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 μg/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 μg/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)
subjects (serum cholesterol <6.2 mmol/l) by ultracentrifugation 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 time 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 U/ml) (vol/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-Aminco SPF-500 spectrofluorometer (SLM Instruments, Inc., SLM-Aminco, 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. The resting tension of 3.5g 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 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\(^{2+}\)] and pH.

As depicted in Figure 1 (panel A, upper recording), LDL 7\(\mu\)g/ml isolated from one donor induced in the presence of calcium a rapid increase in [Ca\(^{2+}\)], of 240 nM (basal value, 118 nM) with a peak value at 15 seconds. The increase of LDL-induced stimulation of [Ca\(^{2+}\)], 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\(\mu\)g/ml caused a dose-dependent increase of [Ca\(^{2+}\)]. LDL concentrations higher than 7\(\mu\)g/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\(\mu\)g/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.
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 Ca2+-free 1 mM EGTA–Krebs-Ringer solution. In the absence of extracellular Ca2+, LDL also induced a contractile response, which was weaker, however, than that generated in the presence of Ca2+ (Figure 4, recording B). Figure 4 (recording C) shows the contractile tension of the aortic ring caused by 40 mM KCl–Krebs-Ringer solution. In the absence of extracellular Ca2+, LDL also induced a contractile response, expressed as percentages of the maximum response to 40 mM KCl–Krebs-Ringer solution are summarized in Figure 5. Half-maximal effect of LDL (EC50) 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,1 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 [Ca2+]i in VSMC, which partly is derived from intracellular stores. Additionally, LDL exerts a biphasic change of pH, 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 [Ca2+], also occurred in the Ca2+-free medium indicates that Ca2+ can be partly released from intracellular stores. Because intracellular Ca2+ mobilization is thought to be triggered by inositol trisphosphate, a degradation product from the phosphoinositol turnover,23 it might be concluded that LDL-mediated calcium release occurs because of the activation of phosphoinositol turnover. Activation of both phosphoinositol turnover and Ca2+ release by LDL was reported in human platelets.24 Because changes in the cytosolic free-Ca2+ concentration in VSMC triggered by hormones and growth factors act as impor-
tient 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 $^{45}$Ca$^{2+}$ influx, compared with the value in the absence of LDL. $^{45}$Ca$^{2+}$ 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 [Ca$^{2+}$], a biphasic shift of the pH$_i$ 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 Ca$^{2+}$ and, consequently, activation of the Ca$^{2+}$-ATPase system, or 3) other yet unknown effects related to cellular acid-base homeostasis.

Changes in cellular behavior such as enhanced DNA synthesis after various stimuli from hormones and growth factors have been attributed to this pH$_i$ shift. The exertion of a biphasic pH$_i$ 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 [Ca$^{2+}$]. Based on these reports, it can be assumed that activation of the Na$^+$-influx/H$^+$-efflux exchanger occurs as a consequence of the LDL-stimulated increase of [Ca$^{2+}$].

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

![Graph](http://hyper.ahajournals.org/)

**FIGURE 3.** Plotting of effect of low density lipoprotein (LDL) on intracellular pH (pH$_i$) in cultured vascular smooth muscle cells (VSMC). Upper recording, LDL (7 μg/ml) was applied to the loaded VSMC and pH$_i$ 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.

**TABLE 1.** Effect of 1–15 μg/ml Low Density Lipoprotein on $[^3]$HThymidine Incorporation Into DNA of Vascular Smooth Muscle Cells

<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>
</table>

Values represent mean±SD of one representative experiment from four independent experiments performed in quadruplicate.
Effects of Low Density Lipoprotein

FIGURE 4. Plotting 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.

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

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⁺], performed in the presence or absence of extracellular calcium in which a reduced increase of [Ca²⁺] was also observed in calcium-free EGTA medium.

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.
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 actual 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.\(^{30,31}\) 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,\(^{32}\) physiological,\(^{33}\) and pharmacological manipulation.\(^{34}\) Additionally, binding and uptake of LDL have been observed after incubation of cultured rat VSMC with 10 \(\mu g/ml\) of homologous LDL.\(^{35,36}\)

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 \(45Ca^{2+}\) 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,\(^37\) 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 alkalinization 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 vasoconstrictive 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 \(pH_4\).

Acknowledgment

We are particularly indebted to Dr. Bruno Marti noni, Institute of Organic Chemistry, ETH-Zürich, Switzerland, for dimethylamiloride synthesis.

References

12. Rasmussen H, Barret PO: Calcium messenger system, an integrated view. \(Physiol Rev\) 1984;64:938–984
15. Lowry OH, Rosebrough NH, Farr AL, Randall RJ: Protein measurements with the folin phenol reagent. \(J Biol Chem\) 1951;193:265–275


31. Spady DK, Bilheimer DW, Dietzchy JM: Rates of receptor-dependent and independent low density lipoprotein uptake in the hamster. *Proc Natl Acad Sci USA* 1985;80:3499–3503


**KEY WORDS** • lipoproteins, LDL • vascular smooth muscle • vasoconstriction
Novel cellular activities for low density lipoprotein in vascular smooth muscle cells.
A Sachinidis, T Mengden, R Locher, C Brunner and W Vetter

Hypertension. 1990;15:704-711
doi: 10.1161/01.HYP.15.6.704

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/15/6_Pt_2/704

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/