Novel Cellular Activities for Low Density Lipoprotein in Vascular Smooth Muscle Cells

Agapios Sachinidis, Thomas Mengden, Rudolf Locher, Claudia Brunner, and Wilhelm Vetter

Hyperlipidemia and hypertension play important roles in the pathogenesis of atherosclerosis. To investigate the underlying intracellular mechanisms, we studied the effect of various concentrations of low density lipoprotein from normolipidemic subjects on concentrations of free intracellular calcium, intracellular pH, DNA synthesis, and vascular tone in vascular smooth muscle cells and rings from rat aortas. Low density lipoprotein in the range of 1–15 \( \mu \)g/ml induced a dose-dependent increase of concentration of free intracellular calcium and a biphasic change of the intracellular pH. Similar concentrations of low density lipoprotein led to an enhanced DNA synthesis. Furthermore, cumulative addition of 1–15 \( \mu \)g/ml low density lipoprotein produced a dose-dependent increase in contractile tension of thoracic aortic rings from rats. The maximal low density lipoprotein–induced contractile response was approximately 70% of that induced by 40 mM KCl. These findings indicate that low concentrations of low density lipoprotein occurring, for example, in the extravascular fluid might contribute to the pathogenesis of cardiovascular diseases by enhancing cell proliferation and vasoconstriction by changing intracellular calcium and intracellular pH. (Hypertension 1990;15:704–711)

Hypertension and elevated levels of low density lipoprotein (LDL) cholesterol are two of the most important risk factors for atherosclerosis and cardiovascular morbidity.\(^1\)\(^2\) Hypertension accelerates the development of atherosclerotic lesions and increases their severity as revealed in several animal studies.\(^3\)\(^4\) Apart from their cumulative effect of causing coronary heart disease, a correlation between blood pressure and serum cholesterol levels was observed in the Framingham study, suggesting that those patients with higher blood pressure values tend to have higher cholesterol levels.\(^1\) 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 cholesterol homeostasis,\(^5\)\(^6\) LDL was recently shown to cause a general cellular activation and enhance proliferation of vascular smooth muscle cells (VSMC).\(^7\)\(^8\)\(^9\) VSMC play a key pathophysiological role in both hypertension and atherosclerosis.\(^10\)\(^11\) It is well known that vasoactive polyopeptides (e.g., angiotensin II or platelet-derived growth factor) are involved in the development of these cardiovascular diseases by enhancing contractility and proliferation in VSMC. Proliferation and cell contractility are probably triggered by changes of the intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) and intracellular pH (pH\(_i\)).\(^12\)\(^13\) To elucidate cellular mechanisms by which LDL could be involved in the development of cardiovascular diseases, the influence of LDL on cellular parameters such as [Ca\(^{2+}\)]\(_i\), pH\(_i\), and DNA synthesis was studied in cultured VSMC from the rat aorta. Additionally, the influence of LDL on vascular tone in rat aortic rings was examined.

Methods

Materials

Fura 2–pentaacetoxymethyl ester (fura 2-AM) and [2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein] pentaacetoxymethyl ester (BCECF-AM) were obtained from Calbiochem (Zürich, Switzerland). Verapamil-HCl was a gift from Knoll AG (Zürich, Switzerland) and Dulbecco's modified Eagle's medium (DMEM), Ham's F-10, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Aniwed (Zürich, Switzerland). EGTA, HEPES, and EDTA were from Merck-Schuchardt (Zürich, Switzerland). Tris(hydroxymethyl)aminomethane (Tris-base) and other chemicals were obtained from Sigma Chemical Co. (Zürich, Switzerland).

LDL (density 1.019–1.063 g/ml) was isolated from the plasma of eight individual normocholesteremic
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subjects (serum cholesterol <6.2 mmol/l) by ultra-centrifugation according to Redgrave et al. 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 ascorbic acid throughout. LDL was stored at 4°C and used within 3 weeks; no changes in activity were observed during this period. Determination of protein was performed by Lowry's method.

VSMC were isolated from rat aorta (female, Wistar-Kyoto strain, 6–8 weeks old) and cultured over several passages according to Ross. The cells were allowed to grow for 4–5 days in 5% CO2 and 95% air at 37°C. The culture medium was DMEM supplemented with 10% fetal calf serum.

For the measurement of [Ca2+]i, confluent cells were detached with a solution containing (mM) HEPES 20, glucose 16, NaCl 130, MgSO4·7H2O 1, CaCl2 0.5, and Tris-base pH 7.4 (HEPES buffer) supplemented with collagenase, soybean trypsin inhibitor, and bovine serum albumin (0.1:0.1:0.3 ml/vol/vol) after 20 minutes at 37°C. Then, cells were incubated with 2 μM fura 2-AM at 37°C for 20 minutes in HEPES buffer. After loading, cells were washed and suspended in HEPES buffer (approximately 2x10⁶ cells/ml). The Ca²⁺-fura 2 fluorescence was measured at 37°C under stirring in an SLM-Amino SPF-500 spectrofluorometer (SLM Instruments, Inc., SLM-Amino, Urbana, Illinois) at excitation wavelengths 340 and 380 nm and at emission wavelength 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 MnCl₂ for minimum fluorescence according to Grynkiewicz et al. The calcium-influx experiments were performed with cells grown in 35-mm culture dishes according to the method of Smith et al. After cells reached confluence, the culture medium was removed by aspiration, and the VSMC were rinsed twice at 37°C with PBS containing 1 mM CaCl₂, 1 mM MgCl₂, 10% trichloroacetic acid, and ethanol/ether (2:1, vol/vol). Phase-contrast microscopy was used to inspect the dishes for evidence of cell detachment or changes in cell morphology. Acid-insoluble [³H]thymidine was extracted into 1 ml/dish 0.5 M NaOH, and 0.2 ml of this solution was mixed with 5 ml scintillator and quantified by using a Packard Instrument liquid scintillation counter Model Tri-Carb 300C (Zürich, Switzerland). Then, 0.1 ml of the residual solution was prepared for the determination of protein by Lowry's method.

The measurements of the aortic ring contractile responses were performed according to Marriot. Three-millimeter-long rings of thoracic aorta from female Wistar-Kyoto rats (300 g) were mounted under resting tension of 3.5 g in a Krebs-Ringer solution of the following composition (mM): NaCl 118.4, KCl 4.75, CaCl₂ 2.5, MgSO₄·7H₂O 1.18, KH₂PO₄ 1.19, NaHCO₃ 25, glucose 11.66, ascorbic acid 0.05, and EDTA 0.01, maintained at 37°C and aerated with 5% CO₂ in O₂. Contractile responses of aortic rings to LDL were also performed under Ca²⁺-free conditions by addition of 1 mM EGTA to Krebs-Ringer solution from which Ca²⁺ had been omitted. Additionally, similar measurements were performed after incubation of the rings with 10 μM of the calcium entry blocker verapamil for 30 minutes. The rings from the aortas had intact endothelium, which was assessed by vasodilation when challenged with 1 μM acetylcholine. The rings were equilibrated for 1 hour before experimentation, and during this period, the bathing fluid was changed every 20 minutes. The contractile response of the rings was measured isotonically in a...
Schuler Organ Bath with a transducer type B 368 (Hugo Sachs Elektronik KG, March-Hugstetten, FRG) after cumulative administration of LDL. The LDL-induced tension response of each aortic ring was expressed as the percentage of its maximum response to 40 mM KCl-Krebs-Ringer solution.

Results

Effect of Low Density Lipoprotein on [Ca\(^{2+}\)]\(_i\), and pH,

As depicted in Figure 1 (panel A, upper recording), LDL 7 μg/ml isolated from one donor induced in the presence of calcium a rapid increase in [Ca\(^{2+}\)]\(_i\) of 240 nM (basal value, 118 nM) with a peak value at 15 seconds. The increase of LDL-induced stimulation of [Ca\(^{2+}\)]\(_i\), declined after this peak toward the resting level within 1.5 minutes. Figure 1 (panel A, lower recording) shows that LDL also stimulated a rapid increase of [Ca\(^{2+}\)] in the Ca\(^{2+}\)-free 1 mM EGTA buffer. This increase, however, was less pronounced than in the presence of Ca\(^{2+}\). Furthermore, the effect of different concentrations of LDL from seven normocholesterolemic donors on [Ca\(^{2+}\)] was investigated. Figure 1 (panel B) shows the concentration-response curve for the LDL-induced maximal increase of [Ca\(^{2+}\)] at 15 seconds. LDL concentrations in the range of 1–7 μg/ml caused a dose-dependent increase of [Ca\(^{2+}\)]. LDL concentrations higher than 7 μg/ml caused only a minor further increase of [Ca\(^{2+}\)]. No differences were observed in the LDL-induced increase of [Ca\(^{2+}\)], when LDL was taken from different donors (variation coefficients for each LDL dose were calculated to be approximately 5–6%).

Figure 2 shows the effect of both 7 μg/ml LDL and K\(^+\) buffer on \(^{45}\)Ca\(^{2+}\) influx measured at 2 minutes. LDL and K\(^+\) buffer caused an approximate 1.8-fold and twofold increase in \(^{45}\)Ca\(^{2+}\) influx, respectively, compared with that found with normal transport buffer.
Incorporation Into DNA

Effect of Low Density Lipoprotein on $[^3H]$Thymidine

...whether the alkalinization phase occurred by activation of the Na$^+$-H$^+$ antiport, experiments were performed in triplicate determinations. Each column represents the mean±SD of one representative experiment from four independent experiments performed in replicate determinations.

Figure 3 (upper recording) illustrates the effect of LDL on pH$_i$ in VSMC. Addition of 7 µg/ml LDL in the presence of Na$^+$ exerted a biphasic effect on pH$_i$ (basal value, 7.03±0.03 [mean±SD], n=12). Initially, a rapid acidification of 0.04±0.015 pH units (n=4) occurred followed by a subsequent prolonged alkalinization of 0.12±0.04 pH units. To investigate whether the alkalinization phase occurred by activation of the Na$^+$-H$^+$ antiport, experiments were performed in the presence of 30 µM DMA, a potent inhibitor of the Na$^+$-H$^+$ antiport (Figure 3, lower recording). DMA at a concentration of 30 µM produced a gradual but sustained decline in pH$_i$. Within 2 minutes after DMA exposure, pH$_i$ values declined by 0.15±0.02 pH units (n=5). Subsequent addition of LDL caused a further decline in pH$_i$. The alkalization effect of LDL in the presence of 30 µM DMA on VSMC was strongly attenuated.

**Effect of Low Density Lipoprotein on $[^3H]$Thymidine Incorporation Into DNA**

One of three representative experiments performed in quadruplicate of the $[^3H]$thymidine incorporation into DNA is illustrated in Table 1. LDL concentrations in the range of 1–5 µg/ml had a pronounced dose-dependent mitogenic effect on VSMC, shown by an increase of $[^3H]$thymidine incorporation from 176±19 (basal value±SD) to 298±31 cpmp/µg protein. This increase corresponds to a 69% stimulation of $[^3H]$thymidine incorporation into DNA by 3 µg/ml LDL. Further augmentations of LDL concentrations did not exert a further proliferative effect of LDL on VSMC.

**Effect of Low Density Lipoprotein on Vascular Tone of Rat Aortic Rings**

Cumulative addition of 1–7 µg/ml LDL to the muscle bath produced a fast concentration-related contractile response of the aortic ring with intact endothelium (Figure 4, recording A). The calcium channel blocker verapamil (10 µM) induced a 23±8.7% (mean±SD, n=5) decrease of the contractile response induced by 7 µg/ml LDL. Figure 4 (recording B) shows the contractile effect of 7 µg/ml LDL on aortic rings in Ca$^{2+}$-free 1 mM EGTA-Krebs-Ringer solution. In the absence of extracellular Ca$^{2+}$, LDL also induced a contractile response, which was weaker, however, than that generated in the presence of Ca$^{2+}$ (Figure 4, recording B). Figure 4 (recording C) shows the contractile tension of the aortic ring caused by 40 mM KCl–Krebs-Ringer solution. Data of the individual contractile responses induced by different LDL concentrations and expressed as percentages of the maximum response to 40 mM KCl–Krebs-Ringer solution are summarized in Figure 5. Half-maximal effect of LDL (EC$_{50}$) was estimated to be 2.2±0.5 µg/ml (n=6).

**Discussion**

Although epidemiological studies have shown a powerful interaction between hypertension and elevated levels of LDL-cholesterol to cause coronary heart disease, little is known about the pathophysiological mechanisms underlying the higher incidence of atherosclerotic lesions in hypertension. Yet, the atherogenic role of LDL is well established and, therefore, it seemed interesting to investigate the intracellular effects of LDL in VSMC.

Our results show that LDL, at concentrations much lower than those physiologically occurring in blood (85–130 mg/100 ml), causes a concentration-dependent increase of [Ca$^{2+}$] in VSMC, which partly is derived from intracellular stores. Additionally, LDL exerts a biphasic change of pH$_i$, in VSMC, an early rapid acidification and a slower Na$^+$-dependent alkalinization. Furthermore, our results document for the first time that low concentrations of LDL occurring, for example, in the extravascular fluid can cause a concentration-dependent contractile response of aortic rings and enhance DNA synthesis rate in VSMC from rats.

The finding that the dose-dependent increase of [Ca$^{2+}$], also occurred in the Ca$^{2+}$-free medium indicates that Ca$^{2+}$ can be partly released from intracellular stores. Because intracellular Ca$^{2+}$ mobilization is thought to be triggered by inositol trisphosphate, a degradation product from the phosphoinositol turnover, it might be concluded that LDL-mediated calcium release occurs because of the activation of phosphoinositol turnover. Activation of both phosphoinositol turnover and Ca$^{2+}$ release by LDL was reported in human platelets. Because changes in the cytosolic free-Ca$^{2+}$ concentration in VSMC triggered by hormones and growth factors act as impor-
Hypertension Vol 15, No 6, Part 2, June 1990

T=" 6.9-
Q. 7.1-
7.0-
6.9-
6.8-
30 |iM Dimethylamiloride
LDL, 7 ng/ml
0 2 4 6 8 10
Time (min)
12 14
FIGURE 3. Plottings of effect of low density lipoprotein (LDL) on intracellular pH (pHi) in cultured vascular smooth muscle cells (VSMC). Upper recording, LDL (7 μg/ml) was applied to the loaded VSMC and pHi was measured as described in Methods. Lower recording, the same experiment in the presence of 30 μM dimethylamiloride (DMA). Arrows indicate addition of LDL and DMA, respectively.

The intracellular signaling mechanisms for various cellular processes, low concentrations of LDL might thus influence cellular responses like contraction and cell proliferation.

Influx experiments show that LDL caused an approximately 1.8-fold increase of 45Ca2+ influx, compared with the value in the absence of LDL. 45Ca2+ influx from extracellular environment was described as activated either by voltage-operated (e.g., K+ buffer) or receptor-operated channels activated by agonist-receptor combination.

In parallel with changes of [Ca2+]i, a biphasic shift of the pHi was observed on stimulation of VSMC with LDL. An early rapid acidification phase preceded a slower phase involving Na+-dependent alkalinization. Our observation that the alkalinization phase was strongly inhibited in the presence of dimethylamiloride, an effective inhibitor of the Na+-influx/H+-efflux exchanger, indicates that the LDL-mediated cytosolic alkalinization occurs by stimulation of the Na+-influx/H+-efflux exchanger.

The cellular acidification of quiescent VSMC after addition of 30 μM DMA could have resulted from 1) a rudimentary Na+-H+-antiport activity, 2) an increase in cytosolic free Ca2+ and, consequently, activation of the Ca2+-ATPase system, or 3) other yet unknown effects related to cellular acid-base homeostasis.

Chromosomal DNA synthesis caused up to 69% from various stimuli from hormones and growth factors have been attributed to this pH shift. The exertion of a biphasic pH change in VSMC is a typical property of vasoactive hormones and several growth factors. From the observation that agonist-mediated calcium mobilization in VSMC is associated with the early acidification phase, it might be concluded that the activation of the Na+-influx/H+-efflux exchanger is possibly regulated by [Ca2+]i.

Low concentrations of LDL (3 μg/ml) enhanced DNA synthesis up to 69%. Higher doses of LDL failed to further increase DNA synthesis. These findings are in accordance with the results of Oikawa.

<table>
<thead>
<tr>
<th>LDL (μg/ml)</th>
<th>cpm/μg protein</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>176±19</td>
<td>0±11</td>
</tr>
<tr>
<td>1</td>
<td>221±21</td>
<td>26±9</td>
</tr>
<tr>
<td>3</td>
<td>298±31</td>
<td>69±10</td>
</tr>
<tr>
<td>7</td>
<td>279±28</td>
<td>59±10</td>
</tr>
<tr>
<td>15</td>
<td>268±24</td>
<td>52±10</td>
</tr>
</tbody>
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Values represent mean±SD of one representative experiment from four independent experiments performed in quadruplicate.
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FIGURE 4. Plottings of low density lipoprotein (LDL)-stimulated contractile responses of rat aorta rings. The contractile response of the rings was monitored isotonically as described in Methods. Recording a): Contractile response of rat aorta rings in Krebs-Ringer solution containing 2.5 mM CaCl₂ to 1, 3, 7, and 15 µg/ml LDL. Recording b): Contractile response in the absence of external Ca²⁺, achieved by Ca²⁺-free Krebs-Ringer solution, containing 1 mM EGTA. Recording c): Contractile response to 40 mM KCl-Krebs-Ringer solution.

FIGURE 5. Plotting showing summarization of the data of the individual contractile responses of aortic rings with intact endothelium, induced by different low density lipoprotein concentrations, expressed as percentages of its maximum responses to 40 mM KCl-Krebs-Ringer solution. Each experimental value is the mean ± SD of five separate individual experiments.

et al., who reported that 25 µg/ml LDL caused an increased DNA synthesis in human arterial smooth muscle cells. Similar results were obtained by Fischer-Dzoga on monkey aortic smooth muscle cells.

LDL-induced contraction of rat aortic rings was maximal in the presence of extracellular calcium in the external medium. This contraction, however, has been found to be attenuated in the presence of EGTA and, therefore, in the absence of calcium in the external medium although maximal doses of LDL have been used. This finding indicates that maximal contraction occurs only in the presence of extracellular calcium. This conclusion is further supported by experiments in which a decrease by about 23% of the 7 µg/ml LDL-induced contraction in the presence of the calcium entry blocker verapamil was observed. Thus, the extent of the contraction is, at least in part, dependent on the influx of calcium from the external medium into the VSMC. These findings are consistent with the results of measurements of [Ca²⁺]ₐ, performed in the presence or absence of extracellular calcium in which a reduced increase of [Ca²⁺] was also observed in calcium-free EGTA medium.

Thus, our findings suggest that LDL, apart from its physiological function as a cholesterol transport molecule, can trigger cellular events in VSMC, which are normally triggered by vasoactive hormones. Care was
taken in both isolation and storage of LDL to minimize its oxidation, and thus formation of peroxides can be excluded.

We, therefore, tend to support the assumption that LDL can partly act as a vasoactive hormone. This assumption is supported by the fact that low concentrations of LDL induce vascular contraction in vitro. Although the real concentrations of LDL in the extracellular fluid are unknown, experiments on the LDL clearance in several tissues indicate that LDL can occur in the extracellular space.\textsuperscript{30,31} The relatively low in vitro concentrations of LDL triggering the aforementioned cellular events probably correspond to in vivo concentrations of LDL in extracellular fluids, which are much lower than in plasma because of the diffusion barrier offered by vascular endothelium. Additionally, there are fluctuations in LDL concentrations caused by dietary,\textsuperscript{32} physiological,\textsuperscript{33} and pharmacological manipulation.\textsuperscript{34} Additionally, binding and uptake of LDL have been observed after incubation of cultured rat VSMC with 10 \( \mu \)g/ml of homologous LDL.\textsuperscript{35,36}

Our findings do not allow us to draw a conclusion on whether the observed intracellular changes are triggered by other up-to-now unknown mechanisms that are independent from the classic LDL receptor pathway. An alternative pathway to the classic pathway could be that LDL directly or indirectly stimulates calcium channels leading to an elevation of free \([Ca^{2+}]_i\). This possibility is supported by our findings showing that LDL induced an approximate twofold increase of the \([Ca^{2+}]_i\) influx.

From our findings, the question arose whether putative artificial LDL-adherent factors were causing the observed intracellular changes. Although in a recent study similar LDL effects in human VSMC were reported,\textsuperscript{37} nondialyzable factors cannot be excluded. It could thus be that these putative factors only in association with the LDL particle have stimulatory effects.

Because both systems, elevation of \([Ca^{2+}]_i\), and cytosolic alkalization in VSMC, are thought to be signals for vasoconstriction and cell proliferation, we conclude that LDL at low concentrations, for example as occurs in the extracellular fluid, can act as a vasoconstriction and growth-promoting factor and thereby contribute to the pathogenesis of cardiovascular diseases by enhancing vasoconstriction and cell proliferation by changing \([Ca^{2+}]_i\), and pH.\textsubscript{c}

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