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Oxidized low density lipoprotein inhibits bradykinin-induced phosphoinositide hydrolysis in cultured bovine aortic endothelial cells

Ken-ichi Hirata, Hozuka Akita and Mitsuhiro Yokoyama

The First Department of Internal Medicine, Kobe University School of Medicine, Kobe 650, Japan

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Vascular endothelial cells, in response to various neurohumoral and physical stimuli, produce an endothelium-derived relaxing factor, a substance which regulates vascular tone. We have demonstrated that oxidized low density lipoprotein (LDL) inhibits endothelium-dependent relaxation. We studied the effect of oxidized LDL on inositol phosphates formation stimulated with bradykinin (BK) in cultured bovine aortic endothelial cells. BK elicited a rapid generation of inositol phosphates from inositol phospholipids. Accumulation of inositol 1,4,5-trisphosphate (IP3) stimulated with BK (0.1 μM) was markedly inhibited by oxidized LDL. However, native LDL had little effect on BK-induced accumulation of IP3. From these results, oxidized LDL inhibits receptor-mediated phosphoinositides hydrolysis and modulates the endothelial function.

Endothelium; Bradykinin; Endothelium-derived relaxing factor; Intracellular calcium; Low-density lipoprotein

1. INTRODUCTION

Vascular endothelial cells play an important role in the regulation of vascular tone by their ability to produce and release endothelium-derived relaxing factor(s) (EDRF) [1]. It is now established that some hormones and neurotransmitters can promote the formation of inositol 1,4,5-triphosphate(IP3) from phosphatidylinositol 4,5-bisphosphate in the plasma membrane and generated IP_3 elicits the discharge of Ca^{2+} from intracellular stores in many types of cells [2]. Recent studies have demonstrated that agonist-induced production of EDRF or prostacyclin is mediated by a phospholipase C-coupled system and Ca^{2+} mobilization in cultured endothelial cells [3,4].

Oxidative modification of low-density lipoprotein(LDL) has been implicated in the pathogenesis of atherosclerosis as a factor in the generation of macrophage-derived foam cells [5]. Recently, we have demonstrated that oxidized LDL inhibits the endothelium-dependent relaxation and modifies vascular tone [6]. The purpose of this study was to investigate the effect of oxidized LDL on phosphoinositides hydrolysis in cultured bovine aortic endothelial cells.

2. MATERIALS AND METHODS

2.1. *Lipoproteins and modification of LDL*

LDL (density = 1.020–1.060) was isolated by ultracentrifugation from freshly harvested normal human plasma collected in EDTA (1 mg/ml) [7]. Protein was determined by the method described by Bradford [8] using bovine serum albumin as a standard. LDL (500 μg·protein/ml) was oxidatively modified by 10 μM copper in PBS(−) for 24 h at 37°C [9]. Control LDL was incubated without copper. Oxidative modification of LDL was confirmed by agarose gel electrophoresis [10].

2.2. *Culture of endothelial cells*

Bovine aortic endothelial cells (BAECs) were obtained by the scraping of the aorta excised from a freshly slaughtered cow, as described previously [11]. They were cultured in Dulbecco's modified Eagle's medium (DMEM) with fetal calf serum (FCS) (15% v/v). The cells were seeded on 25-cm^2 flasks and incubated at 37°C under an atmosphere of 5% CO_2 and 95% air. The medium was changed on the following day and later every third day. After 4 or 5 days, the primary cultures formed a confluent monolayer and could be subcultured. Cultures used in the present study were from the third to 12th passage. Endothelial cells cultured showed typical morphology and homogeneous staining for factor VIII antigen [12].

2.3. *Assay condition for phospholipase C reaction*

The cells were plated on a 35-mm dish. After 2 days the cultured cells formed a confluent monolayer. The final cell density on the day of the assay was 1×10^6 cells/dish. For assay of the phospholipase C reaction, the cells were rinsed three times with 2 ml of phosphate-buffered saline with 0.9 mM Ca^{2+} and 0.8 mM Mg^{2+}(PBS+) and labeled with myo-[2-^{3}H]inositol(10 μCi/ml/dish) at 37°C for 24 h in inositol-free DMEM without FCS. After the labeling, the cells were washed three times with 2 ml DMEM to remove unincorporated [^{3}H]inositol. The cells were preincubated with 0.9 ml of a HEPES-buffered solution composed of (mM) 130 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 20 HEPES (pH 7.4) containing 10 mM LiCl [13] on a tray in a water bath at 37°C for 15 min. Then, the cells were stimulated with 0.1 ml of the various agents. The reaction was stopped by rapid aspiration of the medium and adding 1 ml ice-cold 15% trichloroacetic acid. The cells were chilled on ice for 1 h to extract water-soluble inositol phosphates. To investigate the effects of oxidized LDL on bradykinin (BK)-induced phosphoinositides hydrolysis, BAECs were preincubated with native or oxidized LDL at 37°C for 30 min and then stimulated with 100 nM BK for 30 s. Cell viability using in this study was greater than 98% by Trypan blue exclusion.

Correspondence address: K. Hirata, The First Department of Internal Medicine, Kobe University School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650, Japan. Fax: (81) (78) 341 1390.

2.4. *Separation of [^{3}H]inositol phosphates*

The extract was transferred to a test tube, and the cells were washed twice with 1 ml distilled water. The washings were combined with the original acid extract. Trichloroacetic acid was removed with 3 ml diethyl ether four times. The final extract was neutralized, applied to columns containing 1 ml Dowex AG 1×8 formate-form resins (Bio-Rad Laboratories, Richmond, CA), and eluted to separate the water-soluble inositol phosphates by the method described previously [13], which was based on that described by Berridge et al. [14]. After application of the sample, the column was washed with 8 ml distilled water. Glycerophosphoinositol was eluted with 165 ml of 5 mM disodium tetraborate/60 mM sodium formate. Inositol 1-monophosphate (IP_1) was eluted with 8 ml of 0.1 M formic acid/0.2 M ammonium formate followed by an 8 ml wash with the same buffer. Inositol 1,4-bisphosphate(IP_2) was eluted with 8 ml of 0.1 M formic acid/0.4 M ammonium formate, followed by an 8 ml wash with the same buffer. IP_3 was eluted with 8 ml of 0.1 M formic acid/1.0 M ammonium formate.

2.5. *Drugs*

BK was purchased from Sigma Chemical Co. (St. Louis, MO). DMEM was from Flow laboratories Inc. (McClean, VA). Myo-[2-^{3}H]inositol (20 Ci/mmol) was from Amersham (Arlington Heights, IL). Concentrations were expressed as final concentration.

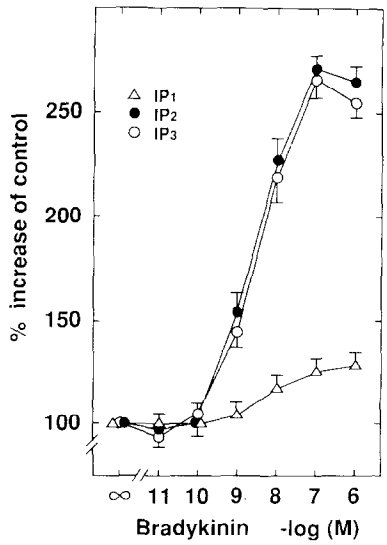

Fig. 2. Dose-response curve of BK-induced accumulation of IP_1 (△), IP_2 (●) and IP_3 (○). BAECs were stimulated with various doses of BK for 30 s. Results were expressed as mean ± SEM of three independent experiments.

3. RESULTS

3.1. *Accumulation of inositol phosphates by bradykinin*

Incubation of cultured BAECs with 1 μM BK induced accumulation of IP_1, IP_2 and IP_3 as shown in Fig. 1. IP_2 and IP_3 production rapidly reached a peak at 30 s with about a 3-fold increase and thereafter declined. The decline of accumulated IP_2 and IP_3 might be due to the conversion of IP_2 to IP_1 and IP_3 to IP_2, as demonstrated in other cell types. On the other hand, without BK, no significant increase in any inositol phosphates was induced in BAECs preincubated with serum-depleted medium even in the presence of LiCl. Fig. 2 shows the dose-response relation of BK for accumulation of IP_3. The threshold concentration and the mean half-maximum effective concentration (ED_{50}) value of BK were 0.1 nM and 3 nM, respectively.

3.2. *Inhibition by oxidized LDL of bradykinin-induced accumulation of inositol phosphates*

We examined the effects of native and oxidized LDL on phospholipase C-mediated reaction in BAECs. Fig. 3 shows the time course of oxidized LDL-induced accumulation of inositol phosphates for 30 min. Oxidized LDL (500 μg·protein/ml) induced a significant amount of accumulation of IP_1 and IP_2 at 20 and 30 min. In

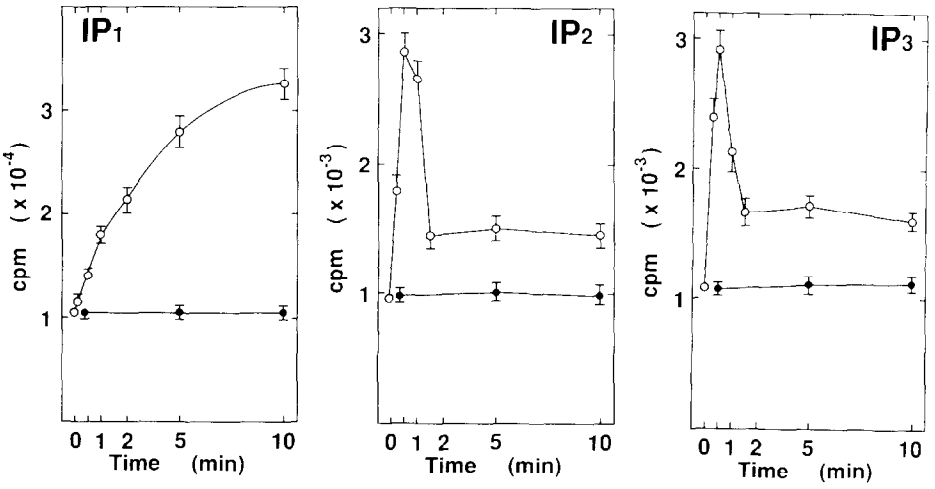

Fig. 1. Time course of BK-induced accumulation of inositol phosphates. BAECs were stimulated with (○) or without (●) 1 μM BK. Panel A: accumulation of IP_1; panel B: accumulation of IP_2; panel C: accumulation of IP_3. Results were expressed as mean + SEM of three independent experiments.

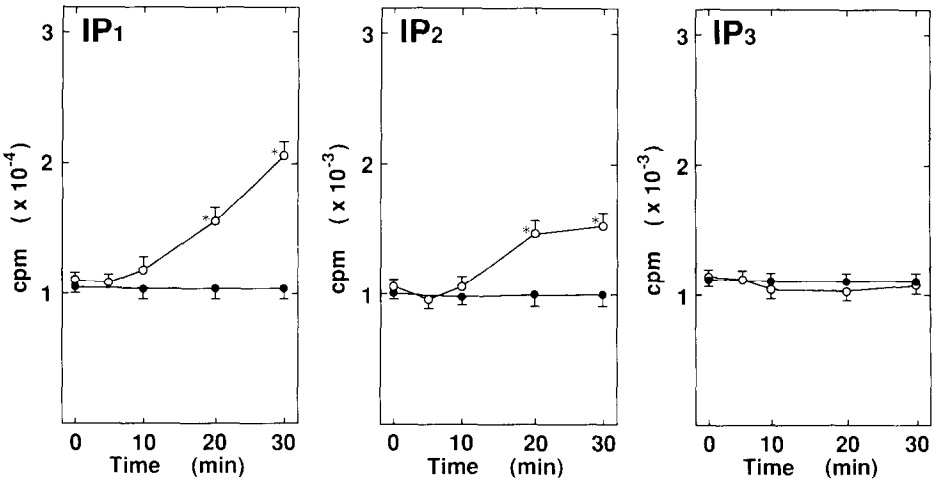

Fig. 3. Time course of oxidized LDL-induced accumulation of inositol phosphates. BAECs were stimulated with native (●) or oxidized LDL (○) at a concentration of 100 μg·protein/ml for various periods of time. Panel A: accumulation of IP_1; panel B: accumulation of IP_2; panel C: accumulation of IP_3. $^*P<0.05$ compared with control. Results were expressed as mean ± SEM of three independent experiments.

contrast, the accumulation of IP_3 was not observed at any time. Native LDL (500 μg·protein/ml) itself had no effect on phospholipase C-mediated phosphoinositides hydrolysis. Pretreatment with oxidized LDL (100–500 μg·protein/ml) for 30 min markedly inhibited the accumulation of inositol phosphates promoted by 100 nM BK. As shown in Fig. 4, the half-maxium inhibitory concentration(ID_{50}) value of oxidized LDL for BK-stimulated IP_3 accumulation was about 100 μg·protein/ml. However, native LDL had little effect on BK-induced accumulation of inositol phosphates.

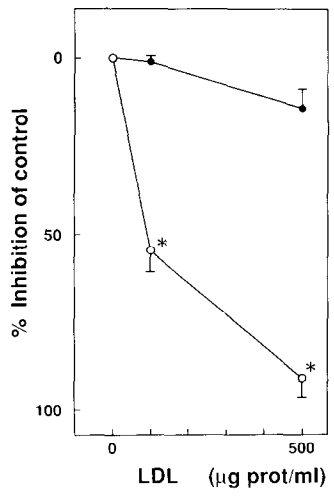

Fig. 4. Dose-dependency of the inhibitory effects of native LDL (●) and oxidized LDL (○) on BK-induced accumulation of IP_3. BAECs were preincubated with various concentrations of oxidized LDL for 30 min and then stimulated with 100 nM BK for 30 s. $^*P<0.001$ compared with control. Results were expressed as mean ± SEM of five independent experiments.

4. DISCUSSION

In the present study, we demonstrated that native LDL had little effect on IP_3 formation by BK, whereas oxidized LDL markedly inhibited BK-induced IP_3 formation in cultured endothelial cells. Oxidized LDL itself did not elicit the accumulation of IP_3 whereas increases of IP_1 and IP_2 stimulated with oxidized LDL were observed. The mechanism of IP_1 and IP_2 formation by oxidized LDL is unknown. The reason for this observation might be related to the non-specific activation of phospholipase C.

Endothelial cells which produce EDRF, prostacyclin and endothelin, regulate vascular tone [1,15]. In atherosclerotic arteries endothelium-dependent relaxation is markedly reduced and the impairment of endothelium-dependent relaxation is thought to play an important role in the pathogenesis of coronary artery spasm [16,17]. Oxidized LDL is proposed to play a significant role in the initiation or progression of atherosclerosis [18]. Recently, we have demonstrated that oxidized LDL inhibits endothelium-dependent relaxation mainly caused by the inhibition of production of EDRF [6,19]. From the present study, inhibition of endothelium-dependent relaxation by oxidized LDL is partly due to its inhibitory action of inositol phosphates formation in endothelial cells. It is reported that oxidized LDL has cytotoxic effect for endothelial cells [20]. In the present study, a leakage of lactate dehydrogenase out of the cells was not observed during the incubation of oxidized LDL (100–500 μg/ml) for 1 h. Furthermore, the viability of endothelial cells was preserved after incubation of oxidized LDL by Trypan blue exclusion. Therefore, irreversible endothelial cell injury by oxidized LDL was not related to its inhibitory effect on phosphoinositides hydrolysis. It is speculated

that impairment of receptor-coupled phosphoinositides hydrolysis was caused by the perturbation of endothelial cell plasma membrane by oxidized LDL. In the present study, oxidized LDL inhibits phosphoinositides hydrolysis and may alter the endothelial cell function including production of EDRF.

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