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VASOCONSTRICTION: A NOVEL ACTIVITY FOR LOW DENSITY LIPOPROTEIN

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SUMMARY Low density lipoprotein plays an important role in the pathogenesis of atherosclerosis. Cumulative addition of 1-30 µg/ml of LDL from normolipidemic subjects produced a dose-dependent increase in contractile tension of thoracic aortic rings from rats. The maximal LDL-induced contractile response was approximately 30 % of that induced by 1 µM norepinephrine. Similar concentrations of LDL induced a dose-dependent transient increase of the concentration of intracellular free calcium, and a biphasic change of the intracellular pH in cultured rat vascular smooth muscle cells.

We conclude that low density lipoprotein occuring for example in the extravascular fluid can mediate vasoconstriction by changes in cytosolic calcium and intracellular pH.

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 receptor and by being internalized by pinocytosis (1).

Besides its physiological role it has recently been reported that LDL, at concentrations corresponding to those of hormones, can cause general cellular activation by stimulation of the phosphoinositol (PI) signaling system in various cell types like platelets or arterial smooth muscle cells (2,3). In this context it should be considered that LDL is found not only in plasma but also in the extravascular fluids, where it is present at much lower concentrations owing to the diffusion barrier offert by the endothelium (4,5). As metabolites of PI-turnover are assumed to trigger cellular processes related with vasoconstriction (6-8), we studied the effect of LDL from normolipidemic subjects on the contractile response of aortic rings from the rat. To elucidate the cellular mechanisms by which LDL induced vascular contractile response, the effect of LDL on intracellular Ca^{2+} $(Ca^{2+})_i$ and pH (pH_i) in vascular smooth muscle cells (VSMC) from rat aorta was investigated.

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. (9). The LDL fraction was dialyzed against 0.15 M NaCl/1 mM EDTA, pH 7.4 and used within 3 weeks. Oxidation of LDL has been prevented by adding 50 µM of ascorbic acid throughout. LDL

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was stored at 4 °C and used within 3 weeks; no changes in activity were observed during this time period.

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, and Ross and Glomset (10,11). 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 medium supplemented with 10% fetal calf serum.

Aortic Ring Contractile Responses. These measurements were performed according to Marriot et al. (12). 3 mm long rings of thoracic aorta from female Wistar-Kyoto rats (300g) were mounted under resting tension of 3.5 g in a Krebs solution of the following composition (in mM): NaCl 118.4, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.18, KH_2PO_4 1.19, $NaHCO_3$ 25, glucose 11.66, ascorbic acid 0.05 and EDTA 0.01, maintained at 37 °C and gased with 5% CO_2 in O_2. Contractile responses to LDL of aortic rings were also performed under Ca^{2+}-free conditions by addition of 1 mM EGTA to Krebs solution from which Ca^{2+} had been omitted. In addition, similar measurements were performed following incubation of the rings with 10 µM of the calcium entry blocker verapamil for 30 min. The rings from aorta had intact endothelium, which was assesed by vasodilatation when challenged with 1 µM acetylcholine.
The rings were equilibrated for 1 h before experimentation and during this period the bathing fluid was changed every 20 minutes. The contractile response of the rings was measured isotonically with a tranducer (Lever transducer type B 368, Hugo Sachs electronic) after cumulative administration of LDL. The LDL-induced tension response of each aortic ring was expressed as the percent of its maximum response to 15 µg/ml LDL.

Measurement of free intracellular $Ca^{2+}[(Ca^{2+})_i]$. Confluent cells were detached with HEPES-buffer solution (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 collagenase, soybean trypsin inhibitor and bovine serum albumin (0.1:0.1:0.3 mg/ml) after 20 minutes at 37 °C. Then cells were incubated with 2 µM fura-2 pentaacetoxymethyl ester at 37 °C for 20 minutes in HEPES-buffer. 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. (13).

Measurement of pH_i. These measurements were performed according to Berk et al.(14,15) 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 (14).

Materials. Fura 2/AM, pentaacetoxymethyl ester and BCECF/AM, [2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein] pentaacetoxymethyl ester were obtained from Calbiochem. Verapamil-HCl was a gift from Knoll AG, Switzerland, Dulbecco's modified Eagles medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline (PBS) were obtained from Amimed. Nutridoma-HU was from Boeringer Mannheim. Other chemicals were from Sigma and Merck-Schuchardt.

RESULTS

LDL induces rat aortic ring contraction. Fig.1a shows a representative experiment of seven individual experiments. Cumulative addition of 1-7 µg/ml LDL to the muscle bath produced a fast concentration-related contractile response of the aorta ring with intact endothelium. No further increase in the contractile response was observed with LDL concentrations of 15 and 30 µg/ml. Fig. 1b shows the contractile tension of the aorta ring

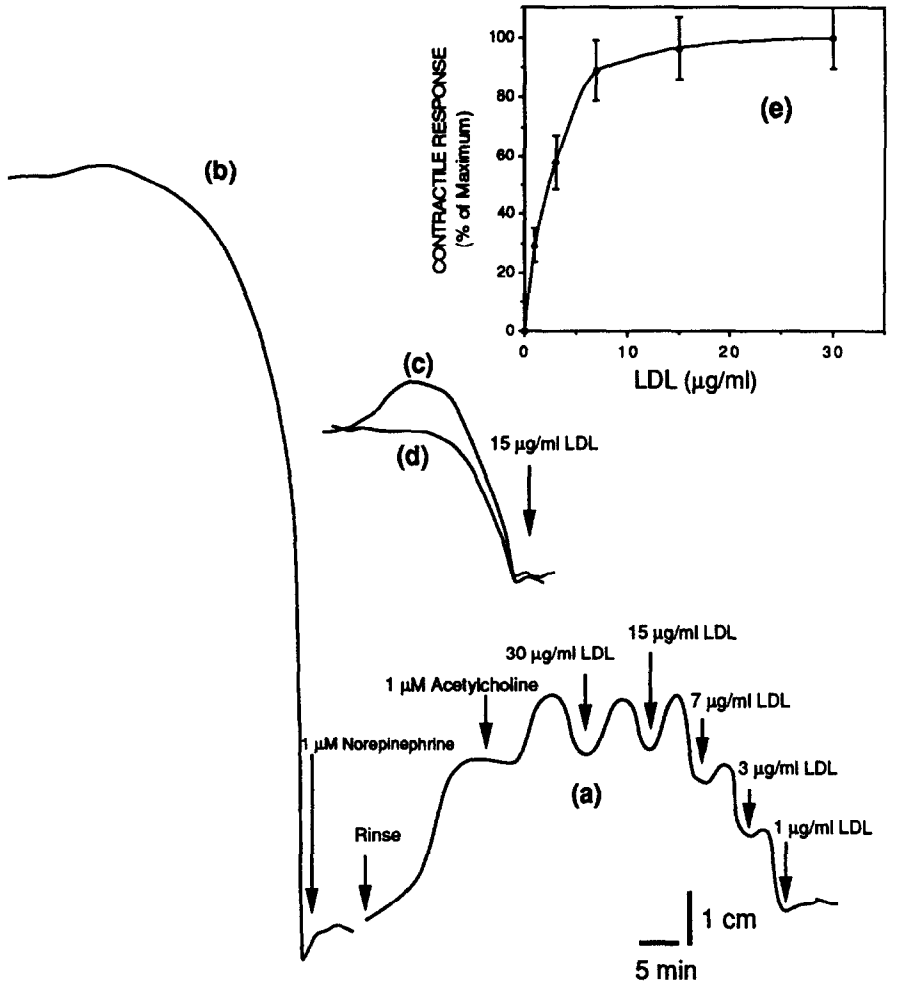

Fig 1. LDL-stimulated contractile responses of rat aorta rings. Contractile response of rat aorta rings in presence of 2.5 mM $CaCl_2$ to (a) 1, 3, 7, 15 and 30 µg/ml LDL, (b) to 1 µM norepinephrine,(c) to 15 µg/ml LDL in the presence of and (d) in the absence of external Ca^{2+}(1 mM EGTA, no added Ca^{2+}). (e) Dose-response curve for LDL-induced aortic contractile responses. Each experimental value is the mean ± SD of 7 separate determinations.

caused by 1 µM norepinephrine. Maximal contractile responses elicited by 15 µg/ml LDL represented 28 ± 5.1 (mean ± SD, n=7)% of the maximum responses elicited by 1 µM norepinephrine. Furthermore, as shown in fig. 1a, addition of 1 µM acetylcholine caused vasodilatation, since aorta rings had intact endothelium. For some experiments, endothelium was intentionally rubbed off, but no change in response to a maximal dose of LDL was observed. Fig 1d shows the contractile response of 15 µg/ml LDL on aorta rings in Ca^{2+}-free 1 mM EGTA Krebs solution. In the absence of extracellular Ca^{2+} LDL induced also a contractile response, which was however weaker than that generated in the presence of Ca^{2+} (Fig. 1c). Verapamil (10 µM) induced a 27 ± 8.7 % (mean ± SD, n=5) decrease of the contractile response induced by 15 µg/ml LDL (data not shown). Data of the individual contractile responses induced by different LDL concentrations are summarized in Fig. 1e. Half-maximal effect (EC50) was estimated to be 2.4 ± 0.6 µg/ml (n = 7).

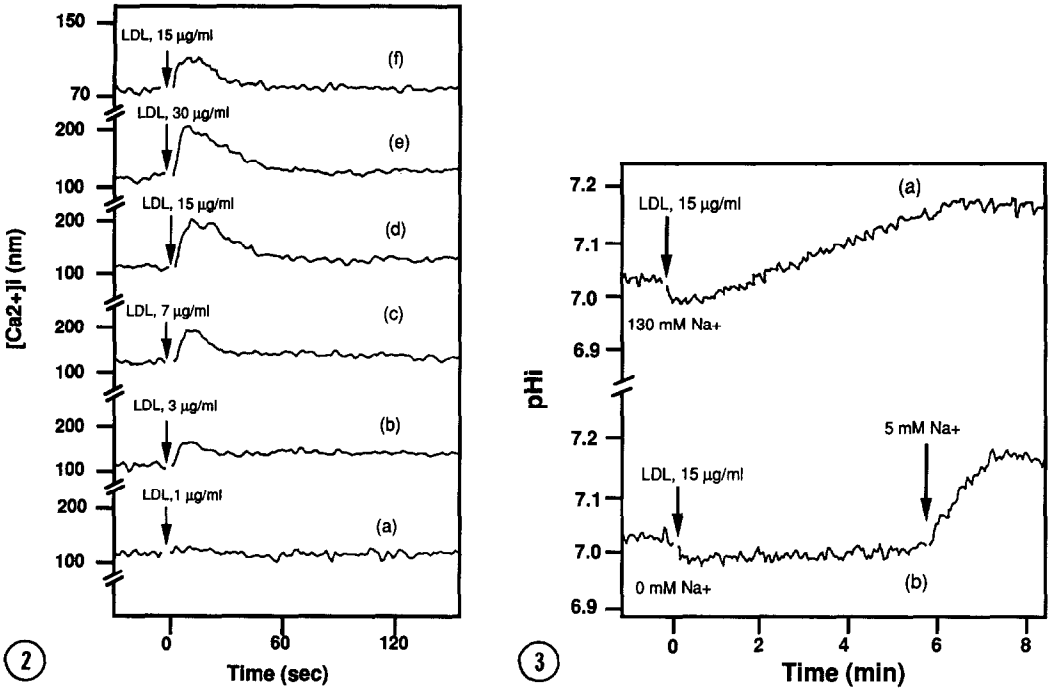

Fig. 2. Effect of various doses of LDL on $(Ca^{2+})_i$ in cultured rat VSMC. Effect of 1, 3, 7, 15, and 30 µg/ml LDL on $(Ca^{2+})_i$ in VSMC in the presence of 1 mM $CaCl_2$ (fig. 2a-2e), and of 15 µg/ml LDL in Ca^{2+}-free medium, achieved by 1 mM EGTA (fig. 2f). $(Ca^{2+})_i$ was measured by the fura 2 method as described in Methods. Arrows indicate addition of LDL.

Fig. 3. Effect of LDL on pH_i in cultured rat VSMC. Effect of 15 µg/ml LDL on pH_i in VSMC in presence of 130 mM Na^+(fig. 3a) or in the presence of 135 mM cholin$^+$ (fig. 3b) (Na^+ was isotonically replaced with cholin$^+$). VSMC were prepared and loaded with BCECF as described in Methods. Arrows indicate addition of LDL and Na^+, respectively.

LDL raises intracellular free Ca^{2+} in VSMC. As depicted in Fig. 2a-e, LDL in the presence of calcium induced a dose-dependent rapid rise in $(Ca^{2+})_i$ (basal value = 112 ± 11 nM, mean ± SD, n=45) 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 1.5 min. As shown in Fig. 2a, 1 µg/ml LDL had a slight effect on $(Ca^{2+})_i$. A small increase to 145 nM $(Ca^{2+})_i$ occured after addition of 3 µg/ml LDL (Fig. 2b). 15 µg/ml LDL induced a maximal rise to 195 nM $(Ca^{2+})_i$, which represents a net increase of 75% ± 13 (n=5) compared to the basal value (Fig. 2d). Increase of LDL concentration more than 15 µg/ml caused no further significant rise in $(Ca^{2+})_i$ (Fig. 2e). Fig. 2f 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 the cation.

LDL changes intracellular pH. Addition of 15 µg/ml LDL in the presence of Na^+ exerted a biphasic effect on pH_i (basal value = 7.03 ± 0.03, n = 15) (Fig. 3a). Initially a rapid acidification of 0.06 ± 0.02 (n=5) pH units occured, followed by a subsequent prolonged alkalinization of 0.15 ± 0.04 pH units. Fig. 3b illustrates the effect of LDL on pH_i in Na^+-free HEPES buffer in which Na^+ had been replaced isotonically by 135 mM choline$^+$. Under these conditions LDL caused a sustained acidification of 0.06 ± 0.02 pH units. Subsequent addition of

5 mM Na^+ caused a rapid alkalinization reaching finally the same pH_i of 7.18 as in the preceding experiments.

DISCUSSION

Our results document for the first time that LDL, at much lower concentrations (1 to 30 µg/ml) than those occuring physiologically in blood (3-4 x 10^3 µg/ml), can cause a concentration-dependent contractile response of aortic rings, an increase of $(Ca^{2+})_i$ and a biphasic change of pH_i in cultured VSMC from rats. The LDL-induced maximal contractility was about 30 % of that induced by 1 µM norepinephrine and was independent of endothelium.

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 28 % of the 15 µg/ml LDL-induced contraction in the presence of calcium entry blocker verapamil. Thus the extent of the contraction is, at least in part, due to influx of calcium from the external medium into the vascular smooth muscle cell. These findings are consistent with the results of measurements of $(Ca^{2+})_i$ performed in the presence or absence of extracellular calcium, in which also a reduced rise of $(Ca^{2+})_i$ was observed in calcium free EGTA medium. As in VSMC in Ca^{2+}-free milieu such a rise in $(Ca^{2+})_i$ is induced by the PI-system (16), it may be concluded that LDL acts on $(Ca^{2+})_i$ via activation of the PI-system. Stimulation of the PI-cycle by LDL has actually been reported in rat VSMC, human platelets and lymphocytes (2,3).

Vascular contractile response to LDL may be explained by two possible mechanisms:

1) LDL induces vascular contraction by directly increasing $(Ca^{2+})_i$ and changing pHi. This pathway is well known for vasoactive hormones like angiotensin II or PDGF (6, 17).

2) The LDL-induced vasoconstriction is indirect and mediated for example by other vasoactive hormones like prostagladins. This rare mechanisms has previously been described for epidermal growth factor (EGF), which may act via a prostagladine intermediate without increasing $(Ca^{2+})_i$ and changing pH_i (14,18). As in our study LDL caused both an increase in $(Ca^{2+})_i$ as well a pH_i-shift, in our opinion the first mechanism seems more likely to explain the underlying intracellular events of LDL-induced vasoconstriction.

In parallel to changes of $(Ca^{2+})_i$, a slow Na^+-dependent biphasic shift of the pH_i was observed upon stimulation of VSMC with LDL. This observation indicates, that the LDL-mediated cytosolic alkalinization occurs via stimulation of the Na^+influx/H^+efflux exchanger. Agonist-mediated calcium mobilization, associated with an early acidification phase has been reported in VSMC (15). Based on these reports it may be assumed that activation of the $Na^+_{influx}/H^+_{efflux}$ exchanger occurs as a consequence of the LDL-stimulated rise of $(Ca^{2+})_i$.

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 may act as a vasoactive hormone. This assumption is enhanced by the fact, that low concentrations of LDL induce contractile response of the vasculature in vitro. Although the real concentrations of LDL in the extracellular space of the vasculature are unknown, experiments on the LDL clearance in several tissues indicate that LDL can occur in the extracellular space (4,5). In addition, binding and uptake of LDL have been observed following incubation cultured rat VSMC with 10 μg/ml of homologous LDL (19).

We thus conclude that LDL may be involved in the pathogenesis of cardiovascular diseases by enhancing vasoconstriction via changing of $(Ca^{2+})_i$ and pH.

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