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LYSOPHOSPHATIDYLCHOLINE: ESSENTIAL ROLE IN THE INHIBITION OF ENDOTHELIUM-DEPENDENT VASORELAXATION BY OXIDIZED LOW DENSITY LIPOPROTEIN

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SUMMARY: Endothelial cells are known to play an important role in the regulation of vascular tone. Here we demonstrate that modified low density lipoprotein(LDL) with copper oxidation or phospholipase A_2 treatment elicits a potent inhibitory action on endothelium-dependent relaxations evoked by acetylcholine, although native LDL does not affect endothelium-dependent relaxations. Phosphatidylcholine of native LDL is converted to lysophosphatidylcholine during these modifications. Furthermore, lysophosphatidylcholine fraction separated from oxidized LDL(0.5mg.protein/ml) by thin layer chromatography abolished endothelium-dependent relaxations, although the remaining lipid fraction had little effects on endothelium-dependent relaxations. These results indicate that lysophosphatidylcholine is the principal substance for the impairment of endothelium-dependent relaxations by oxidized LDL and phospholipase A_2 treated LDL.

The vascular endothelium, in response to pulsatile flow and various vasoactive agents including acetylcholine(ACh), produces the endothelium-derived relaxing factor, a substance which regulates vascular tone(1). In atherosclerotic arteries endothelium-dependent relaxations are markedly reduced and the impairment of endothelium-dependent relaxations is thought to play an important role in the pathogenesis of coronary spasm(2)(3). Recently, it was reported that pathophysiological concentrations of low density lipoprotein(LDL) inhibited endothelium-dependent relaxations through a receptor-dependent mechanism, whereas chemically modified LDL was inactive(4). The precise mechanisms involved in the observed effects are, however, still unknown. Oxidized LDL can cause foam cell formation via the macrophage

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"scavenger" receptor and play an important role in atherosclerotic processes. The purpose of this study is to evaluate the inhibitory effect of modified LDL with copper oxidation and phospholipase A_2 treatment on endothelium-dependent relaxations and to clarify the mechanism of modified LDL-induced inhibition of endothelium-dependent relaxations.

MATERIALS AND METHODS

Lipoproteins and modification of LDL LDL(density=1.020-1.060) was isolated by ultracentrifugation from freshly harvested normal human plasma collected in EDTA(1mg/ml)(5). Protein was determined by the method described by Bradford(6) using bovine serum albumin as a standard. Phospholipase A_2 treated LDL and copper-oxidized LDL were prepared by the method of Quinn et al(7). Native LDL(1mg) was incubated in 1.0ml of 100mM Tris buffer(pH7.4) containing 2mM $CaCl_2$ and 10 units of naja naja venom phospholipase A_2(Sigma) for 2hr at 37°C. LDL(500μg/ml) was oxidatively modified by 10μM copper in PBS(-) for 24hr at 37°C. Control LDL was incubated without enzyme and copper. Modifications of LDL with phospholipase A_2 treatment and copper oxidation were determined by agarose gel electrophoresis(8).

Lipid extractions and analysis Lipid extractions were carried out according to the method of Bligh and Dyer(9). The chloroform phase was evaporated under N_2. Lipids were fractionated on 20x20cm silica gel plates(Merck) that had been activated for 1hr at 110°C. Plates were developed in $CH_3Cl/CH_3OH/H_2O$, 65:35:6 (vol/vol/vol). Lipid bands were detected by lightly staining with I_2 vapor. The plates were then divided into appropriate zones, which were scraped off and eluted three times with 5ml of CH_3Cl/CH_3OH, 1:1(vol/vol). Samples were then dried under N_2 and inorganic phosphorus was measured according to the method of Bartlett as modified by Marinetti(10). Lipid extracts by these methods were dispersed by sonication for 3min in calcium free phosphate-buffered saline and used in the isometric contraction experiment.

Isometric contraction experiments Japanese white rabbits(2.5-3.5 kg) were anesthetized with pentobarbital sodium(30mg/kg, iv) and descending thoracic aortas were isolated, cleaned of surrounding tissues, and cut into helical strips approximately 2mm wide and 15mm long. For recording isometric force, strips were suspended in 30ml muscle chambers containing buffer of the following compositions(mM): NaCl,118; KCl,4.0; $CaCl_2$,1.5; $MgSO_4$,1.2; $NaHCO_3$,25; glucose,5 and EDTA,0.03, and equilibrated for 90min at 37°C with a 95%O_2-5%CO_2 gas mixture(11). Final pH was approximately 7.38. One end of the strip was attached to the bottom of the chamber, the other to a Statham 4C-2 force transducer, which was connected to a Nihonkoden amplifier/recorder system. An initial preload of 1.5g for aorta was applied. The strips were then contracted with 0.3μM phenylephrine and subsequently relaxed by the cumulative addition of drugs. In some experiments endothelium was removed mechanically by rubbing the intimal surface with filter paper moistured with the buffer. To examine the effects of native LDL, modified LDL and lipids on vasorelaxation, the strips were preincubated with selected concentrations of native LDL, modified LDL, lipids extracted from LDL and synthetic phospholipids for 30min. Lipids were dispersed

with sonication for 3min in phosphate-buffered saline before the use.

Drugs The following drugs were used in the present study. Phenylephrine hydrochloride, acetylcholine chloride, indomethacin(Sigma). Nitroglycerin(Nihonkayaku). 1-palmitoyl-2-oleoyl-phosphatidylcholine(PC), 1-palmitoyl-lysophosphatidylcholine(LPC), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine and 1-palmitoyl-lysophosphatidylethanolamine were purchased from Avanti.

Statistical analysis All values are reported as means±SEM. Comparisons were performed by paired or unpaired t-test. P value<0.05 was considered as indicating a significant difference.

RESULT

Fig 1-a shows a typical recording of dose-dependent relaxations evoked by ACh. The relaxations were unaffected by pretreatment with indomethacin(10μM) and therefore not due to the release of cyclooxygenase products. Furthermore, the removal of endothelium abolished the relaxations induced by ACh(Fig.1-b). The effect of LDL treatment on endothelium-dependent relaxations induced by ACh(0.01-1μM) was investigated. The preincubation of the strips with native LDL(0.5-2mg.protein/ml) had no effects on ACh-induced endothelium-dependent relaxations(n=6-7 at each concentration) (Fig.1-c), whereas phospholipase A_2 teated LDL at a concentration of 0.01mg.protein/ml attenuated relaxations evoked by ACh and they were completely abolished by phospholipase A_2 treated LDL at a concentration of 0.1mg.protein/ml(Fig.1-d). Neither phospholipase A_2(1-2unit/ml) itself, which elicited weak endothelium-dependent relaxations in the precontracted strips with phenylephrine, nor $CuSO_4$(0.1-1μM) affected endothelium-dependent relaxations evoked by ACh. Oxidized LDL also inhibited ACh-induced relaxations in a dose-dependent manner(Fig.1-e). Control LDL incubated under same conditions without phospholipase A_2 or copper had no effects on endothelium-dependent relaxations evoked by ACh. The impairment of endothelium-dependent relaxations induced by phospholipase A_2 treated LDL and oxidized LDL was completely recovered by washing with Krebs buffer containing defatted albumin(BSA)(1mg/ml), indicating the functional integrity of endothelium after exposure to modified LDL. On the other hand, oxidized LDL and phospholipase A_2 treated LDL did not inhibit the relaxations induced by nitroglycerin(0.3nM-1μM), an endothelium-independent vasodilator(Fig1-f,g). Pretreatment of aortic strips with indomethacin(10μM) did not affect the inhibitory effect of oxidized LDL and phospholipase A_2 treated LDL on endothelium-dependent relaxations. Therefore, arachidonic acid products

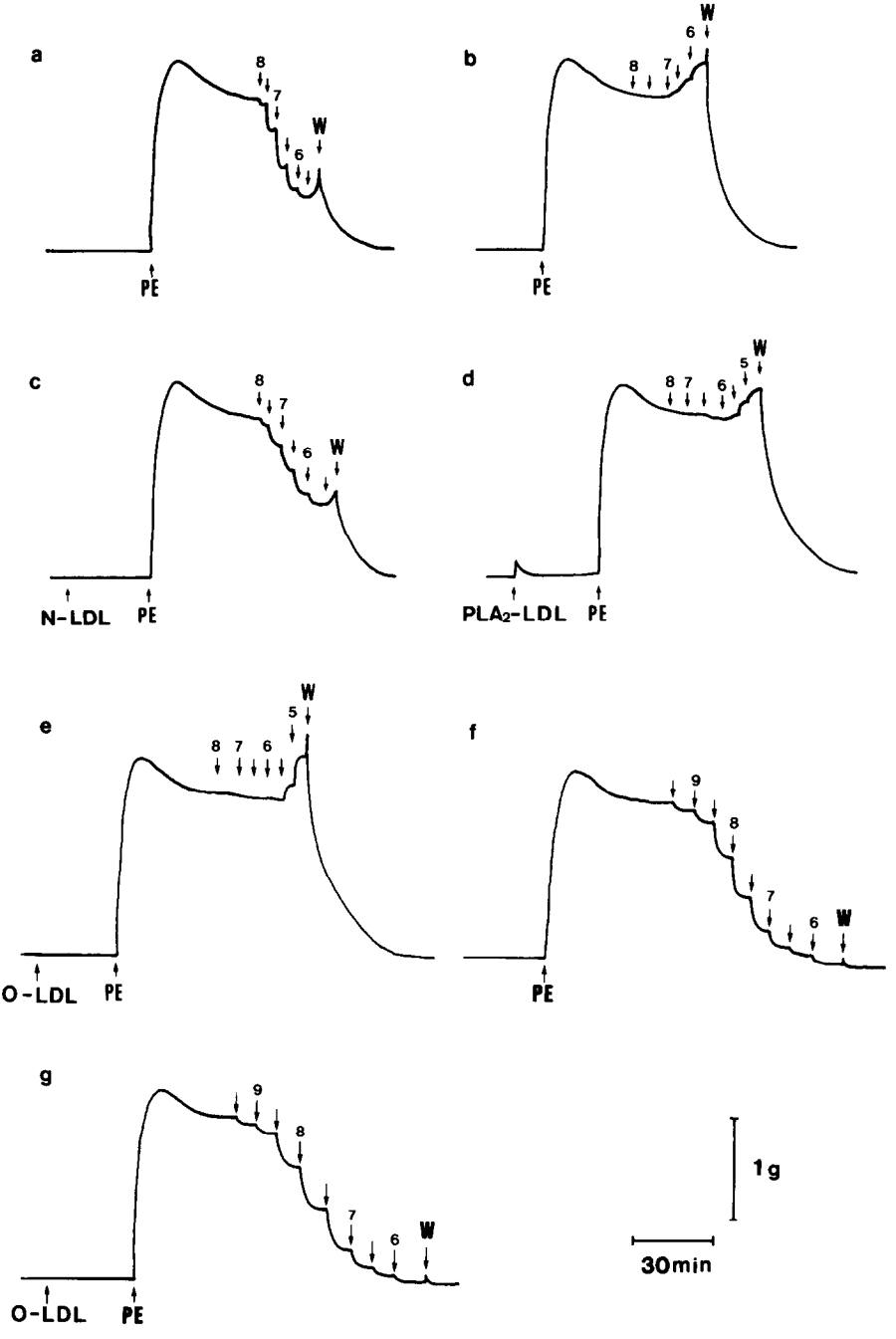

Fig.1 Tracing(a) is a typical example of acetylcholine(ACh)-induced relaxation in a helical strip of rabbit aorta. The removal of endothelium abolished the relaxation induced by ACh(b). The pretreatment with native LDL(2mg.protein/ml) had no effects on ACh-induced relaxation(c). Phospholipase A_2 treated LDL (0.1mg.protein/ml) and oxidized LDL(0.5 mg protein/ml) abolished ACh-evoked relaxation(d and e). Tracing(f) is a typical example of nitroglycerin(TNG)-induced relaxation. Oxidized LDL (0.5mg.protein/ml) had no effects on TNG-induced relaxation(g). PE: 0.3μM phenylephrine, W: wash, N-LDL: native LDL, PLA_2-LDL: phospholipase A_2 treated LDL, O-LDL: oxidized LDL

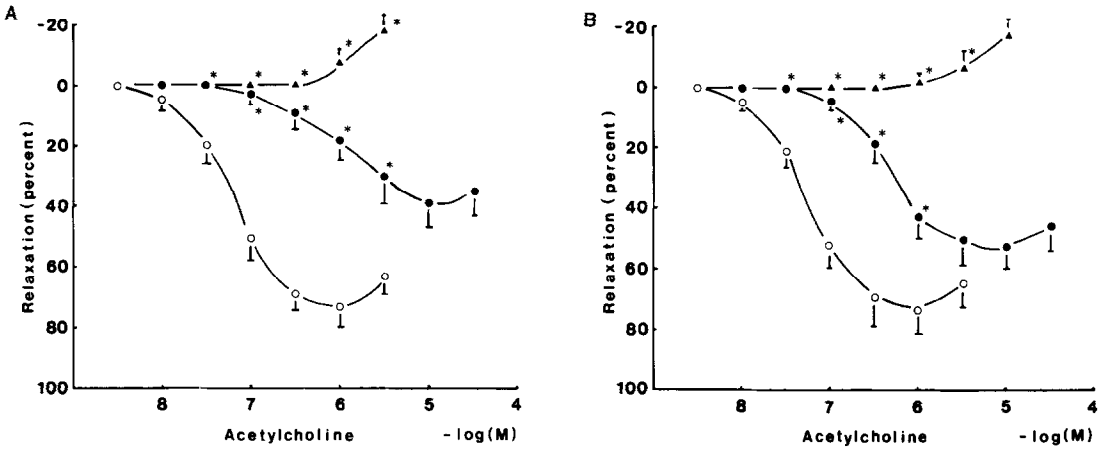

Fig.2 Dose-response curves demonstrating the effects of phospholipase A_2 treated LDL(A) and oxidized LDL(B) on endothelium-dependent relaxations evoked by acetylcholine in intact rabbit aortic strips precontracted with 0.3μM phenylephrine. Each curve is shown from the strips relaxed with cumulative concentrations of acetylcholine after treatment for 30min with Krebs buffer(○) or phospholipase A_2 treated LDL at a protein concentration of 0.01mg/ml(●) or 0.1mg/ml(▲)(A) and oxidized LDL at a protein concentration of 0.1mg/ml(●) or 0.5mg/ml(▲)(B). Each point is the mean of 5-6 observations. Vertical bars indicate SEM.

through the cyclooxygenase pathway in the vascular cells are not the inhibitors of endothelium-dependent relaxations.

We examined the effects of lipid extracts from native, oxidized and phospholipase A_2 treated LDL.(Fig. 2). Lipid extracts from native LDL (2mg.protein/ml) had no effects on endothelium-dependent relaxations evoked by ACh in intact rabbit aorta, whereas lipids from oxidized LDL(0.5mg.protein/ml) and phospholipase A_2 treated

Table 1. Conversion from PC to LPC during modification of LDL (Pi μg/mg LDL protein)

	PC	lyso-PC
native LDL	25.8±3.2	1.3±0.8
oxidized LDL	14.3±2.5*	12.5±2.2*
PLA_2 LDL	1.2±0.5*	23.3±3.1*

Values are mean±SEM. * $p<0.01$ compared to native LDL
PLA_2 LDL: phospholipase A_2 treated LDL
See the text for details.

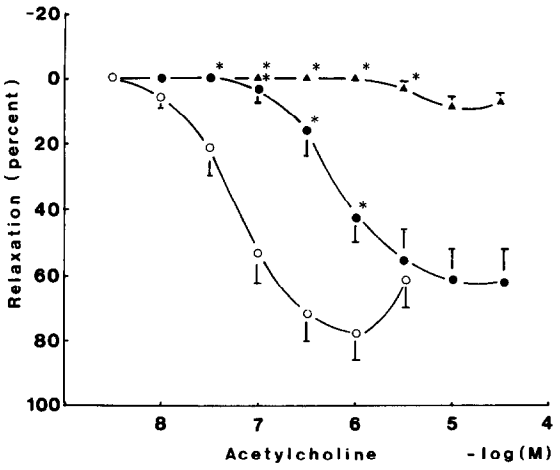

Fig.3 Dose-response curves demonstrating the effects of lysophosphatidylcholine(LPC) on endothelium-dependent relaxations in rabbit aortic strips. Each curve is shown from the strips relaxed with cumulative concentrations of acetylcholine after treatment for 30min with Krebs buffer(○) or 1μg/ml(●) and 10μg/ml(▲) of LPC. The results were identical in these vehicles. The vehicles alone had no effects on endothelium-dependent relaxations. Each point is the mean of 5-6 observations. Vertical bars indicate SEM.

LDL(0.1mg·protein/ml) inhibited almost completely endothelium-dependent relaxations induced by ACh. From measurement of inorganic phosphorus as much as 40% of PC in LDL was converted to LPC during the oxidative modification and almost complete conversion of PC to LPC was achieved during incubation with phospholipase A_2(Table 1). PC and LPC contents of native and modified LDL in 1mg protein correspond to approximately 600 and 20μg in native LDL, 340 and 220μg in oxidized LDL, and 30 and 400μg in phospholipase A_2 treated LDL, respectively. Furthermore, LPC fraction separated from oxidized LDL(0.5mg·protein/ml) by thin layer chromatography abolished endothelium-dependent relaxations, although the remaining lipid fraction including oxidized free fatty acids, sphingomyelin and other phospholipids had no effects on endothelium-dependent relaxations. Exogeneous administration of synthetic 1-palmitoyl-2-oleoyl-PC(1-10μg/ml) had little effects on endothelium-dependent relaxations evoked by ACh. On the other hand, synthetic 1-palmitoyl-LPC(1-10μg/ml) had a potent inhibitory effect on endothelium-dependent relaxations induced by ACh(Fig.3). Synthetic 1-palmitoyl-2-oleoyl phosphatidylethanolamine and 1-palmitoyl-lysophosphatidylethanolamine had no effects on endothelium-dependent relaxations.

DISCUSSION

In the present study, we demonstrated that native LDL freshly obtained from normal volunteers elicited no effects on endothelium-dependent relaxations evoked by ACh, whereas modified LDL with copper oxidation or phospholipase A_2 treatment markedly inhibited endothelium-dependent relaxations in rabbit aortas. The observation that native LDL had no effects on endothelium-dependent relaxations evoked by ACh in intact rabbit aortas is different from the previous data described by Andrews et al(4). The reasons of the difference between these studies are unknown. We prepared human LDL from fresh plasma by differential ultracentrifugation as the $1.020<d<1.060$ fraction to prevent the contamination. On the other hand, fractions prepared by Andrews et al. was 1.006-1.063 which contained intermediate low density lipoporotein. They found that LDL did not produce significant oxidation LDL when tested by fluorescence measurements. The differences of density fractions in LDL preparation might be one of the possibilities to explain this discrepancy.

Oxidatively modified LDL is proposed to play a significant role in the pathogenesis of atherosclerosis(12,13). LPC that is generated during oxidative modification of LDL can alter membrane fluidity and functions and produce various biological activities including a potent chemotactic factor for human monocyte(7). The wide difference in the concentrations of LPC in modified LDL and exogeneous synthetic one to induce comparable effects may partly reflect the limited availability of free LPC from modified LDL particles in the buffer and the difference in its fatty acid constituents. The present study indicates that oxidized LDL also plays an important part in the impairment of agonist-induced endothelium-dependent relaxations and that LPC is the principal substance for its inhibitory effect.

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