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Oxidized low-density lipoproteins stimulate adhesion of monocytes to endothelial cells[1]

NIU Xi-Lin, YAN Xiang-Dong, GUO Zhao-Gui[2]
(*Laboratory of Molecular Pharmacology, Hunan Medical University, Changsha 410078, China*)

KEY WORDS vascular endothelium; monocytes; LDL lipoproteins; adhesions

AIM: To study the effects of oxidized low-density lipoproteins (ox-LDL) on the adhesiveness of monocytes to endothelial cells. **METHODS**: LDL was obtained from healthy human plasma by ultracentrifugation, and oxidized by $CuSO_4$ 10 μmol $\cdot L^{-1}$. The assay of adhesion was performed using cultured bovine aortic endothelial cells (BAEC) and human peripheral blood monocytes. **RESULTS**: Pretreatment BAEC with ox-LDL enhanced monocyte adhesion to BAEC in time- and dose-dependent manner. ox-LDL as little as 10 mg $\cdot L^{-1}$ and 30 min of preincubation stimulated monocyte adhesion. Cycloheximide (Cyc, a protein synthesis inhibitor) 1 mg $\cdot L^{-1}$ and staurosporine (Sta, a PKC inhibitor) 20 nmol $\cdot L^{-1}$ abolished the effect of ox-LDL (60 mg $\cdot L^{-1}$), but dextran sulfate 20 mg $\cdot L^{-1}$ had no effect on monocyte adhesion. Phorbol 12-myristate 13-acetate (PMA) 1 nmol $\cdot L^{-1}$ and lysophosphatidylcholine (Lys) 6 μmol $\cdot L^{-1}$ mimicked the effects of ox-LDL and potentiated monocyte adhesion. Sta also suppressed the augmentative effects of Lys and PMA. **CONCLUSION**: ox-LDL enhances the adhesion of monocytes to BAEC through the activation of PKC.

Among the earliest events in atherogenesis is the binding of monocytes to the endothelium and their entry into the vessel wall, where they differentiate into macrophages. This process is an important initiating mechanism in atherogenesis[1]. Increased adhesion of monocytes to endothelium was observed in those areas that lesions first develop in hypercholesterolemic animals[2]. But the underlying mechanism remains unclear.

Oxidized low-density lipoproteins (ox-LDL) was identified in atherosclerotic lesions[3]. It is expected that ox-LDL may play a role in the altered interactions between monocytes and endothelium in the atherosclerotic arteries. The purposes of present study were to examine whether adhesiveness of monocytes to endothelium may be enhanced by treating endothelium with ox-LDL, and to determine the mechanisms by which ox-LDL may affect the endothelial susceptibility to monocytes.

MATERIALS AND METHODS

Cell culture Bovine aortic endothelial cells (BAEC) were harvested and cultured in M199 medium with 10 % heat-inactivated fetal bovine serum. All cultures showed typical morphology[4]. The endothelial cells was identified by electron microscopy. Experiments were performed with cells from passage 4 to 10.

Isolation of monocytes The leukocyte-rich buffy coats was obtained by centrifugation of blood taken with citrate as anticoagulant and incubated in a slightly hypertonic solution at 37 ℃ for 30 min, during which the lymphocytes became more dense, whereas the monocytes were comparatively resistant to hypertonic condition. The monocytes were separated from lymphocytes using Ficoll-Hypaque[5]. The isolated populations were routinely >85 % pure by Wright-Giemsa stain. This method has the advantages of being rapid and not involving adherence of monocytes, which may cause activation.

Preparation of LDL LDL was isolated from fresh normal human plasma by ultracentrifugation at 119 000 × g in the presence of edetic acid 0.2 mmol $\cdot L^{-1}$. Then LDL was filtered (0.2 mm) aseptically into dialysis tube and dialyzed in phosphate-buffer solution (PBS) at 4 ℃ for 36 h. Dialyzed LDL was concentrated and stored at 4 ℃ in dark. LDL thus prepared was referred to as native LDL (n-LDL)[6]. Before oxidation, n-LDL was dialyzed against PBS to remove edetic acid. Then it was oxidized by exposure to $CuSO_4$ 10 μmol $\cdot L^{-1}$ at 37 ℃ for 18 h. The entire procedure was performed under sterile conditions. The ox-LDL extent was estimated by measuring increases in thiobarbituric acid reacting substance content, absorption at 234 nm, and electrophoretic

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[2] Correspondence to Prof GUO Zhao-Gui
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mobility on agarose gel[7].

Assay for the monocyte adhesion The confluent monolayer of BAEC was prepared[4]. Cells were incubated with 1 mL medium containing ox-LDL, n-LDL, bovine serum albumin (BSA) or PBS for a period of time. Then 4 $\times 10^5$ monocytes were added to each well, incubated for an additional 60 min. In 0.5 h thereafter, the culture plates were gently agitated to ensure even dispersal of non-adherent monocytes. The nonadherent monocytes were washed off by rinsing 3 times with 1 mL medium, and once with PBS at 37 ℃. The adherent cells were fixed in 1 % glutaraldehyde at 4 ℃ overnight. To facilitate the recognition of the monocytes, these cells were labeled by staining for endogenous peroxidase activity by the following procedure. The wells were incubated with a mixture of diaminobenzidin ·4HCl 1 g·L^{-1} and H_2O_2 0.02 % in PBS for approximate 3 min and washed in PBS. The attached monocytes were counted in 20 microscopic fields by phase contrast microscopy[8]. The data were expressed as the ratio of the adherent monocytes in the lipoprotein-treated BAEC cultures to that in the PBS-treated cultures.

The effect of ox-LDL on monocyte adhesion to BAEC was assessed as compared to that of n-LDL, BSA all at 50 mg protein·L^{-1} or in same volume of PBS after 6-h preincubation.

Experimental protocol The effect of ox-LDL on monocyte adhesion was examined in comparison with n-LDL and BSA relative to PBS. To analyze the underlying mechanism, 1) Cycloheximide (Cyc) pretreatment to inhibit protein synthesis to identify the connection between adhesion and protein synthesis. 2) Dextran sulfate as a scavenger receptor antagonist was used to test the role of these receptors in ox-LDL induced monocyte adhesion. 3) Staurosporine (Sta) and phorbol 12-myristate 13-acetate (PMA) were used to observe the effect of PKC activation or antagonism on this event. 4) Pretreatment of lysophosphatidylcholine (Lys) to examine monocyte adhesion compared with ox-LDL.

Chemicals M199 medium, bovine serum albumin, diaminobenzidin · 4HCl, Cyc, Sta, dextran sulfate, edetic acid, PMA, and Lys were purchased from Sigma.

Statistics The results were expressed as $\bar{x} \pm s$, and assessed by ANOVA and t test.

RESULTS

Effects of ox-LDL on monocyte adhesion

ox-LDL induced 2-fold increases in monocyte adhesion ($P < 0.01$ *vs* PBS, $n = 6$), whereas n-LDL, and BSA had no significant effect ($P > 0.05$ *vs* PBS, $n = 6$). As little as ox-LDL 10 mg ·L^{-1} stimulated monocyte adhesion, and the effect was maximal at 60 mg·L^{-1}(Fig 1).

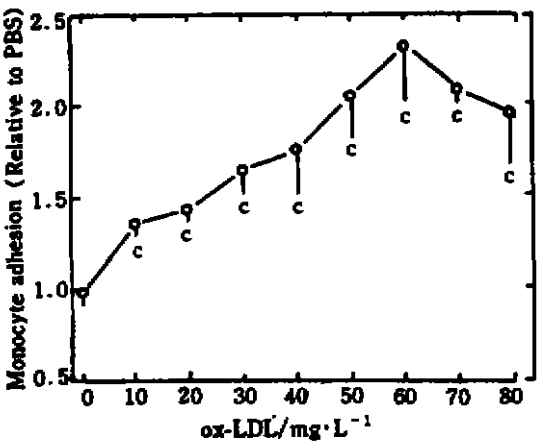

Fig 1. ox-LDL induced monocyte adhesion to BAEC. $n = 4$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

The kinetics of ox-LDL-induced enhancement of monocyte adhesion was also assessed, 30 min of preincubation was necessary for ox-LDL 60 mg·L^{-1} to induce increase of adhesion, which reached the maximum at 4 h and was sustained for >48 h (Fig 2).

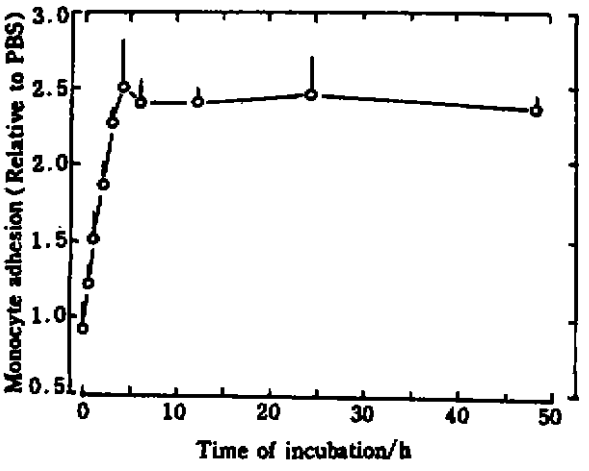

Fig 2. Effect of ox-LDL 60 mg·L^{-1} on monocyte adhesion to BAEC. $n = 4$, $\bar{x} \pm s$.

Therefore, following experiments were performed using ox-LDL 60 mg·L^{-1}, and preincubation time was 6 h. BSA was chosen as control.

Role of protein synthesis Pretreatment of the BAEC with Cyc, a protein synthesis inhibitor, 1 mg ·L^{-1} for 1 h before adding ox-LDL abolished the effects of ox-LDL (60 mg · L^{-1}) to stimulate

adhesion of monocytes to BAEC (ox-LDL + Cyc: 0.96 ± 0.14 *vs* ox-LDL: 2.10 ± 0.21, $P < 0.01$, $n = 6$).

Role of scavenger receptor Coincubation with dextran sulfate 20 $mg \cdot L^{-1}$ during pretreatment of the BAEC with ox-LDL (60 $mg \cdot L^{-1}$) exerted no effect on increased adhesion of monocytes to BAEC (2.06 ± 0.20 *vs* ox-LDL; 2.17 ± 0.35, $P > 0.05$, $n = 6$).

Role of PKC activation Coincubation with Sta 20 $nmol \cdot L^{-1}$ during pretreatment of the BAEC with ox-LDL (60 $mg \cdot L^{-1}$) suppressed the augmentative effects of ox-LDL on monocyte adhesion. Pretreatment of the BAEC with PMA 1 $nmol \cdot L^{-1}$ induced an increase of adhesion, which was abolished by Sta (Tab 1).

Tab 1. Inhibitory effects of staurosporine (Sta 20 $mg \cdot L^{-1}$) on PMA 1.0 $nmol \cdot L^{-1}$, Lys 6 $\mu mol \cdot L^{-1}$, and ox-LDL 60 $mg \cdot L^{-1}$ induced monocyte adhesion to BAEC. $n = 6$, $\bar{x} \pm s$. $^{a}P > 0.05$, $^{c}P < 0.01$ *vs* control.

Pretreatment	Adhesion of monocytes to BAEC, relative to PBS
Control	0.99 ± 0.17
ox-LDL	2.20 ± 0.21^{c}
ox-LDL + Sta	1.20 ± 0.18^{a}
PMA	2.78 ± 0.21^{c}
PMA + Sta	0.96 ± 0.07^{a}
Lys	1.54 ± 0.10^{c}
Lys + Sta	1.04 ± 0.14^{a}

Effects of Lys on monocyte adhesion Pretreatment of BAEC with Lys 6 $\mu mol \cdot L^{-1}$ increased monocyte adhesion. Sta suppressed the augmentative effects of Lys (Tab 1).

DISCUSSION

In present study, we demonstrated that the pretreatment of BAEC with ox-LDL increased adhesion of monocytes to BAEC. The effects of ox-LDL was not mediated by the scavenger-receptor-dependent mechanism, but was attributed to PKC activation and *de novo* protein synthesis in endothelial cells.

Increased adhesion of monocytes to the artery wall endothelium is one of the earliest events in the development of atherosclerotic plaque[1]. The mechanisms that initiate this process are not fully understood. ox-LDL has been found to be present in atherosclerotic plaques, and in the blood of human and experimental animals at risk to develop lesions[9]. It is commonly accepted that ox-LDL plays a key role in the development of atherosclerosis[10]. In present study, we found that pretreatment of BAEC with ox-LDL increased adhesion of monocytes to BAEC, this increased adhesion was not dependent upon scavenger receptor, because dextran sulfate, a scavenger receptor blocker, did not affect the effect of ox-LDL, indicating protein moiety of ox-LDL is not involved in these effects. Furthermore, Lys, an important active component in ox-LDL[11], at concentration referred to as that deprived from an equivalent quantity of ox-LDL 60 $mg \cdot L^{-1}$ [12], also induced an increased adhesiveness of monocytes to BAEC, indicating that Lys in ox-LDL was at least partly responsible for the effect of ox-LDL.

Several lines of evidences have suggested that during the oxidative modification of LDL, phosphatidylcholine (PC) was hydrolyzed by an intrinsic LDL-associated phospholipase A_2 to become Lys[11], Lys in ox-LDL could be transferred and incorporated into endothelial cell surface membrane in an apoprotein-independent manner. The transferred Lys activates PKC in the endothelial cell surface membrane and modulates various endothelial function[12,13]. The present study showed that staurosporine, PKC inhibitor, suppressed Lys and ox-LDL-induced increased monocyte adhesion. This result is in agreement with this observed in polymorphonuclear leukocytes[14]. Furthermore, PMA, a specific PKC activator, enhanced the adhesion of monocytes to BAEC, closely mimicking the results obtained by ox-LDL and Lys. These results reasoned that PKC activation by Lys in ox-LDL may at least in part be involved in the enhanced adhesion of monocytes to BAEC.

The interaction between ox-LDL and the endothelial cells appears to be necessary to induce an increase in monocyte adhesion. When ox-LDL was added simultaneously with monocytes, no effect on monocyte adhesion was seen, whereas, preincubation of BAEC with ox-LDL for 30 min resulted in a significant increase of adhesion. This finding, plus

our further results that treatment of BAEC with Cyc, an inhibitor of protein synthesis, abolished the effect of ox-LDL to stimulate adhesion of monocytes, suggesting that *de novo* protein synthesis in the endothelial cells is required for the effect of ox-LDL. It is reasonable to speculate that certain kind of adhesion molecule synthesized in BAEC during incubation with ox-LDL is responsible for increased adhesion of monocytes to BAEC induced by ox-LDL.

In conclusion, ox-LDL enhances the adhesion of monocytes to BAEC, PKC activated by Lys in ox-LDL may play an important role in the mechanisms of the enhanced adhesion of monocytes to BAEC.

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氧化型低密度脂蛋白促进单核细胞与血管内皮细胞粘附作用[1]

牛喜林，晏向东，郭兆贵[2]
(湖南医科大学分子药理研究室，长沙 410078，中国)

关键词 血管内皮；单核细胞；低密度脂蛋白类；粘连

目的：研究氧化型低密度脂蛋白(ox-LDL)对单核细胞与血管内皮细胞粘附作用. 方法：用超速离心方法分离健康人血浆低密度脂蛋白，以 10 μmol·L^{-1} $CuSO_4$ 氧化. 观察 ox-LDL 对人外周血单核细胞(MC)与小牛主动脉血管内皮细胞(BAEC)粘附作用的影响. 结果：ox-LDL 能以时间和剂量依赖性方式促进 MC 与 BAEC 粘附作用. 放线菌酮 D 和 staurosporine (Sta)能取消这种促进作用，但硫酸葡聚糖对此作用无影响. PMA 和 6 μmol·L^{-1}溶血性磷酯酰胆碱也显著增强 MC 与 BAEC 的粘附作用，且此增敏作用也可被 Sta 取消. 结论：ox-LDL 能通过激活蛋白激酶 C 促进 MC 与 BAEC 的粘附作用.