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Lysolecithin actions on vascular smooth muscle cells

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Summary Oxidation of low density lipoprotein increases its atherogenic potential. During oxidation there is an extensive conversion of lecithin to lysolecithin. In rat aortic smooth muscle cells, 2-25 μg/ml lysolecithin elevated cytosolic calcium concentration up to 560%. Lysolecithin (10-20 μg/ml) increased [^{3}H]thymidine incorporation from 15 cpm/mg cell protein (controls) up to 189 cpm/mg cell protein. Lysolecithin (10 μg/ml) potentiated the PDGF-induced (50 ng/ml) [^{3}H]thymidine incorporation up to 6.3 times. The results indicate that lysolecithin could induce mechanisms, by which oxydized low density lipoproteins could promote cell growth and thus contribute to atherosclerosis.

Elevated levels of low density lipoprotein (LDL) cholesterol are an important risk factor for atherosclerosis and cardiovascular morbidity (1–4). LDL is considered to be the main atherogenic class of lipoproteins and contains 60-70% of the total serum cholesterol. Apart from its physiological role as a transport vehicle and its regulatory function for cellular cholesterol homeostasis, LDL has been shown to cause a general activation of human thrombocytes and rat vascular smooth muscle cells (VSMC) (5,6). In addition, LDL caused a time-dependent induction of both c-myc and c-fos (7) of human VSMC and enhanced proliferation of rat VSMC (8).

During its incubation with cells, the LDL particle undergoes a large number of physical and chemical changes that alters its metabolism in important ways (11-15) .The extensive conversion of LDL lecithin to lysolecithin is one of these changes. Lysolecithin concentration was 10-fold elevated in copper-oxidized LDL and impaired the endothelium-dependent relaxation of the vasculature (16). In addition, lysophosphatidic acid in the micromolar range was suggested to cause calcium release and aggregation of platelets (17).

Since the active principle(s), by which LDL activates VSMC and consequently could exert a pathophysiological role in atherosclerosis (18,19) is (are) are still unknown, we studied the action of lysolecithin on the intracellular free calcium concentration ($[Ca^{2+}]_i$) as well as thymidine incorporation into DNA.

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Materials and Methods

Dulbecco's modified Eagle's medium, and Ham's F-10 medium were from Amimed, Muttenz, Switzerland. Fetal bovine serum was purchased from Seromed, Basel, Switzerland. FURA-2 (2',7'-biscarboxyethyl-5(6)-carboxyfluorescein) and its esterized form were from Calbiochem, Luzern, Switzerland. Lysolecithin grade I was obtained from Lipid Products, South Nutfield, Great Britain, and L-α-lysopalmitoyllecithin was from Sigma, Buchs, Switzerland. The $PDGF_{AB}$ isoform was from Boehringer, Rotkreuz, Switzerland [Methyl-^{3}H]thymidine (2 Ci/mmol) was purchased from Amersham Corp. int., Great Britain.

Cell culture

The procedures for isolation and culture of smooth muscle cells (VSMC) isolated from rat aortic medial tissue explants (normal female rats, 180-200 g, strain ZUR:SIV) has been described in detail previously (18). Cultured cells were fed every other day with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 100 U/ml of penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. For experiments cultured VSMC from passages 3–12 were used throughout.

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Confluent cells were detached by collagenase treatment (0.1 mg/ml collagenase, 0.1 mg/ml soybean trypsin inhibitor, 0.3 mg/ml bovine serum albumin in Hanks balanced salt solution (HBS)) for 20 min at 37℃. After washing with HBS supplemented with 1% BSA, VSMC were resuspended in the same buffer to a concentration of 10^6 /ml and loaded with 1 µmol/l FURA 2/AM for 30 min in a 37℃ shaking water bath. Aliquots of 1 ml of the cell suspension were spun down at 800xg, and the supernatant was removed. Cells were washed in HBS buffer-1% BSA and centrifuged at 800xg. Immediately before use, the supernatent was removed and the cells were resuspended in HBS buffer. Fluorescence of cells was measured at 37℃ under stirring in a quartz cuvette placed in an SLM-Aminco SPF-500C spectrofluorometer. Excitation wavelengths were set at 340 and 380 nm, emission wavelength was 505 nm. Excitation bandwith was set at 7.5 nm and the emission bandwith at 5 nm. Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated using 0.5 % Triton X-100 for maximum fluorescence followed by the addition of 1.0 mol/l Tris, 300 mM EGTA, pH < 8.8 for minimum fluorescence as described (19).

Measurement of DNA synthesis rate

The proliferative effect of lysolecithin was examined by a slight modification of the method described by Nemecek et al. (20). VSMC were seeded in petri dishes (30 mm diameter) and cultivated in DMEM until confluent. Then the medium was replaced by a serumfree starvation medium consisting of a mixture of DMEM and Ham's F-10 medium (1:1, v/v), supplemented with 10 µg/ml transferrin and 1 µmol/l insulin. Following another 24 h culture, cells were exposed to either lysolecithin or L-α-lysopalmitoyllecithin, both in the absence or presence of 50 ng/ml of PDGF. After 20 h, 3 µCi/ml of [^{3}H]thymidine was added. Four hours later the reaction was terminated by aspirating the medium and subjecting the cultures to sequential washes with Dulbecco's phosphate buffered saline (PBS) containing 1 mmol/l CaCl2 and 1 mmol/l MgCl2; 10% trichloroacetic acid; and ethanol/diethyl-ether (2:1, v/v). Acid-insoluble [3H]thymidine was extracted into 1 ml/dish 0.5 mol/l NaOH. Aliquots of 0.2 ml of this solution were used for liquid scintillation counting and another 100 µl for determination of protein concentration by Lowry's method (21).

Statistics Data are presented as mean±SD. Statistical evaluation was done with Student's t-test for paired data. A probalitiy of $p<0.05$ was considered significant.

Results

Baseline concentration of cytosolic free calcium was 91±12.2 (mean ± SD, n=72). Incubation of cells with lysolecithin caused a dose-dependent rise in $[Ca^{2+}]_i$, with a sharp increase in $[Ca^{2+}]_i$ in the range 6–12.5 µg/ml lysolecithin. (Fig. 1). The maximal $[Ca^{2+}]_i$ was reached within 40 s. The subsequent decline in $[Ca^{2+}]_i$ to almost baseline seen in agonist-receptor-coupled elevations in $[Ca^{2+}]_i$ was not observed, when lysolecithin in the range 14-20 µg/ml was added. As a positive control of cell activity, VSMC were stimulated with 100 nmol/l of angiotensin II, which caused an increase in $[Ca^{2+}]_i$ up to 312 ± 41 nmol/l (n=12). This value corresponded to a mean net increase of $[Ca^{2+}]_i$ by 240%.

Elevation of $[Ca^{2+}]_i$ remained unaffected, when cells were preincubated for 30 min with nifedipine (14 µmol/l) or the intracellular Ca^{2+} antagonist TMB-8 (11.6 µmol/l) and subsequently with increasing concentrations of lysolecithin (data not shown). However, there was no increase in $[Ca^{2+}]_i$ in the presence of 1 mmol/l extracellular EGTA

Incubation of VSMC in the presence of [^{3}H]thymidine revealed that lysolecithin could stimulate DNA synthesis. Baseline value in unstimulated cells was 15.4 ± 2.4 cpm/µg cell protein

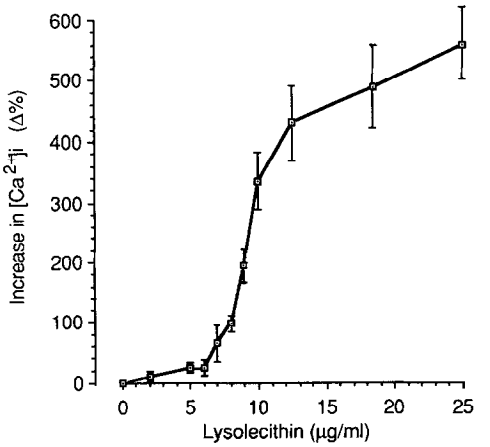

Fig. 1. Elevation of cytosolic free calcium concentration in vascular smooth muscle cell suspensions (10^6 cells/ml). Cells loaded with the FURA 2–AM fluorescent calcium chelator were stimulated with increasing doses of lysolecithin (from egg yolk) dissolved in PBS. Values are means±SD (n=6).

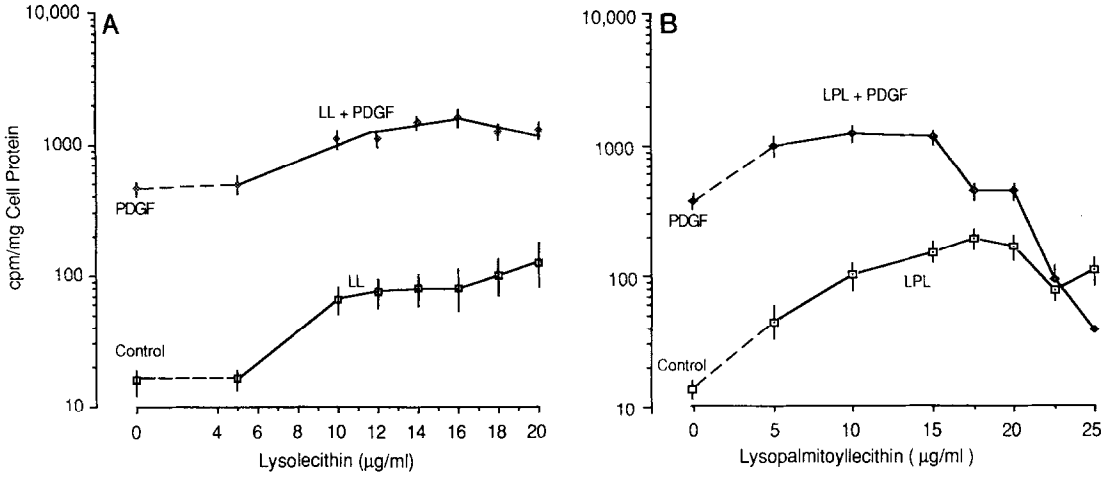

Fig. 2. Augmentation of PDGF-induced [^{3}H]thymidine incorporation by increasing doses of lysolecithin or L-α-lysopalmitoyllecithin. Either PDGF (50 ng/ml) or PBS-vehicle was added to confluent cells precultured in quiescing medium. One hour later, increasing doses of either lysolecithin (LL) or L-α-lysopalmitoyllecithin (LPL) was added and cells were incubated for 23 h at 37°C. [^{3}H]thymidine pulse was given during the last 4 h of incubation. Experiments were carried out in quadruplicate. Values are means±SD (n=5 for each lysolecithin).

(n=10). Increasing concentrations of both lysolecithin and lysopalmitoyllecithin stimulated [^{3}-H] thymidine incorporation into DNA in a dose-dependent manner (Fig. 2).Maximal stimulation by lysolecithin was 125±32 and that by lysopalmitoyllecithin 189±39 cpm/mg cell protein. Addition of 50 ng/ml of PDGF in the absence of lysolipid caused [^{3}H]thymidine incorporation of 461±68 (n=5, $p<0.01$) and 373±55 cpm/µg cell protein (n=5, $p<0.01$), respectively (Fig. 2). In the presence of 50 ng/ml PDGF, the lysolecithin–induced [^{3}H]thymidine incorporation was further augmented and peaked at 16 µg/ml lysolecithin (Fig. 2A) and at 10 µg/ml lysopalmitoyllecithin (Fig. 2B). At higher lysolecithin concentrations, a decline of [^{3}H]thymidine incorporation was observed. The 24–h hour incubation with lysolecithin compounds (5-20 µg/ml) did not influence significantly cellular protein synthesis.

Discussion

Our results showed that in addition to known activities of lysolecithin, e.g. impairement of the endothelium–dependent relaxation of the vasculature, this compound has the capacity to induce relatively longlasting elevations in intracellular free calcium concentration in vascular muscle cells in vitro.

Since increase of $[Ca^{2+}]_i$ is a prerequisite for the initiation of a number of cellular activities, such as smooth muscle contraction (22) or enzyme activation, lysolecithin could interfere with biochemical processes dependent on cytosolic free calcium as a second messenger. This conclusion is confirmed by the lysolecithin–induced DNA synthesis in vascular muscle cells.

The ability of relatively low *in vitro* doses of lysolecithin to activate cell activity without remarkable concomitant cytotxic effects was confirmed by its potential to induce DNA synthesis in VSMC. The presence of PDGF markedly enhanced the DNA formation capacity of lysolecithin. Since calcium antagonists failed to affect the lysolecithin-induced rise in $[Ca^{2+}]_i$, it is assumed that lysolecithin caused influx of calcium without the participation of calcium channels, which are blocked by 1,4 dihydropyridine Ca-channel blockers. The lack of $[Ca^{2+}]_i$ elevation in the presence of extracellular EGTA as well as the independence of $[Ca^{2+}]_i$ elevation on TMB-8 indicates that no intracellular calcium sources contributed to the $[Ca^{2+}]_i$ rise. Thus it seems obvious to suggested that in VSMC lysolecithin by exerting some ionophor properties promotes calcium influx.

Indeed, ionophore properties have been discussed for the action of lysophosphatidic acids in platelet aggregation and calcium flux (17,23). ^{45}Ca release in both platelets and platelet membrane vesicles was induced by lysophosphatidic acid per se and augmented after addition of lysophosphatidic acid to synthetic diacylglycerols (23,17). Based on these findings it was doubted that in platelets lysophosphatidic acids are acting as simple calcium ionophores. Our results support these suggestions, since in cultured VSMC lysolecithin not only induced calcium influx but had also potentiating effects on PDGF-induced thymidine incorporation.

Low density lipoprotein gently extracted from human and rabbit atherosclerotic lesions greatly resembled in physical, chemical, and biological properties LDL, that has been oxidatively modified *in vitro* (24). During oxidation of LDL, there is an extensive conversion of lecithin to lysolecithin and an increased diene formation of fatty acids. In addition, it was found that LDL isolated from atherosclerotic human lesions had a higher concentration of lysolecithin as compared to that from plasma LDL (24). There is also evidence, that oxidized LDL is more atherogenic than native LDL (16,25). This was confirmed by a very recent study showing that oxidized LDL stimulated the elevation in $[Ca^{2+}]_i$ more than native LDL (26). As in the present study lysolecithin induced a dose-dependent increase in $[Ca^{2+}]_i$, it is conceivable that this component might be responsible for the direct influence of LDL and its oxidized derivatives on cellular calcium metabolism. It is thus tempting to associate the reinforced biological properties of oxidized LDL with its lysolecithin content. However it remains to show, whether other constituents of LDL, e.g. cholesterol derivates, can induce VSMC responses comparable to those induced by lysolecithin.

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