**Superoxide-Dependent Stimulation of Leukocyte Adhesion by Oxidatively Modified LDL In Vivo**

Hans-Anton Lehr, Matthias Becker, Stefan L. Marklund, Christoph Hübner, Karl E. Arfors, Alfred Kohlschütter, and Konrad Messmer

Low density lipoprotein modified by oxidation (Ox-LDL) causes adhesion of leukocytes to the endothelium, a feature common in early atherogenesis. Because leukocyte adhesion under various pathophysiological conditions involves superoxide generation, we explored the possibility that superoxide is likewise involved in leukocyte adhesion in response to Ox-LDL. For our studies, we used the dorsal skin fold chamber model for intravital microscopic observation of leukocyte-endothelium interactions in hamsters.

We show here that injection of human LDL (4 mg/kg LDL cholesterol oxidatively modified by incubation in 7.5 µM Cu²⁺ for 18 hours at 37°C) elicited in control hamsters (n=7) the rolling and adhesion of circulating leukocytes along the endothelium of arterioles and postcapillary venules. This adhesion was significantly attenuated when hamsters were pretreated with bovine copper-zinc-superoxide dismutase (CuZn-SOD, 0.25 mg/kg, n=7) or heparin (2,000 IU/kg, n=7). The CuZn-SOD infusion and the heparin-induced release of extracellular SOD from endothelial cell surfaces to plasma resulted in nearly equal plasma SOD activities. Further inhibition of Ox-LDL-induced leukocyte adhesion could not be achieved by increasing the dose of CuZn-SOD to 5 mg/kg (n=6). Pretreatment of the hamsters with inactivated CuZn-SOD showed no effect. These results indicate that Ox-LDL stimulates leukocyte adhesion through a superoxide-dependent step, and they indicate a possible mechanism by which antioxidants might inhibit the onset of experimental and clinical atherosclerosis. (Arteriosclerosis and Thrombosis 1992;12:824–829)

**KEY WORDS** • low density lipoproteins • oxidation • leukocyte-endothelium interaction • superoxide dismutase • microcirculation • atherosclerosis • animal models

---

**Methods**

**Animal Model**

For our study we used Syrian golden hamsters (6–8 weeks old; weight, 60–80 g) that were fed standard lab...
cholesterol) was injected intravenously as a bolus into the hamsters via a permanent jugular catheter. Cu²⁺ was added after oxidation, Ox-LDL (4 mg/kg body wt LDL at 37°C) of the LDL suspension (1–1.5 ml) in 7.5 μM Cu²⁺ solution. Oxidative modification was achieved by incubation (18 hours, 37°C) in 7.5 μM Cu²⁺-supplemented PBS. EDTA-anticoagulated blood (1.5 mg K₂EDTA/ml blood) was collected from the aorta after laparotomy of pentobarbital-anesthetized hamsters. Plasma, erythrocytes, and leukocytes were separated by density gradient centrifugation. Total lipids were extracted, and total lipid fatty acids were determined by capillary gas chromatography as previously described. The data are expressed as weight percent of total identified fatty acids.

Statistical Analysis

Statistical analysis was performed using SAS software (SAS Statistics, version 5, Cary, N.C.). Values were tested for parametric distribution. Although parametric distribution was not uniformly found in all datasets, the data in the figures are given as mean±SD to facilitate rapid interpretation. Probability values were calculated using the Mann-Whitney U test or the Wilcoxon test with Bonferroni correction. Probability values of <0.05, <0.01, and <0.001 were considered significant and are denoted in the figures by one, two, and three asterisks, respectively.

Results

Oxidation of LDL In Vitro

Before injection into the animals and investigation of the microvascular changes, human LDL was incubated (18 hours, 37°C) in 7.5 μM Cu²⁺-supplemented PBS. According to previously published criteria, oxidation of LDL was verified by the demonstration of a decrease in the relative content of cis-polyunsaturated fatty acids.
Intravenous injection of heparin (2,000 IU/kg body wt) led to a prompt increase in plasma SOD activity (Figure 1). Postheparin plasma SOD activity was maintained at high levels and showed only a slight decline during the experiment (Figure 1). This initial increase in SOD activity by heparin corresponds to the increase in heparin-vascular endothelium interaction was encountered preferentially at arterial vessel bifurcations involving the lateral leading edges and the distal area of the flow divider, where the laminar flow is disrupted. No stimulation of leukocyte-endothelium interaction was observed after injection of equivalent amounts of native LDL (data not shown). The higher total number of sticking leukocytes per square millimeter of endothelial surface of postcapillary venules compared with arterioles (Figures 2 and 3) may be due to differences in local shear force conditions, with low shear forces prevailing in venules and high shear forces in arterioles.

When injected into heparin- and CuZnSOD-pretreated hamsters, Ox-LDL elicited an immediate onset of leukocyte rolling along the endothelial lining and subsequent firm adhesion to the endothelial surfaces of both arterioles and postcapillary venules (Figures 2–4). Both rolling and adhesive leukocytes tended to form aggregates of three and more cells. Although the fluorescent marker acridine orange does not stain platelets, the loose consistency of the leukocyte aggregates suggests that platelets were involved in their formation. Ox-LDL-stimulated leukocyte–endothelium interaction was observed preferentially at arterial vessel bifurcations involving the lateral leading edges and the distal area of the flow divider, where the laminar flow is disrupted. No stimulation of leukocyte–endothelium interaction was observed after injection of equivalent amounts of native LDL (data not shown). The higher total number of sticking leukocytes per square millimeter of endothelial surface of postcapillary venules compared with arterioles (Figures 2 and 3) may be due to differences in local shear force conditions, with low shear forces prevailing in venules and high shear forces in arterioles.

When injected into heparin- and CuZnSOD-pretreated hamsters, Ox-LDL elicited an increase in the fraction of rolling leukocytes in postcapillary venules comparable with the increases seen in untreated control animals (Figure 4). In contrast, the adhesion of leukocytes to the endothelium was significantly attenuated in arterioles and postcapillary venules in animals pretreated with heparin or CuZnSOD (Figures 2 and 3). No inhibition of Ox-LDL–induced leukocyte–endothelium interaction was observed in animals pretreated with 5 mg/kg body wt inactivated SOD. Neither the time course nor the maximal extent of leukocyte adhesion (52±7 cells/mm² and 192±49 cells/mm², 15 minutes

**FIGURE 1.** **Plot of superoxide dismutase (SOD) activity in hamster plasma.** Heparin (2,000 IU/kg body wt, n=6 animals) and bovine CuZn-SOD (0.25 mg/kg, n=7 animals or 5 mg/kg, n=7 animals) were injected via indwelling venous catheters. SOD activity was assessed by a direct spectrophotometric method in blood samples taken from arterial catheters before and 5, 10, and 20 minutes after injection. Data given in the figure are mean±SD.

**Plasma Superoxide Dismutase Activity**

Intravenous injection of heparin (2,000 IU/kg body wt) led to a prompt increase in plasma SOD activity (Figure 1). Postheparin plasma SOD activity was maintained at high levels and showed only a slight decline during the experiment (Figure 1). This initial increase in SOD activity by heparin corresponds to the increase in SOD activity as elicited by intravenous infusion of CuZn-SOD (0.25 mg/kg; Figure 1). However, the plasma activity decreased rapidly after CuZn-SOD injection: 20 minutes after SOD administration, plasma SOD activity had returned to levels that were no longer significantly different from baseline values (Figure 1). A 20-fold higher dose of CuZn-SOD (5 mg/kg) resulted in a 20-fold-higher increase in plasma SOD activity, showing a clearing pattern similar to that observed after low-dose SOD administration (Figure 1). The pharmacokinetics of CuZn-SOD observed in our study (plasma half-life, 6.5 minutes) is consistent with a previous report in which the plasma half-life of CuZn-SOD in rodents was reported at about 7 minutes.

**Microcirculatory Changes After Injection of Ox-LDL**

Under normal conditions as represented by the baseline situation, the majority of leukocytes did not interact with either arteriolar or venular endothelium. Intravenous injection of Ox-LDL (4 mg/kg LDL cholesterol) elicited an immediate onset of leukocyte rolling along the endothelial lining and subsequent firm adhesion to the endothelial surfaces of both arterioles and postcapillary venules (Figures 2–4). Both rolling and adhesive leukocytes tended to form aggregates of three and more cells. Although the fluorescent marker acridine orange does not stain platelets, the loose consistency of the leukocyte aggregates suggests that platelets were involved in their formation. Ox-LDL–stimulated leukocyte–endothelium interaction was encountered preferentially at arterial vessel bifurcations involving the lateral leading edges and the distal area of the flow divider, where the laminar flow is disrupted. No stimulation of leukocyte–endothelium interaction was observed after injection of equivalent amounts of native LDL (data not shown). The higher total number of sticking leukocytes per square millimeter of endothelial surface of postcapillary venules compared with arterioles (Figures 2 and 3) may be due to differences in local shear force conditions, with low shear forces prevailing in venules and high shear forces in arterioles.

When injected into heparin- and CuZnSOD-pretreated hamsters, Ox-LDL elicited an increase in the fraction of rolling leukocytes in postcapillary venules comparable with the increases seen in untreated control animals (Figure 4). In contrast, the adhesion of leukocytes to the endothelium was significantly attenuated in arterioles and postcapillary venules in animals pretreated with heparin or CuZnSOD (Figures 2 and 3). No inhibition of Ox-LDL–induced leukocyte–endothelium interaction was observed in animals pretreated with 5 mg/kg body wt inactivated SOD. Neither the time course nor the maximal extent of leukocyte adhesion (52±7 cells/mm² and 192±49 cells/mm², 15 minutes

**FIGURE 2.** **Bar graph showing leukocyte adhesion to the endothelium of arterioles after injection of oxidized low density lipoprotein (Ox-LDL).** Leukocyte adhesion was assessed in five arterioles in each observation chamber before injection of Ox-LDL and during the time course thereafter. For contrast enhancement, leukocytes were stained in vivo with acridine orange. Measurements were performed in control hamsters (n=7) and hamsters pretreated with either heparin (2,000 IU/kg body wt L; n=7) or bovine CuZn-superoxide dismutase (SOD; 0.25 mg/kg body wt L; n=7; or 5 mg/kg body wt L; n=6). Adherent leukocytes are expressed as number of cells per square millimeter of endothelial surface. Data given in the figure are mean±SD. *p<0.05. **p<0.01 vs. corresponding values in control animals (Wilcoxon test with Bonferroni correction).
after Ox-LDL injection in arterioles and postcapillary venules, respectively; mean±SD) were different from the data obtained in untreated control hamsters.

Neither control nor heparin- or CuZn-SOD-treated animals showed changes in red blood cell velocity and vessel diameter in postcapillary venules after injection of Ox-LDL (data not shown). This implies that the observed protection from Ox-LDL-stimulated leukocyte-endothelium interaction by heparin or CuZn-SOD was not secondary to changes in local hemodynamic and hence wall shear conditions. Because of technical limitations of the video system, the assessment of higher red blood cell velocities in arterioles cannot be determined by dual-slit cross correlation. However, no obvious differences were noted between control hamsters and heparin- or CuZn-SOD-pretreated hamsters before and at any time after injection of Ox-LDL, nor did we observe differences in arteriolar vessel diameters between the experimental groups (data not shown).

Fatty Acid Composition in Plasma, Leukocyte, and Erythrocyte Lipids

To examine whether injection of Ox-LDL elicited a generalized peroxidative attack to plasma or circulating blood cell lipids, we assessed the fatty acid composition of total lipids in plasma, leukocytes, and erythrocyte ghosts before and 15 minutes after injection of Ox-LDL. We observed no significant changes in the profile of single fatty acids, in the content of cis-polyunsaturated fatty acids, or in the ratio of saturated to cis-polyunsaturated fatty acids between untreated and Ox-LDL-treated hamsters (data not shown).

Discussion

Previous studies from our laboratory have shown that systemic administration of Ox-LDL (4 mg/kg LDL cholesterol) to awake Syrian golden hamsters elicits the adhesion of leukocytes to the endothelium of postcapillary venules and arterioles in vivo. These observations are consistent with the response-to-injury hypothesis holding that leukocytes, accumulating and adhering to the endothelium, are involved in the inception and progression of atherosclerotic lesions. In this study we demonstrated that Ox-LDL–induced leukocyte adhesion can be inhibited by increasing SOD plasma activity with low doses of either bovine CuZn-SOD (0.25 mg/kg) or heparin (Figures 2 and 3).

EC-SOD exists in the vasculature in equilibrium between the plasma phase and heparan sulfate proteoglycan in the glycocalyx of endothelial cell surfaces. Heparin has a higher affinity for EC-SOD than heparan sulfate and thus displaces the enzyme to the plasma. Because endothelial-bound EC-SOD should interfere with the Ox-LDL–induced leukocyte adhesion to the endothelium of postcapillary venules after injection of oxidized low density lipoprotein (Ox-LDL). Leukocyte rolling was assessed in five postcapillary venules in each observation chamber before injection of Ox-LDL and in the time course thereafter. For contrast enhancement, leukocytes were stained in vivo with acridine orange. Measurements were performed in control hamsters (n=7) and hamsters pretreated with either heparin (2,000 IU/kg body wt i.v.; n=7) or bovine CuZn-superoxide dismutase (SOD; 0.25 mg/kg body wt i.v.; n=7; or 5 mg/kg body wt i.v.; n=6). Rolling leukocytes are expressed as a percentage of all nonadherent cells passing through the observed vessel segment within a period of 30 seconds. Data given in the figure are mean±SD.
endothelium, the dramatic effect of heparin may seem surprising. However, the endothelial-bound EC-SOD is very unevenly distributed between organs. The major part exists in the liver, kidney, and spleen, and only minimal amounts are found in other organs. Injection of heparin results in an equal distribution of the enzyme in the vasculature, although all of it will exist in the plasma phase. The net result appears to be an improved protection against Ox-LDL–induced leukocyte adhesion. Injection of heparin into hamsters in this study resulted in a sixfold increase over baseline EC-SOD plasma levels (Figure 1). These data agree with values obtained in a previous, independent study with Syrian golden hamsters. A similar increase in plasma SOD activity was observed after injection of a low dose of CuZn-SOD (0.25 mg/kg; Figure 1). Pretreatment of the hamsters with either CuZn-SOD or heparin resulted in a comparable inhibition of Ox-LDL–induced leukocyte adhesion (Figures 2 and 3). Although injection of a higher dose of CuZn-SOD (5 mg/kg) resulted in considerably higher plasma SOD activities (Figure 1), no stronger inhibition of Ox-LDL–induced leukocyte adhesion was seen (Figures 2 and 3). These results suggest that maximal inhibition of Ox-LDL–induced leukocyte adhesion was achieved by moderate increases in plasma SOD activity, from about 80 to 500 units/ml (Figure 1). The basal plasma EC-SOD activities vary widely between species. It is notable that in rabbits, which have a very high plasma EC-SOD activity (approximately 850 units/ml), Ox-LDL fails to elicit leukocyte adhesion (H.A. Lehr, unpublished data). On the other hand, the low plasma SOD activity prevailing in humans (20 units/ml; see References 13 and 26) makes the phenomenon likely to occur during human atherogenesis. However, the maximal heparin-induced release of EC-SOD results in plasma SOD activities of about 80 units/ml, which suggests that heparin may not inhibit the Ox-LDL effect in humans as efficiently as it does in the hamster model. Although this study indicates that heparin did suppress Ox-LDL–induced leukocyte adhesion through the release and action of EC-SOD, it must be acknowledged that heparin exerts a variety of antiatherogenic effects, some of which may have contributed to the observed inhibition of Ox-LDL–induced leukocyte adhesion.

The mechanisms of action by which SOD inhibits Ox-LDL–induced leukocyte–endothelium interaction are unclear. SOD has been shown to inhibit leukocyte adhesion not only in response to Ox-LDL but also in inflammation, ischemia/reperfusion injury, and after administration of platelet-activating factor. This suggests that SOD affects the process of leukocyte adhesion rather than the specific pathological stimulus. In all these studies, endothelial cells were proposed as the source of superoxide radicals. Because Ox-LDL–induced leukocyte adhesion involves not only monocytes but also neutrophils and because both cell types are capable of producing superoxide radicals, particularly after activation and adhesion, the source of superoxide radical in our model remains a matter of speculation. It can be assumed that adherent neutrophils contribute to the further recruitment of leukocytes to the endothelium through the generation and release of degranulation products and radicals. Inhibition of such a neutrophil-dependent propagation of leukocyte recruitment could have contributed to the observed suppression of Ox-LDL–induced leukocyte adhesion by SOD.

Inhibition of leukocyte adhesion by SOD, irrespective of the pathological stimulus, could furthermore involve the generation and/or action of adhesion-promoting leukotrienes or the function of adhesion receptors. Taking the time course of Ox-LDL–induced leukocyte adhesion into account, the most likely candidate would be granule membrane protein–140 (GMP-140), which is translocated within minutes to the endothelial surface from its intracellular pool, the Weibel-Palade body. Both translocation of GMP-140 and its prolonged expression on the cell surface are induced by low concentrations of oxygen radicals and can be blocked effectively by antioxidants. Additionally, expression of GMP-140 on activated platelets and their interaction with circulating leukocytes may explain the occurrence of leukocytic aggregates after injection of Ox-LDL.

In addition to this direct action of SOD on leukocyte–endothelium interaction, SOD could inhibit the radical-mediated initiation and propagation of lipid peroxidation after introduction of Ox-LDL into the hamster organism. Although we did not detect any signs of generalized lipid peroxidation in the plasma or circulating blood cells, such an event could occur at the endothelial wall and result in the local generation of chemotactic and adhesion-promoting factors, including lysophosphatidylcholine and leukotrienes.

In summary, the results of the present study demonstrate that Ox-LDL stimulates leukocyte–endothelium interaction by a superoxide-dependent step. The finding that this event can be inhibited by CuZn-SOD or heparin may help to clarify the mechanism of action by which antioxidants and heparin exert their protective role in experimental and clinical atherogenesis. The identification of the mechanism of action underlying Ox-LDL–induced leukocyte adhesion and the susceptibility of this phenomenon to inhibition by clinically available therapies may open the way for novel prophylactic and therapeutic strategies in clinical settings characterized by a high risk of atherogenesis, such as hyperlipidemia and cardiac allograft transplantation.

Acknowledgments
The excellent technical assistance of B. Sahringer-Mansour and I. Wernicke is gratefully acknowledged.

References
11. Carew TE, Schwenke DC, Steinberg D: Antithrombogenic effect of probuloc unrelated to its hypolipoproteinemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc Natl Acad Sci U S A* 1987;84:7725-7729
Superoxide-dependent stimulation of leukocyte adhesion by oxidatively modified LDL in vivo.

H A Lehr, M Becker, S L Marklund, C Hübner, K E Arfors, A Kohlschütter and K Messmer

doi: 10.1161/01.ATV.12.7.824

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/7/824

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answerdocument.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/