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Oxidized low-density lipoproteins induce apoptosis in aortic and endocardial endothelial cells[1]

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KEY WORDS LDL lipoproteins; endothelium; aorta; endocardium; apoptosis; DNA fragmentation; Hoe-33258; butylated hydroxytoluene; dextrans; cycloheximide

AIM: To examine whether oxidized low-density lipoproteins (ox-LDL) might induce apoptosis in bovine aortic and endocardial endothelial cells (BAEC and BEEC). **METHODS:** Low-density lipoproteins (LDL) were isolated from healthy human plasma by ultracentrifugation and oxidized by $CuSO_4$ 10 $\mu mol \cdot L^{-1}$. BAEC and BEEC were incubated in a medium containing ox-LDL, LDL, or phosphate-buffer solution (PBS) as control. DNA fragmentation was visualized by agarose gel electrophoresis and determined quantitatively using Hoechst-33258 fluorochrome. **RESULTS:** Ox-LDL, not LDL, elicited typical apoptotic changes and DNA fragmentation in BAEC and BEEC. In BAEC, dextran sulfate, and cicloheximide (Cic) exhibited no effect on DNA fragmentation induced by ox-LDL. Butylated hydroxytoluene (BHT) 20 $\mu mol \cdot L^{-1}$ completely inhibited Cu^{2+}-mediated oxidation of LDL as well as the apoptosis-inducing effect of Cu^{2+}-exposed LDL. Lysophosphatidylcholine (LPC) did not elicit DNA fragmentation in BAEC and in BEEC. DNA fragmentation induced by ox-LDL in BAEC and in BEEC was blocked by chelating the calcium of the culture medium by egtazic acid. **CONCLUSION:** Ox-LDL induces apoptosis in BAEC and BEEC without involving the LPC.

Oxidized low-density lipoproteins (ox-LDL) is a potent atherogenic agent and can be regarded as endogenously biosynthesized cytotoxin[1]. Its cytotoxic effects to various kinds of cells including endothelial cells were well documented[2]. Apart from necrosis, there is an another type of cell death, and many pathological conditions and chemical agents, such as anticancer drugs, hyperthermia, and viral infection can induce programmed cell death (PCD)[3]. Oxidized LDL induces a sustained rise of cytosolic calcium in endothelial cells[4], which, in turn, activates calcium-dependent endonuclease potentially involved in DNA fragmentation leading to apoptosis[5]. In addition, ox-LDL was also shown to be able to trigger PCD in lymphoblastoid cells[6] and macrophages[7]. This study was aimed to investigate whether ox-LDL might induce apoptosis in vascular and endocardial endothelial cells.

MATERIALS AND METHODS

Cell culture BAEC were harvested and cultured in M199 medium with 10 % heat-inactivated fetal bovine serum. All cultures showed typical morphology. The endothelial cells were identified by transmission electron microscopy and the presence of factor VIII-related antigen. Experiments were performed with cells from passage 4 to 10. For BEEC culture, BEEC were isolated from bovine ventricles by use of 0.1 % collagenase and plated in M199 medium supplemented with 10 % heat-inactivated fetal bovine serum. BEEC were also confirmed by the presence of factor VIII-related antigen and transmission electron microscopy. Cells were used from passage 5 - 10 and 1 - 2 d after confluence[8].

Preparation of LDL LDL and ox-LDL[7].

Apoptosis After treatment, the apoptotic cells were examined microscopically by hematoxylin-eosin stain.

DNA fragmentation At selected times BAEC and BEEC were harvested and spun at 200 × *g* for 10 min, and lysed for 15 min in 1.5 mL lysis buffer (Triton X-100 5 % vol/vol and edetic acid 20 $mmol \cdot L^{-1}$, Tris 5 $mmol \cdot L^{-1}$, pH 8.0). The lysed cells were spun at 15 000 × *g* for 20 min to separate intact DNA from fragmented DNA. The pellet was then sonicated for 10 s in another 1.5 mL lysis buffer. DNA in supernatant and pellet was determined according to our method[7]. The data were expressed as the % of DNA appeared in supernatant to the total cellular DNA.

DNA electrophoresis At the end of incubation period, the DNA of BAEC or BEEC was extracted and ectrophoretically

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fractionated on 1.5 % agarose gel and visualized by ethidium bromide according to our method[7].

Statistical evaluation All results were expressed as $\bar{x} \pm s$ and analyzed by ANOVA and t test.

RESULTS

Morphological changes induced by ox-LDL

Ox-LDL-treated BAEC underwent morphological changes in cell structure typical of apoptosis. The cell volume was reduced, indicating shrinkage of cytoplasm. The nucleus marginated to the periphery of the cell membrane. Some apoptotic cells were phagocytosed by neighboring normal cells (Fig 1, Plate 2).

DNA fragmentation elicited by ox-LDL

DNA electrophoresis results showed that preincubating BAEC with ox-LDL 200 mg protein · L^{-1} elicited a "ladder" of DNA bands representing integer multiples of the internucleosomal DNA length (about 180 bp) in a time-dependent manner (Fig 2), whereas n-LDL had no such effect up to 36 h. The dose-dependent results demonstrated that significant DNA fragmentation was first seen after 24 h of incubation in the presence of ox-LDL 100 mg · L^{-1}, and reached near 50 % with ox-LDL 200 mg · L^{-1} (Tab 1).

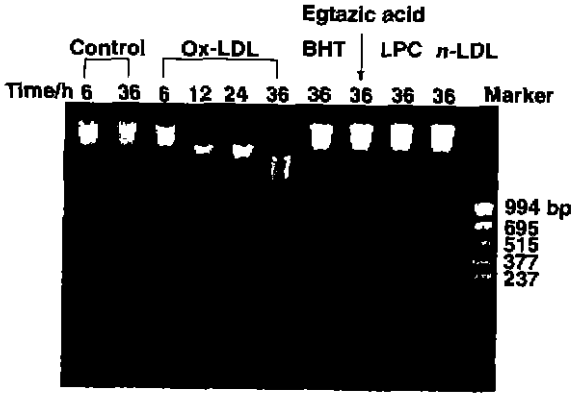

Fig 2. Kinetics of ox-LDL-induced DNA fragmentation in BAEC.

Protein synthesis and scavenger receptor

Pretreatment of the BAEC with Cic 1 mg · L^{-1}, a protein synthesis inhibitor, for 1 h before adding ox-LDL did not affect the effect on DNA fragmentation pretreated with ox-LDL for 24 h (200 mg · L^{-1}) (Tab 1).

Coincubation with dextran sulfate (DS) 20 mg · L^{-1}, a scavenger receptor antagonist, during pretreatment of the BAEC with ox-LDL (200 mg · L^{-1}) for 24 h exerted no effect on the DNA fragmentation (Tab 1).

Tab 1. Effects of ox-LDL, dextran sulfate (DS), cycloheximide (Cic), and BHT on DNA fragmentation in BAEC induced by ox-LDL.
$n = 6$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* PBS.

Pretreatment	DNA fragmentation/ %
PBS	4.5 ± 0.5
ox-LDL 50 mg · L^{-1}	4.9 ± 0.9
ox-LDL 100 mg · L^{-1}	30.4 ± 6.5^c
ox-LDL 200 mg · L^{-1}	51.5 ± 4.9^c
ox-LDL 200 mg · L^{-1} + DS	52.5 ± 6.8^c
ox-LDL 200 mg · L^{-1} + Cic	50.4 ± 5.6^c
ox-LDL 200 mg · L^{-1} + BHT	55.1 ± 6.3^c

LPC Pretreatment of BAEC with LPC at concentration even up to 50 mmol · L^{-1} for 36 h did not elicit DNA fragmentation (Fig 2).

Effect of BHT BHT 20 mmol · L^{-1} completely inhibited Cu^{2+}-mediated increase in LDL TBARs material (Tab 2), as well as the DNA fragmentation-inducing effect of Cu^{2+}-exposed LDL (Fig 2), and abolished morphological change elicited by Cu^{2+}-exposed LDL. But BHT, when added with ox-LDL simultaneously to the medium, did not exert any protective effect on the DNA fragmentation induced by ox-LDL (Tab 2).

Tab 2. Effect of antioxidant (BHT) on oxidation of LDL induced by Cu^{2+}.
$n = 5$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* PBS.

Pretreatment	MDA equivalent, μmol/g protein
n-LDL 8 g · L^{-1}	2.15 ± 0.33
$CuSO_4$ 10 μmol · L^{-1}	6.33 ± 0.29^c
$CuSO_4$ 10 μmol · L^{-1} + BHT	2.23 ± 0.28

$[Ca^{2+}]_i$ When calcium of the culture medium (1.8 mmol · L^{-1}) was chelated by adding egtazic acid (2.5 mmol · L^{-1}) at 6 h after ox-LDL, the subsequent DNA fragmentation of BAEC was abolished (Fig 2).

Effect of ox-LDL on BEEC Similarly with BAEC, preincubating BEEC with ox-LDL 200 mg · L^{-1} also elicited morphological changes in cell

structure typical of apoptosis and DNA fragmentation which was also abolished by egtazic acid. Pretreatment of BEEC with LPC at concentration even up to 50 mmol · L^{-1} for 36 h did not elicit DNA fragmentation (Fig 3).

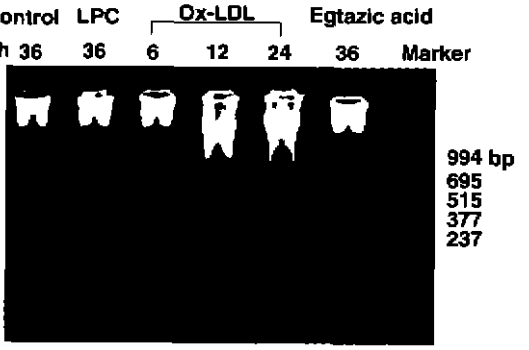

Fig 3. Kinetics of ox-LDL-induced DNA fragmentation in BEEC.

DISCUSSION

Cytotoxicity of ox-LDL on endothelial cells plays an important role in initiating atherosclerosis[9,10], but its mechanism is still unclear. In this study we have verified that the endothelial cell injury induced by ox-LDL is related to its apoptosis-inducing effect. Apoptosis is considered to be a controlled mechanism of cell death, and has been best characterized biochemically by the cleavage of genomic DNA into nucleosomal size fragments of 180 bp or multiples of thereof, which are detected by gel electrophoresis as a DNA ladder[6]. Results in this study demonstrated that pretreating BAEC with ox-LDL elicited DNA fragmentation and typical apoptotic morphological changes including shrinkage of cytoplasm, condensation of chromatin and margination of nucleus in BAEC. These results provide further evidence that ox-LDL plays a critical role in the pathogenesis of atherosclerosis.

The fact that scavenger receptor blocker and protein synthesis inhibitor did not affect the effect of ox-LDL on DNA fragmentation reasoned that apoptosis-inducing effect of ox-LDL was not dependent upon scavenger receptor, and that *de novo* protein synthesis in the BAEC was not required. These results plus our further finding that antioxidant BHT completely inhibited Cu^{2+}-mediated oxidation of LDL as well as the apoptosis-inducing effect of Cu^{2+}-exposed LDL suggest that lipid peroxidation products of LDL might be an important factor for the action of ox-LDL. However BHT, when added to medium simultaneously with ox-LDL, did not inhibit DNA fragmentation. Thus it was most likely that an oxidized lipid in ox-LDL and not a free radical or intermediate substance generated during preincubation with ox-LDL was responsible for the toxicity.

LPC in ox-LDL could be transferred and incorporated into cell surface membrane in an apoprotein-independent manner. The transferred LPC can modulates various cell function[11,12]. However, the present study showed that LPC at concentration even up to 50 mmol · L^{-1} did not elicit DNA fragmentation, indicating that LPC is not responsible for the effects of ox-LDL. In agreement with previous reports that calcium-dependent endonuclease is involved in DNA fragmentation leading to apoptosis[5], our results showed that when the calcium of the medium was chelated by adding egtazic acid, subsequent DNA fragmentation was abolished. This suggests that the rise in $[Ca^{2+}]_i$ elicited by ox-LDL may be involved in the activation of endonuclease.

In addition, we also reported for the first time that ox-LDL induced apoptosis in endocardial endothelial cells, indicating that ox-LDL may have certain effect on myocardial performance by modulating endocardial endothelial cell release function[13].

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氧化型低密度脂蛋白诱导主动脉和心内膜内皮细胞凋亡[1]

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关键词 低密度脂蛋白类; 内皮; 主动脉; 心内膜; 细胞凋亡; DNA 断片; Hoe-33258; 丁羟甲苯; 右旋糖苷类; 环己米特

目的: 研究氧化型低密度脂蛋白(ox-LDL)诱导血管和心内膜内皮细胞凋亡. **方法**: 用超速离心法分离健康人血浆低密度脂蛋白(LDL), 以 $CuSO_4$ 10 $\mu mol \cdot L^{-1}$氧化. 观察 ox-LDL 对培养新生小牛主动脉内皮细胞及心内膜细胞的损伤作用. 琼脂糖凝胶电泳和 Hoechst 33258 荧光密度法定性与定量分析 DNA 降解. **结果**: ox-LDL 诱导血管内皮细胞及心内膜细胞典型凋亡形态学改变、DNA 降解呈时间和剂量依赖性. 环己米特和硫酸葡聚糖对此作用无影响. BHT 20 $\mu mol \cdot L^{-1}$可取消 DNA 降解, 溶血性磷脂酰胆碱 50 $\mu mol \cdot L^{-1}$无诱导凋亡作用. ox-LDL 诱导的 DNA 降解可被依他酸取消. **结论**: ox-LDL 诱导血管内皮细胞及心内膜细胞凋亡.

书 讯

由金正均、王永铭和苏定冯三位教授主编的《药理学进展·1997》一书, 已由人民卫生出版社出版发行. 本书是由从事药理学与毒理学研究的 32 位专家结合自己的研究成果编写而成. 全书约 35 万余字, 包括 24 个专题, 概括了近年来国内外药理学的主要进展, 提供了大量的信息和知识、对药理学工作者的教学和科研极有指导价值、也可作为研究生和本科生的参考书. 《药理学进展》计划每年出版一本、形成系列、请勿错过. 每册 20 元(含邮资).

由陈修教授主编的《心血管药理学》第一版于 1989 年出版后, 得到广大读者好评. 《心血管药理学》第二版增加了陈维洲与曾贵云两位著名的心血管药理学家为主编, 增聘了 16 位心血管临床与心血管药理学专家参加编写. 新版增加了许多新内容. 介绍了近几年细胞与分子生物学研究在心血管药理学的新成果等、是目前我国唯一的一本心血管药理学专著. 全书 100 多万字、精装. 定价 90 元(含邮资). 欲购上述两本书者请从邮局汇款到: 200433, 上海市翔殷路 800 号、第二军医大学基础部药理学教研室, 苏定冯教授收.