lag time prior to the initiation of lipid oxidation that is used as an index of endogenous antioxidant activity. A shorter lag time reveals that the antioxidant components of the particle have been depleted via oxidative reactions and that the level of endogenous lipid peroxides has increased, thus enhancing the susceptibility to oxidation. The lag time for oxidation was modestly prolonged in lipoprotein samples

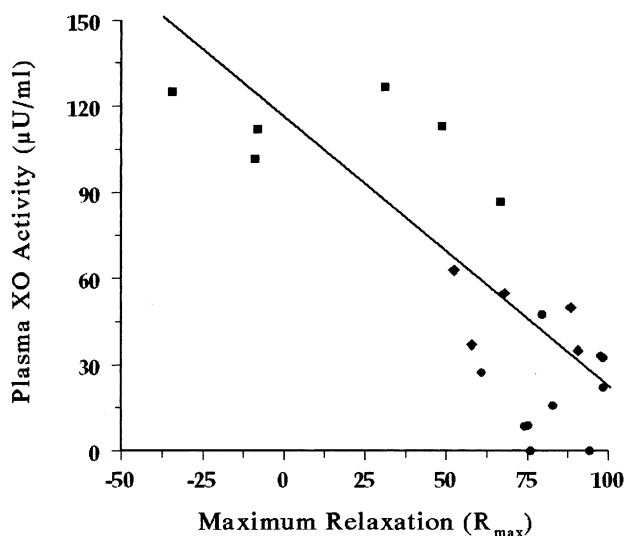


Fig. 3. Relationship between plasma XO activity and relaxant capacity of isolated aortic ring segments. Data points depict responses of control ( $\bullet$ ,  $n=10$ ), HC ( $\blacksquare$ ,  $n=6$ ) and L-arginine-treated HC rabbits ( $\blacklozenge$ ,  $n=6$ ). Negative values on the abscissa reflect contractile responses to ACh in HC rabbits.

from L-arginine-treated HC rabbits ( $95 \pm 10$  min,  $n = 8$ ,  $P < 0.05$ ) compared to untreated HC rabbits ( $62 \pm 6$  min,  $n = 7$ ).

#### 4. Discussion

Endothelial dysfunction is a hallmark of atherosclerosis in both animal models of hypercholesterolemia and humans [2,5]. The enzyme XO binds to the endothelium in the HC rabbit, may be expressed at greater levels in vascular cells and is a source of both of  $O_2^{\bullet -}$  and  $H_2O_2$  [12]. In support of the hypothesis that oxidants derived from XO contribute to vascular dysfunction, several studies showed that the XO inhibitor allopurinol improves endothelial function in experimental models of cardiovascular disease [12,13,24]. Other data suggest that the development of endothelial dysfunction in humans and animal models of atherosclerosis is due to a depletion of L-arginine substrate, the precursor for NO synthesis [2,25], which may also account for the diminished vasodilator response of isolated vascular preparations to calcium-mobilizing agonists such as ACh. While chronic dietary supplementation of HC rabbits with L-arginine has been shown to restore endothelium-dependent relaxation [20], acute treatment of isolated tissues with L-arginine has shown mixed effects on the restoration of endothelial NO production, suggesting that substrate availability may not be a rate-limiting factor [7,26,27]. An additional vasoprotective mechanism proposed for L-arginine is the stimulation of insulin release that in turn directly promotes vasorelaxation by enhancing NO formation [27]. Herein, it is reported that chronic treatment of HC rabbits with L-arginine significantly restores endothelium-dependent vasodilator function in arteries of HC rabbits, with an L-arginine-induced reduction in plasma XO and its oxidizing products strongly correlating with improved ACh-mediated relaxation. While direct measurements of  $O_2^{\bullet -}$  were not carried out in the current studies, our previous findings show that increases in plasma XO are associated with enhanced vascular  $O_2^{\bullet -}$  generation [12], supporting that the restoration of endothelium-dependent relaxation in L-arginine-treated HC rabbits is due to diminished reaction of  $O_2^{\bullet -}$  with NO, and thus less ONOO<sup>-</sup> production.

Numerous studies have used LDL oxidation measurements as a surrogate endpoint to show that specific therapeutic interventions decrease atherosclerosis. L-Arginine treatment did not alter plasma total cholesterol in HC rabbits but was associated with a modest reduction in the oxidizability of  $\beta$ -VLDL, suggesting that L-arginine indirectly preserves endogenous levels of antioxidant molecules in lipoproteins and/or limits the formation of pre-formed lipid peroxides [28]. The increase in lag time was approximately 33 min in L-arginine-treated HC rabbits, and it is not clear whether such a small effect can significantly alter the progression of atherosclerosis. In contrast to the effects of L-arginine, chronic treatment of HC rabbits with the antioxidant probucol yields a lag time for LDL oxidation that is increased more than 860 min over control [29]. Our results therefore suggest that beneficial effects of L-arginine in the prevention of endothelial dysfunction in hypercholesterolemic rabbits are largely independent of the susceptibility of  $\beta$ -VLDL to oxidation.

In the current study, the enhanced antioxidant activity of L-arginine was associated with the limitation of hepatocellular release of ALT and XO and improvement of endothelium-

dependent vasodilator function. The liver is an important source of circulating XO under pathologic conditions. It is hypothesized that a major protective effect of L-arginine under conditions of hypercholesterolemia is to minimize hepatic injury and concomitant XO release into the circulation. In support of this precept, L-arginine treatment induced a reduction in plasma levels of ALT, a hepatic enzyme associated with XO release from the liver [30]. Results of other studies support a hepatoprotective role for L-arginine. In this regard, it was shown that L-arginine infusion reduces liver injury associated with ischemia–reperfusion [31]. The protective effect of L-arginine was reversed by NOS inhibitors, implicating enhanced NO synthesis as an important component of this response [31]. The mechanism(s) underlying the protective effect of L-arginine in our model may thus involve a reduced hepatotoxic response to elevated cholesterol in the rabbit. Chronic L-arginine treatment may also improve hepatic blood flow by an NO-dependent mechanism, thereby reducing regional ischemic injury associated with cholesterol deposition. A reduction in XO release from the liver and circulating levels of the enzyme would then be expected under these conditions. We previously reported an increase in circulating and vessel-bound XO in SCD patients and knockout-transgenic SCD mice [32]. This response was associated with an increase in the hepatocellular release of ALT [17] and was viewed to be a consequence of episodes of hepatic hypoxia–reoxygenation in SCD patients. Hepatic XO release and its subsequent binding to vascular elements may thus represent a common mechanism for tissue injury in atherosclerosis, SCD and possibly other vascular diseases. In summary, our results show that an important response to chronic L-arginine treatment in HC rabbits is the improvement of vasodilator function via a mechanism that is associated with reduced circulating levels of XO.

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