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LOW-DENSITY LIPOPROTEIN ELEVATES INTRACELLULAR CALCIUM AND pH IN VASCULAR SMOOTH MUSCLE CELLS AND FIBROBLASTS WITHOUT MEDIATION OF LDL RECEPTOR

Agapios Sachinidis, Rudolf Locher, Thomas Mengden and Wilhelm Vetter

Department of Internal Medicine , University Hospital
Rämistrasse 100, 8091 Zürich, SWITZERLAND

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Low-density lipoprotein (7 µg/ml) induced in the absence or in the presence of 7, 35, 70 µg/ml monoclonal antibodies against the specific Low-density lipoprotein receptor an elevation of intracellular Ca^{2+} from 105 to approximately 210 nM in vascular smooth muscle cells from rat aorta. Moreover, in both human cultured fibroblasts from normocholesterolemic individuals and from patients with familial hypercholesterolemia homozygote class 1, Low-density lipoprotein (7 µg/ml) induced a rise of free intracellular calcium and a biphasic change of intracellular pH. Low-density lipoprotein (1,7,15,30 µg/ml) had no significant influence on the phosphatidylinositol-turnover in vascular smooth muscle cells and fibroblasts. Since homozygote class 1 fibroblasts lack specific Low-density lipoprotein receptors, and as antibodies against this receptor did not attenuate the Low-density lipoprotein-induced elevation of cytosolic calcium and pH, we conclude that these intracellular changes are independent from the classical Low-density lipoprotein receptor. © 1990 Academic Press, Inc.

Increased levels of Low-density lipoprotein (LDL) constitute one of the major risk factors for cardiovascular morbidity and mortality. LDL, the major cholesterol-carrying lipoprotein of human plasma, delivers cholesterol to cells by binding to specific cell surface receptors and consecutive internalization by endocytosis (1).

It has recently been reported that LDL, besides its physiological role, at concentrations corresponding to those of hormones, can cause general cellular activation by stimulation of the phosphatidylinositol (PtIns)-turnover cellular signal system in vascular smooth muscle cells (VSMC) (2,3). LDL is found not only in plasma but also in the extravascular fluids, where its concentrations are much lower owing to the diffusion barrier offered by the endothelium (4,5). Recently, we demonstrated that LDL (1-30 µg/ml) can induce elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$), a biphasic change of intracellular pH (pH_i) via the Na^+/H^+ exchanger in cultured VSMC and vasoconstriction of thoracic aortic rings from rat (6). Consequently, the question arose whether these intracellular changes are mediated by the LDL receptor via stimulation of the PtIns-turnover cellular signal system. For this purpose, we investigated the effect of LDL in VSMC on PtIns-turnover cellular signal system as well as on $[Ca^{2+}]_i$ and pH_i in the presence of monoclonal LDL receptor antibodies (I_gGC_7)(7). Moreover we studied the effect of LDL on $[Ca^{2+}]_i$ and pH_i in LDL receptor negative cultured fibroblasts from patients with familial hypercholesterinemia (FH) homozygote class 1 mutations. Genes from these mutations produce either no LDL receptor protein or only trace amounts as determined by reaction with monoclonal antibodies (1).

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MATERIALS AND METHODS

LDL isolation. LDL (density, 1.019-1.063 g/ml) was isolated from the plasma of 5 individual normocholesteremic subjects by ultracentrifugation according to Redgrave et al. (8). The LDL fraction was dialyzed against 0.15 M NaCl/1 mM EDTA, pH 7.4 and used within 3 weeks. Oxidation of LDL was prevented by adding 50 µM of ascorbic acid throughout. LDL was stored at 4°C and used within 3 weeks; no changes in activity were observed during this time period. Determination of protein was performed by Lowry's method (9).

Culture of Vascular Smooth Muscle Cells. Vascular smooth muscle cells were isolated from rat aorta (female, strain Wistar-Kyoto, 6-8 weeks old) and cultured over several passages according to Ross (10). The cells were allowed to grow for 4 - 5 days in 5% CO_2, 95% air at 37° C. The culture medium was Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal calf serum.

Culture of Fibroblasts. Normal and FH-homozygote fibroblasts were obtained from Human Genetic Mutant Cell Repository Institute for Medical Research, USA, and cultured over several passages after detachment of the confluent cells with a Puck's Saline A physiological solution containing 0.04% trypsin/0.02% EDTA buffer. The cells were allowed to grow as described for the VSMC.

Measurement of free intracellular Ca^{2+} $[Ca^{2+}]_i$. Confluent cells were detached with 0.04% trypsin/0.02% EGTA/Puck's Saline A physiological solution after 5 to 10 minutes at 37° C. Then cells were incubated with 2 µM fura-2 pentaacetoxymethyl ester at 37°C for 20 minutes in HEPES-buffer (20 mM HEPES, 16 mM glucose, 130 mM NaCl, 1mM $MgSO_4 .7 H_2O$, 0.5 mM $CaCl_2$,Tris-base, pH, 7.4) supplemented with 1% Bovine Serum Albumin (BSA). After loading, cells were washed and suspended in HEPES-buffer (approximately 2 x 10^6 cells/ml). The Ca^{2+}-fura-2 fluorescence was measured at 37° C under stirring in a SLM-Aminco SPF-500 spectrofluorometer (excitation wavelengths: 340 and 380 nm; emission: 505 nm). Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated using 0.5 % Triton X-100 for measurement of maximum fluorescence followed by the addition of 2 mM $MnCl_2$ for minimum fluorescence according to Grynkiewicz et al. (11).

Measurement of pH_i. These measurements were performed according to Berk et al.(12) with the fluorescence pH indicator [2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein] (BCECF). Cells were loaded as described for the fura-2 loading method in HEPES-buffer with 2 µM BCECF-pentaacetoxymethyl ester for 20 minutes at 37°C. For the fluorescence measurements the following wavelengths were set: Excitation wavelengths: 492 and 438 nm; emission wavelength 525 nm. The calibration curve was performed by permeabilizing the cells with 30 µM digitonin as previously described (12).

Measurement of inositolphosphates. Cells were seeded in petri dishes (60 mm diameter) and grew in the presence of myo-[2-3H]inositol (4 µCi/ml) for 3 days. During this time the cells reached confluence. They were washed three times with HEPES-buffer, followed by a 2h incubation in DMEM without FCS. After 2 other washes with buffer, cells were incubated for 20 minutes in DMEM containing 20 mM LiCl in order to inhibit inositol-1 monophosphatase activity. Then cells were stimulated with LDL for various time periods. The reaction were terminated by the addition of icecold 8% trichloroacetic acid (TCA). Samples were kept on ice for 30 minutes before removing TCA by extracting the samples with diethyl ether (13). Radiolabelled inositolphospates were analyzed and quantified by standardized anion-exchange HPLC (14). The 1.25 ml fractions were transferred to scintillation vials and radioactivity was determined in the liquid scintillation counter.

Materials. Fura 2/AM, pentaacetoxymethyl ester and BCECF/AM, [2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein] pentaacetoxymethyl ester were obtained from Calbiochem. Dulbecco's modified Eagles medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline (PBS) were obtained from Amimed. Monoclonal anti-low density lipoprotein antibodies as well as myo-[2-3H]inositol, and standard 3H-Inositolphosphates were obtained from Amersham International, U.K. Normal and FH-homocygote fibroblasts Class 1 were obtained from Human Genetic Mutant Cell Repository Institute for Medical Researche, U.S.A. Other chemicals were from Sigma and Merck-Schuchardt.

RESULTS

Effect of LDL on $[Ca^{2+}]_i$ in VSMC in the presence of monoclonal antibodies against the LDL receptor (I_gGC_7). To examine if the LDL-induced rise of $[Ca^{2+}]_i$ is triggered by the specific LDL surface receptor, we

investigated the effect of LDL on $[Ca^{2+}]_i$ 30 min after preincubation of VSMC with 1,7, 35, and 70 µg/ml I_gGC_7 at 4° C. Then cells were stimulated with 7 µg/ml LDL. I_gGC_7 in the given concentrations did not abolish or attenuate the LDL-induced elavation of $[Ca^{2+}]_i$. Fig 1 shows one representative experiment from a total of four. The maximal 7 µg/ml LDL-induced elevation of $[Ca^{2+}]_i$, occurring at 20 sec, was 210 ± 22 nM in the absence and 218 ± 25 in the presence of 35 µg/ml I_gGC_7 (mean ± SD, n=4, basal value 105 ± 16 nM, n=8). I_gGC_7 in the used concentrations had no effect on $[Ca^{2+}]_i$.

LDL changes $[Ca^{2+}]_i$ in normal and receptor negative fibroblasts

Fig. 2a shows the effect of 7 µg/ml LDL on $[Ca^{2+}]_i$ in receptor negative FH-homozygote fibroblasts. LDL induced in the the presence of extracellular Ca^{2+} a rapid rise in $[Ca^{2+}]_i$ (basal value =86 ± 12 nM, mean ± SD, n=10) with a maximum at 15 s. The rise of LDL-induced stimulation of $[Ca^{2+}]_i$ declined after 15 s towards the resting level within 1.0 min. The maximal LDL induced elevation of $[Ca^{2+}]_i$, occuring at 15 sec, was 205 ± 22 nM (n=4). Fig. 2b shows that LDL also stimulated a rapid rise in $[Ca^{2+}]_i$ in the Ca^{2+}-free buffer. This rise, however, was less pronounced than in the presence of extracellular Ca^{2+}. Fig. 2c,d show the effect of 7 µg/LDL on $[Ca^{2+}]_i$ in normal fibroblasts in the presence (c) and absence (d) of extracellular Ca^{2+}. The maximal LDL-induced elevation of $[Ca^{2+}]_i$ was 196 ± 25 nM (n=5). These results clearly indicate that the LDL induced elevation of $[Ca^{2+}]_i$ is not triggered by the specific LDL receptor.

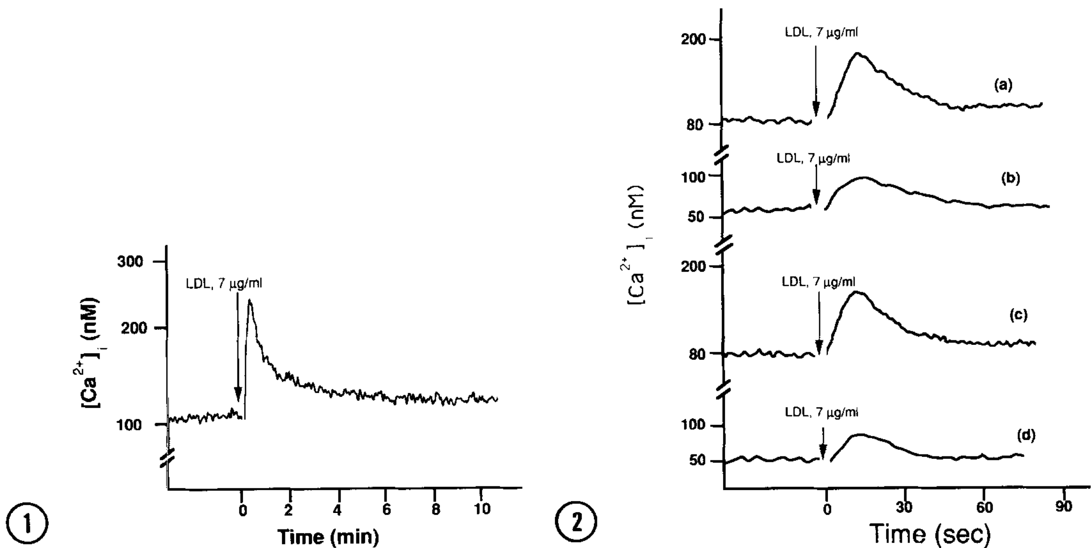

Fig. 1. Effect of 7 µg/ml LDL on $[Ca^{2+}]_i$ in cultured rat VSMC pretreated with the LDL receptor antibodies. Fura-2-loaded VSMC in HEPES buffer in cell suspension were incubated for 30 min at 4° C with 35 µg/ml monoclonal LDL receptor antibodies. Then cells were stimulated with LDL (7 µg/ml) and changes in fluorescence were monitored for 3 min. After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio by the emission wavelength of 505 nm were converted into corresponding levels of $[Ca^{2+}]_i$.

Fig. 2. Effect of 7 µg/ml LDL on $[Ca^{2+}]_i$ in cultured human fibroblasts in the presence and absence of extracellular Ca^{2+}. LDL (7 µg/ml) was applied to fura-2-loaded LDL receptor negative fibroblasts in HEPES buffer, containing 1 mM $CaCl_2$ (a) and in Ca^{2+}-free HEPES-buffer, containing 1 mM EGTA (b). Similar measurements were performed in normal fibroblasts in the presence (c) or absence of extracellular Ca^{2+} (d). Changes in fluorescence were monitored for 3 min. After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio by the emission wavelength of 505 nm were converted into corresponding levels of $[Ca^{2+}]_i$.

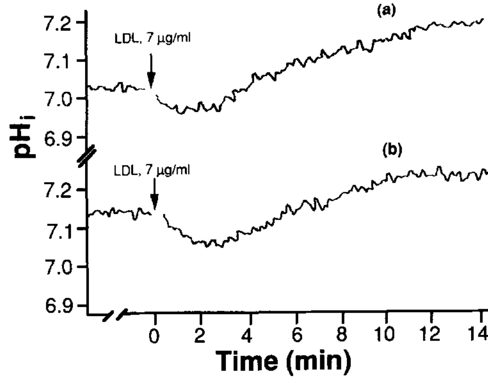

Fig. 3. Effect of LDL on pH_i in cultured human fibroblasts. LDL (7 μg/ml) was applied to BCECF-loaded LDL receptor negative (a) and normal human fibroblasts (b). Changes in fluorescence were monitored for 20 min in HEPES-buffer. After calibration of the fluorescence signal by permeabilizing the cells with 30 μM digitonin, changes in 492/ 438 nm excitation wavelength ratio by the emission wavelength 525 nm were converted into corresponding levels of pH_i.

LDL changes pH_i in normal and receptor negative fibroblasts. Fig. 3a illustrates the effect of LDL on pH_i of normal and receptor negative fibroblasts in HEPES buffer. Addition of 7 μg/ml LDL exerted in both cell typs a biphasic effect on pH_i (basal value = 7.04 ± 0.03, n = 10). Initially, within 2 min a rapid acidification of 0.06 ± 0.02 (n=5) pH units occured, followed by a subsequent prolonged alkalinization of 0.12 ± 0.04 pH units. These results clearly indicate that the LDL induced biphasic change of pH_i is independent of the existence of the specific LDL receptor.

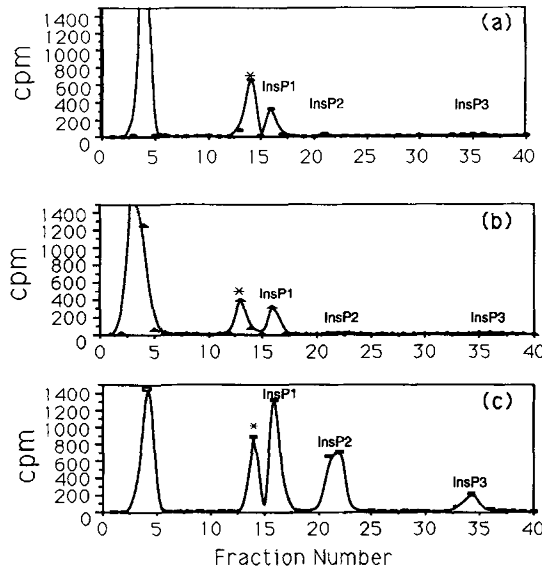

Fig. 4. Analysis of inositolphosphates by HPLC. Inositolphosphates were isolated as described in Method. HPLC was performed by anion exchanger Partisil SAX column. Elution conditions: Flow rate, 1.25 ml/min/ fraction, 0-6 min, 100% water; 7-30 min a linear gradient from water to 100% 1.0 M ammonium formate/orthophosphoric acid pH 3.7; 31-35 min 100% 1.0 M ammonium formate/orthophosphoric acid pH 3.7; 36-37 min a linear gradient from 1.0 M ammonium formate/orthophosphoric acid pH 3.7 to 100% water; 38-48 min 100% water. Identification of inositolphosphates was performed with standard ^{3}H-Inositolphosphates. * Unidentified substance.

LDL has no effects on inositolphosphate formation. In order to investigate whether LDL can stimulate the PtIns-turnover the formation of radiolabeled inositol-1 monophosphate ($InsP_1$), inositol-1,4-phosphate ($InsP_2$) and inositol-1,4,5-trisphosphate ($InsP_3$) in VSMC by using standardized anion HPLC was determined. Peak identification of $InsP_1$, $InsP_2$, and $InsP_3$ were performed by using standard radiolabeled [^{3}H]inositolphosphates. Cells were stimulated with 1, 7 and 15 μg/ml LDL for 20 sec, 1, 3, 5, 10, 20 min. Positive control experiments were performed by stimulation of the cells with 100 nM Angiotensin II (AII) and 50 ng/ml PDGF for 20 sec and 10 min. No significant formation of $InsP_1$, $InsP_2$, and $InsP_3$ could be observed after different stimulation periods of 20 sec, 1, 3, 5, 10, 20 min with 1, 7 and 15 μg/ml LDL compared to the unstimulated cells (n=5). PDGF or AII induced strong stimulation of the PtIns-turnover. Fig. 4 shows the HPLC separation of the radiolabeled $InsP_1$, $InsP_2$, and $InsP_3$ isolated from unstimulated VSMC (a), from VSMC after 10 min stimulation with 7 μg/ml LDL (b) and from cells after 10 min stimulation with 50 ng/ml PDGF. Our result document that LDL could not stimulate the PtIns-turnover, in contrast to paralell experiments with 50 ng/ml PDGF, which induced an approximately 3.5, 25, and 4-fold higher formation of $InsP_1$, $InsP_2$, and $InsP_3$ respectivelly, compared to unstimulated cells.

DISCUSSION

We have recently reported that 1-30 μg/ml LDL induced elevation of $[Ca^{2+}]_i$ in VSMC (6). This elevation was partly due to mobilization of calcium from intracellular stores. In addition, LDL caused pH_i shifts in VSMC, probably via activation of Na^+/H^+ exchange. Since such intracellular changes are mainly triggered by the PtIns-turnover signal system (2,3), it was assumed that LDL induces these intracellular changes via activation of the PtIns-turnover mediated by the specific LDL receptor. Our results, however, document that the LDL induced elevation of $[Ca^{2+}]_i$ and the biphasic change of pH_i in VSMC from rat aorta and human fibroblasts are not mediated by the classical LDL receptor via stimulation of the PtIns-turnover signal system.

This conclusion is well documented by the following observations. Preincubation of the VSMC cultures with monoclonal antibodies (IgGC7) against specific surface LDL receptor (7) could not abolish or attenuate the intracellular effects of LDL. These results gave reason for the assumption that the LDL-induced elevation of $[Ca^{2+}]_i$ and pH_i are not triggered by the specific LDL receptor. To further examine this assumption, the effect of LDL on the formation of the PtIns-turnover metabolites $InsP_1$, $InsP_2$, and $InsP_3$, was investigated.The metabolits were separated by HPLC method (13). However, stimulation of the cells with LDL caused no inositolpolyphoshates formation, whereas both PDGF and AII caused an up to 5-fold and an up to 25-fold increase of $InsP_3$ and $InsP_2$ formation, respectively. Our results are thus in contrast with those recently reported (2), showing that 30 μg/ml LDL induced an 50% net increase of both InsP1 and $InsP_2$, and a 100% increase of $InsP_3$ in VSMC within 10 minutes. Compared to the strong effects of classical vasoactive agonists on inositol phosphate formation, these increases appears to be of less importance triggering cellular responses. In another study, LDL-induced increase of $InsP_3$ was reported (3). A comparison of the present data with these findings is difficult since only $InsP_3$ formation was measured. The differences in the results of our study with that of others (2,3) may reflect VSMC differences, depending e.g on the isolation procedure, strain and age of the animal, cultivation conditions and number of passages. Another possible mechanism would be the much higher sensitivity of the HPLC

method in separating inositolphosphates (15) compared to the customary method (13) using Dowex columns. It is thus conceivable to find different reports about the effect of LDL on PtIns-turnover.

The decisive proove that LDL-induced intracellular changes are not mediated by the classical LDL receptor is given by experiments with normal and LDL receptor negative fibroblasts. In these experiments, similar LDL-induced changes of $[Ca^{2+}]_i$ and pH_i in normal and receptor negative fibroblasts were observed, indicating that the observed LDL-induced intracellular changes are independent of the specific LDL receptor. Furthermore, LDL had again no effect on PtIns-turnover in both cell types.

Recently, we showed that LDL induced an approximately 2-fold increase of the $^{45}Ca^{2+}$ influx compared to that in unstimulated cells (16). It thus can be assumed that LDL stimulates Ca^{2+} channels directly or indirectly via opening non-specific cation channels, which induce depolarization of the membrane and thus triggers the activity of Ca^{2+} channels, leading to an elevation of $[Ca^{2+}]_i$. An indirect mechanism is documented by endhothelin (17). LDL-induced mobilization of Ca^{2+} from intracellular stores may be brought about by an up to now unknown mediator. This assumption is based on reports indicating that except of Ins-1,4,5-trisphosphate, GTP is also able to mobilize Ca^{2+} from intracellular stores (18-19). According to another report (19), indicating that a caffein-induced influx of extracellular Ca^{2+} mobilized Ca^{2+} from intracellular stores in VSMC, it can be assumed that the LDL-induced mobilization of Ca^{2+} from intracellular stores may be triggered by a similar mechanism.

In conclusion our findings suggest that LDL, apart from its physiological function as a cholesterol transport molecule, can also contribute to the pathogenesis of cardiovascular diseases by elevating $[Ca^{2+}]_i$ and pH_i without involving the specific LDL receptor.

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