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THE INFLUENCE OF OXIDATIVELY MODIFIED LOW DENSITY LIPOPROTEIN ON PARAMETERS OF ENERGY METABOLISM AND CONTRACTILE FUNCTION OF ARTERIAL SMOOTH MUSCLE

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Recently published results provide evidence of the importance of oxidatively modified LDL in the development of atherosclerosis. Several typical characteristics of this disease can be ascribed to the effects of oxidized LDL on the different cells involved in lesion formation. In various cell culture systems oxidized LDL was found to be cytotoxic. Therefore we were interested in its influence on parameters of energy metabolism such as glycogen and ATP content as determined for aortic segments *in vitro*. The results show that oxidized LDL leads to sharp decreases in both parameters, indicating an activation of cellular energy metabolism. Findings obtained from contraction experiments in which oxidized LDL shows a contraction-enhancing effect on arterial segments suggest that the oxidized lipoprotein facilitates cellular Ca^{2+} liberation. This seems to be a common signal leading to its effects on energy metabolism and contraction and could also explain its cytotoxicity if cells are exposed to it for longer periods.

KEY WORDS: Arterial smooth muscle, ATP, contraction, cytotoxicity, glycogen, oxidatively modified LDL.

INTRODUCTION

Recent investigations support the hypothesis that oxidatively modified LDL (oxLDL) plays an important role in the development of atherosclerosis.[1] The relevant findings concern its rapid cellular uptake to form foam cells,[2] and its chemoattractant activity for circulating monocytes.[3] Furthermore, evidence was provided that LDL-oxidation occurs *in vivo*,[4] and modified forms of LDL were found in human plasma and in atheromas of rabbits.[5,6] The cytotoxicity of modified LDL[7] might be important in generating injuries of endothelial cells or, in advanced lesions, necrosis within the atheroma.[8]

In this respect, we were interested in the effects of oxidatively modified LDL on the metabolism of arterial smooth muscle. Since we have shown in an animal model of atherosclerosis that atheromatous necrosis is preceded by a decrease of cellular ATP levels,[8] we now determined the influence of oxLDL on parameters of energy metabolism in arterial smooth muscle *in vitro*. In order to gain some insight into the basic mechanisms, we additionally determined the effects on smooth muscle contraction as a physiological indicator of intracellular Ca^{2+} levels.

MATERIALS AND METHODS

Arterial segments of normally fed New Zealand rabbits (male, 2.5–3.5 kg body

weight) were used for the experiments. Effects on energy metabolism were determined in rings of thoracic aorta, while influences on contraction were measured in segments of carotid arteries. Dissection of tissue samples and the methods used have already been published.[8-10] In short, aortic intima-media preparations (0.5 cm length) were incubated in 100 ml of air-equilibrated physiological salt solution (PSS) (composition see[10]) for 30 min at 37° C in a shaking water bath. LDL or oxLDL (see below) was added in concentrations of up to 2 mg/ml (given in total mass/ml). In 3 experiments, the tissue samples were then incubated in PSS again for another 60 min. After that, the tissues were shock-frozen with metal clamps that were precooled in liquid N_2 and lyophilized. In the homogenates, glycogen was determined after enzymatic degradation by fluorimetric methods, ATP by the luciferase reaction.[8] The results were expressed in relation to the dry weight of the samples.

Technical equipment and the procedure for measuring alterations in the tone of arterial segments under isometric conditions have been described elsewhere.[9] The influence of LDL and oxLDL was determined either in relaxed segments of carotid artery or in segments costimulated with a KCl-enriched solution (KCl increased to 60 mM under equimolar reduction of NaCl in the salt solution). For stimulation, LDL was supplied to the continuously superfused vessels in a solution volume of 100 ml (PSS or 60 mM KCl, perfusion rate 10 ml/min). Alterations in the contractile force were recorded. Results were evaluated as force or increase in force, respectively, as induced by the lipoproteins in relaxed or costimulated segments.

The LDL used for oxidative modification was isolated from rabbit plasma by two step ultra-centrifugation (density 1.020–1.050 g/ml), without the additon of oxidation preventing agents. Oxidation was achieved as described by Esterbauer *et al.*[11] LDL was dialysed against the oxygenated solution in a concentration five-fold higher than the one finally used and thereafter appropriately diluted. OxLDL was characterized with agarose gel electrophoresis, which showed increased mobility of the modified form, as well as with the thiobarbituric acid reaction as described by Uchiyama and Mihara.[12] Malondialdehyde equivalents were found to range for 1.1–1.5 nmol/mg in the oxidized form.

For control experiments LDL was prepared under oxidation preventing conditions i.e. EDTA (2.5 mM) and butylated toluene (20 μM) were added during ultracentrifugation. Prior to use, this preparation was dialysed under nitrogen equilibration. The malondialdehyde content under these conditions was found to be in the range of 0.15–0.25 nmol/mg LDL, which is similar to that given by Esterbauer for native LDL.[11] This LDL will be designed as native LDL, the oxidized form as oxLDL.

RESULTS

The results of the incubation experiments with aortic rings are given in Table I. It is obvious that incubation in PSS for 30 min causes a decrease in ATP and glycogen. Addition of native LDL, prepared under oxidation preventing conditions, induces a slight but not significant decrease when compared with the controls. However, the administration of oxidatively modified LDL results in a strong decrease of glycogen and ATP content indicating a severe impairment of energy metabolism of the aortic wall. The results obtained with those tissue samples which were further incubated under normal conditions show a slow recovery of the tissue contents of ATP and glycogen. These alterations indicate at least a partial metabolic regeneration after

TABLE I

Influence of native LDL and oxLDL on ATP and glycogen content of intima media preparations from rabbit aorta during 30 min incubation.

	fresh tissue	incub. in PSS	incub. with native LDL	incub. with oxLDL	incub. with oxLDL +60 min reincub. in PSS (*)
ATP	2.2 ± 0.5	1.9 ± 0.6	1.6 ± 0.8	0.4 ± 0.3 (°)	0.8 ± 0.4
glycogen	12.9 ± 3.6	10.3 ± 2.5	9.5 ± 2.4	2.6 ± 1.9 (°)	3.2 ± 1.5

All values are given in μmol/g dw. (and represent mean values ± S.D., $n = 5$; (*)$n = 3$).Concentration of LDL (native and oxidized) was 2 mg/ml in each case.

(°) Difference to incubation with native LDL is significant with $p < 0.05$ (Student's t-test).

withdrawal of oxLDL. However, it is conceivable that the reversibility of the influence of oxLDL on energy metabolism depends on the duration of the cellular interaction.

Table II summarizes the effect of oxidized LDL as determined in the contraction experiments. It is obvious that high concentrations of the modified lipoprotein are able to induce contraction in relaxed arterial segments. The time course of the contraction response shows a slow increase in force development, reaching a maximum usually at the end of the perfusion time of 10 min. After withdrawal of oxLDL, active force gradually decreases to the initial tone of the relaxed vessel segments.

Repeatedly applied (3 times), oxLDL concentrations of 1 mg/ml evoked identical responses within the same segments, indicating the reversibility of the effects on smooth muscle contractility. Native LDL and rabbit serum albumin only mildly influence contraction in relaxed arterial vessels.

In the costimulation experiments, a dose-dependent, increasing effect of oxLDL on depolarisation-induced contraction was found. Compared with the results of Table IIa, it is evident that at least at low concentrations of oxLDL, this effect is superaddi-

TABLE II

Influence of native LDL and oxLDL on contractile behavior of segments of rabbit carotid artery

a) Maximal contraction forces evoked in relaxed arterial segments during 10 min of stimulation

Stimulation with	Contraction force (mN)
native LDL (2m mg/ml)*	0.6 ± 0.3
oxLDL (0.5 mg/ml)	2.7 ± 0.8
oxLDL (1 mg/ml)	6.3 ± 1.5
oxLDL (2 mg/ml)	10.5 ± 3.7
rabbit serum albumin (1 mg/ml)	0.3 ± 0.2

b) Increase in contraction force by oxLDL during stimulation of arterial segments with KCl (60 mM)

Addition/Stimulus	Relative contraction force (%)
KCl (60 mM)	100%
+ oxLDL (0.2 mg/ml)	110 ± 5
+ oxLDL (0.5 mg/ml)	120 ± 15
+ oxLDL (2 mg/ml)	128 ± 10
+ native LDL (0.5 mg/ml)*	102 ± 3

The values represent mean values ± S.D. ($n = 4$; *$n = 3$). In the costimulation experiments (b), the contraction responses of each segment evoked in the presence of LDL were related to those induced by KCl alone (= 100%). The absolute value of the mean basal contraction amplitude corresponds to 54 ± 12 mN. The increasing effects of oxLDL in a) and b) are significant with $p < 0.05$ beginning at concentrations of 0.5 mg/ml (a) and 0.2 mg/ml (b), respectively (t-test).

tive: the basal contraction amplitude (54 ± 12 mN) evoked by KCl (60 mM) is increased by 5 and 11 mN (mean values) with oxLDL concentrations of 0.2 and 0.5 mg/ml, respectively. This phenomenon represents a facilitation of contraction. Again, the effects were observed repeatedly in the same vessel segment, indicating a reversible influence on the contraction-inducing mechanism stimulated by depolarization. Comparing the actions of oxLDL and native LDL applied at the same concentration (0.5 mg/ml), it is evident that the native form has only a mild effect.

DISCUSSION

It is assumed that the cytotoxic effect of oxidized LDL, which was shown in various cell culture systems,[7] is also responsible for necrobiosis in advanced lesions.[1] In a rabbit model of atherosclerosis it was found that development of intimal necrosis was preceded by ATP depletion within the plaque.[8] These events were only seen in animals in which plasma LDL was raised by cholesterol-feeding. Since under these conditions elevated degradation products of lipid peroxidation were found in plasma,[13] probably related to LDL oxidation, we were interested in the action of oxidized LDL on tissue samples of arterial walls.

As shown here, oxidized LDL has strong effects on parameters of energy metabolism as well as on contractility of arterial smooth muscle. These actions are clearly dependent on LDL oxidation. Although care was taken to avoid LDL oxidation during preparation of the native form, complete absence of oxLDL cannot be guaranteed. Furthermore, it is reasonable to assume that cell contact-mediated LDL oxidation occurs during the incubation experiments. For these reasons it is impossible to decide whether the mild effects observed with native LDL are due to the action of the native form or of small amounts of instantaneously produced oxLDL.

With respect to parameters of energy metabolism, there is a rapid decrease in the contents of ATP and glycogen induced by high concentrations of oxLDL. This effect is not caused by an irreversible blockade of ATP synthesis since there is a slow recovery after withdrawal of oxLDL. Nevertheless, the impairment of ATP metabolism during longer incubation times could be one basis of the described cytotoxic effect of oxLDL.[14] For example, in cell culture, a significant cytotoxic effect was observed with oxLDL concentrations of 0.1–0.2 mg/ml after a period of 66 h,[7] and it is reasonable to assume that in the rabbit model of atherosclerosis, oxLDL accumulating in the plaque is involved in the hypercholesterolemia-induced decrease in ATP and glycogen.[8]

The decrease in glycogen can be explained on the one hand by its function as an energy store. On the other hand, since it was shown that detoxification of organic hydroperoxides via the glutathione peroxidase - glutathione reductase system induces glycogenolysis in aortic rings,[10] it is conceivable that the fatty acid hydroperoxides present in oxLDL have similar effects.

Although LDL cytotoxicity is a well described phenomenon,[14] little is known about the underlying mechanism. Nevertheless, the results presented here support the idea, that, among other possible mechanisms, depletion of glycogen and ATP representing an impaired energy metabolism may contribute to this effect. An indication of how oxLDL exerts this effect is provided by the observation that, depending on the concentration, it induces or facilitates contraction. Since muscle contraction is a sensitive indicator of cytoplasmic Ca^{2+} these results suggests an oxLDL-induced Ca^{2+}

release or at least a facilitated Ca^{2+} liberation. Under this assumption, the effect of oxLDL on energy metabolism cannot be seen in a blockade of ATP regeneration but in an increase of the energy turnover by activating contraction and cation transport processes. However, since contraction induced by depolarization alone did not alter cellular ATP levels in aortic segments of rabbits (Heinle, unpublished results), a second aspect should be considered: the complete detoxification of hydroperoxides via glutathione peroxidase requires $NADPH_2$, provided by the glucose-6-phosphate metabolizing pentose phosphate cycle. It is known that hydroperoxides activate glycogen degradation[10] as well as the pentose phosphate cycle.[15] Therefore it might be that in arterial smooth muscle the presence of oxidized LDL, which contains a large spectrum of fatty acid hydroperoxides and other products of lipid peroxidation,[11] causes a limitation in glucose- /glycogen metabolism which cannot maintain the fluxes for simultaneous supply of energy as well as reducing equivalents.

Due to the central importance, direct intracellular measurements should confirm the Ca^{2+} liberating effect of oxLDL in intact arterial smooth muscle. A recent paper showed increased phosphatidyl inositol turnover and increased intracellular Ca^{2+} levels as a result of LDL metabolism in platelets and isolated smooth muscle cells.[16] However, it was not investigated whether this effect was dependent on extra- or intracellular processing of the lipoprotein via lipid peroxidation.

Further investigations should clarify whether the cellular interactions inducing activation of the energy metabolism and contraction in the arterial smooth muscle depend on a receptor-mediated uptake, on a lipase-catalyzed liberation of free fatty acid hydroperoxides or on free radical reactions initiated in the lipoprotein and thus impairing the cells.

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