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Effects of human low-density lipoproteins on the nucleotide patterns of cultured pig aortic endothelial cells

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Cultured pig aortic endothelial cells display significant changes in their nucleotide patterns after incubation with LDL-cholesterol purified from normal human plasma as determined by HPLC. Incubation at 70 mg/dl LDL-cholesterol for 24 h at 37°C caused a significant decrease ($P<0.001$) in ATP from a control value of 14.0±0.4 nmol/mg protein to 6.6±0.9 nmol/mg protein ($n=4$) with a concomitant increase in ADP and AMP. At higher LDL concentrations these effects were even more pronounced but still reversible. Akin to adenine nucleotides, the guanosine and uridine phosphates as determined by HPLC were changed. In contrast to LDL, HDL and VLDL were ineffectual.

Endothelial cell; Nucleotide; Cholesterol; Lipoprotein

1. INTRODUCTION

In the initiation and development of atherosclerosis the uptake of cholesterol-rich lipoproteins by endothelial cells plays an important role [1,2]. Investigations with the aim to reveal the mechanism(s) of the action(s) exerted by these lipoproteins on endothelial cells have yielded marked changes in the activities of lysosomal hydrolases, lactate dehydrogenase and pyruvate kinase in intima-media homogenates from thoracic aorta [3]. Moreover, they have disclosed perturbations of energy-dependent processes such as proteoglycan synthesis [4], and the endothelium-dependent relaxation [5,6].

To our knowledge, the possibility that cholesterol-rich lipoproteins affect the energy state of endothelial cells has not yet been investigated. Therefore, the nucleotide contents of endothelial cells kept in culture in the absence and presence of various lipoprotein fractions have been analyzed in the present study.

2. EXPERIMENTAL

2.1. *Endothelial cells*

Endothelial cells were obtained by a modification of the method originated by Pearson et al. [7]. Porcine abdominal aortas were transferred from the slaughter house to the laboratory at 4°C. After ligating lumbal arteries, the aortas were perfused with phosphate-buffered saline supplemented with 5 mM glucose (Merck), 100 μg/ml streptomycin and 100 U/ml penicillin (Biochrom) (medium 1) to wash out the blood. After clamping the distal ends, the aortas were filled with medium 1, fortified with 1 mg/ml collagenase (Sigma, Lot 99F6803) and incubated for 30 min at 37°C. After incubation, the collagenase medium was removed and the cells were collected by centrifugation at 260 × *g* for 20 min. The pellet was resuspended in 50 ml medium 199 (Boehringer) supplemented with 20 mM HEPES, 4 mM $NaHCO_3$, 100 mg/dl albumin (Boehringer), 100 U/ml penicillin and 100 μg/ml streptomycin at pH 7.4, prior to centrifugation at 260 × *g* for 10 min and resuspending in 50 ml medium 199 containing 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20% fetal calf serum (Gibco) and 30 μg/ml endothelial cell growth supplemented (Sigma) (medium 2). Cells were preincubated in vials (Greiner) in an atmosphere of 95% O_2 and 5% CO_2 at 37°C for 5 days when subconfluent densities were reached. Medium 2 was changed every 48 hours. Endothelial cells were incubated with fresh medium 2 supplemented with VLDL, LDL, HDL or control medium under the conditions detailed in the tables. Cells were dissociated by treatment with trypsin/EDTA (0.05/0.02% (w/v), Biochrom) for 5 min and centrifuged at 260 × *g* for 5 min prior to nucleotide analysis.

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2.2. *Extraction and analysis of nucleotides*

Nucleotide extracts were prepared by mixing the endothelial cell pellet with 0.2 ml ice-cold 0.4 M $HClO_4$ and incubating at 4°C for 15 min prior to centrifugation for 5 min at 8000 × *g*. The supernatant was neutralized with 5 M K_2CO_3 at 4°C before any insoluble material was removed by centrifugation. Nucleotide determination by HPLC-analysis was performed according to de Korte et al. [8].

2.3. *Lipoproteins*

Lipoproteins were prepared from human plasma of healthy blood donors using the standard density gradient technique of [9] except that the centrifugation media had been fortified with 100 μM vitamin E and that the density of the third centrifugation step was 1.055 g/ml instead of 1.063 g/ml, in order to exclude Lp_a contamination of the LDL fraction.

The lipoprotein fractions were dialyzed for 48 h at 4°C against 0.15 M NaCl containing 0.3 mM EDTA and 100 μM vitamin E, at pH 7.4, to prevent oxidative modification of LDL [10]. Electrophoretic examination of lipoprotein preparations and cholesterol quantitation were performed by routine methods.

2.4. *Protein determination*

Protein of the extracted cell pellets from endothelial cells was determined by the BCA method [11] according to the supplier's instructions. The protein content of endothelial cells was about 25 μg/10^5 cells.

Table I

Nucleotide contents of cultured endothelial cells as a function of LDL-cholesterol concentration.

Endothelial cells obtained from pig aorta were kept under cell culture conditions as detailed in section 2 prior to incubation for 24 h in the presence of the different LDL-cholesterol concentrations stated below. Data are given as means ± SEM

LDL-cholesterol (mg/dl)	Nucleotide contents (nmol/mg protein)								
	ATP	ADP	AMP	GTP	GDP	GMP	UTP	UDP	UMP
20	14.0	8.6	*n.d.	3.6	0.53	1.5	9.2	2.9	n.d.
(n=4)	±0.4	0.3		0.2	0.2	0.6	0.5	0.4	
70	6.6	12.9	9.5	1.9	2.0	1.5	4.3	2.1	5.8
(n=4)	±0.9	0.4	0.5	0.2	0.2	0.3	0.3	0.2	1.1
140	n.d.	7.5	16.4	n.d.	2.9	3.6	n.d.	5.0	14.8
(n=4)		±0.3	1.1		0.3	0.3		0.3	1.4
200	n.d.	0.71	29.2	n.d.	2.2	4.6	n.d.	3.1	11.7
(n=3)		0.1	1.0		0.03	0.3		0.5	1.3

*not detectable (<0.1 nmol/mg protein)

3. RESULTS AND DISCUSSION

The aim of this study was to investigate the possibility of (a) direct effect(s) of lipoprotein fractions of various cholesterol contents on the energy state of endothelial cells. Using the HPLC method of de Korte et al. [8] the tri-, di- and monophosphates of adenosine, guanosine and uridine have been determined in cultured pig aortic endothelial cells. As endothelial cells respond to changes in cell density with reciprocal changes in LDL metabolism [12], the cells used in this study were grown until subconfluent densities.

Our ATP levels as related to the number of endothelial cells found in the absence of LDL are in line with the values reported [13,14], whereas the ADP levels were found to be somewhat higher. Information on the amounts of guanosine and uridine phosphates in endothelial cells has not yet been presented in the literature.

Incubation of endothelial cells for 24 h in the presence of various concentrations of LDL caused marked changes in the nucleotide pattern (Table I). Elevation in the culture medium from 20 mg/dl LDL-cholesterol to 70 mg/dl led to a decrease in ATP by about 50%, an increase in ADP by about 50% and a marked elevation in AMP (from trace amounts to 9.5 nmol/mg protein). Similar changes as in the adenosine phosphates were observed in guanosine and uridine phosphates. From the results summarized in Table I, it appears that the effect of LDL on the nucleotide pattern is concentration-dependent.

As to the specificity of the LDL-effect, experiments with different lipoprotein fractions revealed that VLDL and HDL were essentially infective (Table II).

The data shown in Table III indicate that the LDL effect on the nucleotide pattern was reversible, because normal nucleotide contents were found when after 12 h

Table II

Effect of LDL, HDL and VLDL on nucleotide contents of endothelial cells.

Endothelial cells obtained from pig aorta were kept under cell culture conditions as detailed in section 2 prior to incubation for 24 h in the presence of the lipoprotein fraction given below at a starting concentration of 100 mg cholesterol/dl.

Experiment	Addition	Nucleotide contents (nmol/mg protein)								
		ATP	ADP	AMP	GTP	GDP	GMP	UTP	UDP	UMP
I	None	15.0	5.5	0.91	3.5	0.84	2.5	8.8	1.1	3.0
	LDL	0.3	12.4	12.9	n.d.	2.5	7.2	0.04	6.6	6.6
	HDL	15.6	5.8	1.2	3.5	0.9	2.9	12.0	1.8	3.3
	VLDL	13.6	8.3	1.8	3.0	1.0	0.4	9.5	1.2	2.0
II	None	14.0	7.1	1.1	3.9	0.88	2.0	7.8	1.4	2.6
	LDL	n.d.	15.8	13.2	n.d.	3.1	6.0	n.d.	6.4	7.0
	HDL	14.2	6.4	1.4	3.7	1.2	0.9	14.9	1.1	3.0
	VLDL	14.0	7.0	1.2	3.6	1.0	1.6	8.9	2.1	2.0

Table III

Reversibility of the LDL effect on the nucleotide contents of endothelial cells.

Endothelial cells obtained from pig aortas were kept under cell culture conditions as detailed in section 2 prior to incubation for 24 h (2 × 12 h) in the presence of LDL at 20 mg and 100 mg cholesterol/dl, respectively; after the first 12 h period medium was replaced and LDL-cholesterol, where appropriate, was lowered to 20 mg/dl and incubation continued for another 12 h period.

Experiment	Incubation time	LDL (cholesterol mg/dl)	Nucleotide contents (nmol/mg protein)								
	(h)		ATP	ADP	AMP	GTP	GDP	GMP	UTP	UDP	UMP
I	0–12	20	14.2	7.4	1.2	4.1	0.55	2.8	9.0	1.6	2.4
	12–24	20	14.0	6.9	1.1	3.4	0.8	3.8	8.7	0.8	2.8
	0–12	100	9.4	12.4	2.1	0.3	1.3	3.8	1.2	2.0	4.6
	12–24	20	15.2	7.3	1.7	2.5	0.74	1.7	7.6	1.3	1.4
II	0–12	20	15.7	7.7	1.4	3.3	0.77	1.7	7.6	1.3	1.4
	12–24	20	17.3	8.3	1.6	4.3	1.1	2.5	9.5	1.3	1.4
	0–12	100	10.3	13.9	3.2	0.4	1.8	3.1	2.0	2.2	6.1
	12–24	20	16.3	7.2	1.6	3.6	0.8	2.4	9.0	1.1	1.5

incubation at 100 mg/dl LDL-cholesterol the concentration was reduced to 20 mg/dl and incubation was continued for another 12 h period. This finding is compatible with the view that the effect of LDL as described here is not an unspecific toxic event [15] but rather a function of the cholesterol concentration employed. In this connection it deserves mentioning that prolonged exposure of endothelial cells to atherogenic concentrations of LDL does not affect endothelial cell viability as assessed by cell count, vital dye exclusion, 51-chromium release and lactate dehydrogenase release, if lipid peroxide formation during LDL isolation has been prevented [16].

The mechanism(s) by which LDL affects cellular nucleotide patterns is at present unknown. As the sum of the cellular nucleotide species appears much less affected than the respective nucleotide fractions, the possibility has to be considered that substrate uptake and/or oxidation by endothelial cells becomes impaired after LDL exposure, possibly being related to the reported [15] decrease in membrane fluidity under this condition.

From the magnitude of effects on a variety of cellular nucleotides it appears reasonable to assume that the changes observed might have pathophysiological significance. Thus it is conceivable that even under conditions where the loss in ATP may not be great enough to markedly lower the cellular energy level, the concomitant increase in nucleoside diphosphates may become an important regulatory factor. For example with intracellular concern, the increase in GDP might affect the microtubule system (and processes depending thereon) by favouring its depolymerisation [17]. On the other hand, the increase in cellular ADP possibly leads to a change in its concentration in the microenvironment of the endothelial cells, thereby favouring platelet adhesion. Although experimental proof for these events to occur is as yet lacking, the data presented here seem to indicate that the effects of LDL on the nucleotide patterns of endothelial cells are of interest for future work towards a better understanding of early events in the development of atherogenesis, including human aortic endothelial cells.

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