Effect of Hypoxia on Lipid Accumulation in Cultured Fibroblasts from Normal Rabbit and Watanabe Heritable Hyperlipidemic Rabbit

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Summary We studied the effect of hypoxia on lipid accumulation in cultured fibroblasts from normal rabbits (normal fibroblasts) and in low density lipoprotein (LDL) receptor-negative fibroblasts from WHHL (Watanabe heritable hyperlipidemic) rabbits (WHHL fibroblasts). These cells were incubated in medium with normolipemic rabbit serum (NRS) or hyperlipemic rabbit serum (HRS). The cells were incubated in a humidified atmosphere of either 20% O₂, 75% N₂, and 5% CO₂ (control cells) or 2% O₂, 93% N₂, and 5% CO₂ (hypoxic cells).

After 48 h incubation of normal fibroblasts in medium with 20% NRS, free fatty acid (FFA) levels were increased slightly and the triglyceride (TG) level markedly in hypoxic cells. In the medium with 20% HRS, in addition to the increased FFA and TG levels, the free cholesterol level was increased slightly and the esterified cholesterol level markedly in hypoxic cells. Moreover, in WHHL fibroblasts, which lack LDL receptors, cellular lipid accumulation was also observed after 48 h incubation in the medium with 20% HRS, and hypoxic incubation enhanced the cellular cholesterol and TG accumulation, as in normal fibroblasts.

These results suggest that under hyperlipemic conditions, non LDL receptor-mediated uptake of lipoproteins plays a major role in cellular lipid deposition and that tissue hypoxia promotes lipid accumulation in peripheral cells by the LDL receptor-independent mechanisms.

Key Words: atherosclerosis, hyperlipemia, hypoxia, LDL receptor, WHHL rabbit

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Cultured skin fibroblasts possess specific high-affinity receptors that bind low density lipoproteins (LDL) on their cell surfaces. It is believed that the receptor-mediated uptake of LDL is the major process by which most extrahepatic cells take up cholesterol [1]. But the role of LDL receptors in lipid deposition in cells remains uncertain.

We previously reported that hypoxia (2% O₂) promoted cholesterol accumulation, especially that of the esterified form, in cultured rabbit aortic smooth muscle cells under hyperlipemic conditions. It was thus suggested that hypoxia plays an important role in atherogenesis [2].

The present study was designed to evaluate the role of LDL receptors in peripheral cells under hyperlipemic conditions, and the effect of hypoxia on lipid accumulation (not only of cholesterol but also of other lipids) in peripheral cells, using cultured skin fibroblasts from normal rabbits (normal fibroblasts) and LDL receptor negative fibroblasts from WHHL (Watanabe heritable hyperlipidemic) rabbits (WHHL fibroblasts) [3–6].

MATERIALS AND METHODS

Serum. Normolipemic rabbit serum (NRS) was obtained from male Japanese White rabbits fed a normal rabbit chow diet, and hyperlipemic rabbit serum (HRS) was obtained from animals fed a rabbit chow diet supplemented with 1% cholesterol for at least one month. Blood was collected from the rabbits after an overnight fast by puncturing the marginal ear vein without the use of anesthesia. Serum was obtained by centrifugation and sterilized by filtration through 0.45- μ m filters (Millipore Japan, Tokyo). Serum cholesterol, triglyceride, and phospholipid were measured by enzymatic techniques, using Determiner TC '5 (Kyowa Hakko Kogyo, Tokyo), triglyceride C II-Test (Wako Pure Chemical Industries, Osaka), and PL kit-K (Nippon Shoji, Tokyo), respectively.

Preparation of lipoprotein-deficient serum (LPDS). LPDS ($d > 1.21$ g/ml) from NRS was isolated by ultracentrifugation, as described by Havel *et al.* [7]. The fraction was dialysed against 0.15 M NaCl containing 0.01% EDTA, pH 7.4, and sterilized by filtration through 0.45- μ m filters.

Cells. LDL receptor positive skin fibroblasts were obtained from a normal Japanese White rabbit, and LDL receptor-negative fibroblasts, from a WHHL rabbit [4–6]. Cells were cultured in a humidified CO₂ (5%) incubator at 37°C in 75-cm² flasks (Corning) containing Eagle's minimum essential medium (Nissui, Tokyo) supplemented with Kanamycin (100 μ g/ml), 1% (v/v) nonessential amino acids (GIBCO), 10% (v/v) newborn calf serum (GIBCO), 20 mM Tricine (P-L Biochemicals) and NaHCO₃ at pH 7.4. The medium was changed every third day. Cells in the stationary phase of growth were subcultured at a 3 : 1 split ratio following detachment by washing with Dulbecco's phosphate-buffered saline (PBS) and incubation with 0.05% trypsin-0.02% EDTA solution for 7–10 min at 37°C. All cells used in the experiments had been grown in monolayers for 7–9 generations.

For the experiments, cells were seeded in 60-mm plastic Petri dishes at a density of 4.5×10^5 cells/dish and incubated for 3 days until subconfluent. After the growth medium was removed, the cells were washed once with PBS and incubated with 3 ml of medium containing LPDS (2.5 mg of protein/ml) for 24 h in order to activate LDL receptors. Then the medium was removed and 3 ml of medium containing 20% NRS or HRS was added to each dish. The cells were transferred either to the incubator conditioned with 20% O₂, 75% N₂, and 5% CO₂ (control cells) or the incubator with 2% O₂, 93% N₂, and 5% CO₂ (hypoxic cells). After a 48 h incubation, the culture medium was removed, and the cells were rinsed twice with PBS and harvested with 0.05% trypsin-0.02% EDTA solution. Cells from these dishes were collected in a conical centrifuge tube and washed three times with 0.9% NaCl solution by repeated centrifugation (2,000 rpm for 5 min each time).

Cellular lipid analyses. Lipids were extracted from the washed cell pellet using the method of Folch *et al.* [8]. Namely, 1 ml of methanol was added to each tube containing a washed cell pellet. After sonication of the cell pellet for 1 min using the microtip of the sonifier (Sonifier B-12 Branson Sonic Power, Danbury Conn. U.S.A.), 2 ml of chloroform were added. After mixing and centrifugation (3,000 rpm for 10 min), the clear supernatant was transferred to a glass tube, and the sediment was washed twice with 1 ml of chloroform-methanol (2 : 1) mixture by repeated centrifugation (3,000 rpm for 10 min). The dry sediment was dissolved in 0.5 N NaOH and an aliquot was taken for protein determination by the method of Lowry *et al.* [9]. One ml of 0.5% NaCl was added to 5 ml of crude lipid extract, and the two liquids were mixed vigorously. After centrifugation (3,000 rpm for 10 min), the lower phase was taken for lipid analysis, and an aliquot was evaporated under reduced pressure. Free and total cholesterol were determined by the enzymatic fluorometric method of Heider and Boyett [10]. The esterified cholesterol content was determined by subtraction of free cholesterol content from that of total cholesterol. Triglyceride (TG) was determined by the method of Fletcher, free fatty acid (FFA) by the modified Itaya-Ui's method, and phospholipid (PL) was measured by Hoeflmayr-Fried's method [11]. All the results were expressed per mg of cell protein, and statistical significance was calculated using the Student's *t*-test.

RESULTS

After 48 h, the hypoxic cultures exhibited growth inhibition, the cellular protein per dish being approximately 60% of the control culture value.

Table 1 shows the lipid contents of the control (20% O₂) and the hypoxic (2% O₂) cells after a 48 h incubation in medium containing 20% NRS or 20% HRS. In the medium containing 20% NRS, the FFA levels of hypoxic cells were 44% higher than those of control cells (in both normal fibroblasts and WHHL fibroblasts). In the medium containing 20% HRS, FFA levels of hypoxic cells from normal and WHHL rabbits were 26% and 22% higher, respectively. The TG

Table 1. Lipid content of cultured normal and WHHL fibroblasts incubated with a medium containing 20% NRS or HRS under either control or hypoxic conditions for 48 h.

	Normal fibroblasts		WHHL fibroblasts	
	20% O ₂	2% O ₂	20% O ₂	2% O ₂
Free fatty acid				
LPDS	27.7±1.3		30.5±1.8	
NRS	23.8±0.8	34.3±1.8***	27.4±0.7	39.4±1.2***
HRS	25.8±1.1	32.6±2.1**	27.4±2.3	32.9±1.2*
Triglyceride				
LPDS	9.4±1.7		9.1±0.8	
NRS	5.4±0.5	127.3±1.5***	6.6±0.2	113.0±5.3***
HRS	67.6±2.1	292.3±9.7***	86.0±1.0	250.3±5.7***
Free cholesterol				
LPDS	12.8±0.7		14.4±0.4	
NRS	10.7±0.1	11.2±0.4	11.5±0.4	9.5±0.3***
HRS	14.6±0.2	21.3±1.1***	15.4±1.0	19.8±1.5*
Esterified cholesterol				
LPDS	1.7±0.6		1.1±0.3	
NRS	0.6±0.2	0.7±0.7	0.4±0.4	0.9±0.2
HRS	14.4±0.8	32.5±2.3***	12.4±1.3	23.2±1.0***
Phospholipid				
LPDS	161±3		149±2	
NRS	168±4	166±2	168±4	166±3
HRS	183±5	184±7	176±3	175±2

Cells were grown to subconfluence in culture medium containing 10% newborn calf serum, rinsed once with PBS, and incubated in medium containing LPDS (2.5 mg protein/ml) for 24 h. Then the medium was removed and medium containing 20% NRS or 20% HRS was added to each dish. The dishes were transferred either to the control (20% O₂, 75% N₂, and 5% CO₂) or the hypoxic (2% O₂, 93% N₂, and 5% CO₂) incubator, in which the cells were incubated for 48 h. The total cholesterol, triglyceride, and phospholipid values of HRS were 2,200 mg/dl, 210 mg/dl, and 592 mg/dl, respectively, and those of NRS were 43 mg/dl, 22 mg/dl, and 57 mg/dl, respectively. All results are expressed in µg/mg of cell protein. Each value represents the mean±SD of triplicate cultures. **p*<0.05; ***p*<0.01; ****p*<0.001 as compared with control cells.

levels of control cells incubated in the medium containing 20% HRS were markedly higher than those of cells incubated in the medium containing 20% NRS. Moreover, there was a much higher TG accumulation in the hypoxic cells. HRS induced cholesterol accumulation, especially that of the esterified form, not only in normal cells but also in WHHL cells. The free cholesterol levels of hypoxic cells incubated with the medium containing 20% HRS were higher than those of control cells by 46% in normal cells and 29% in WHHL cells. Moreover, the esterified cholesterol levels of hypoxic cells incubated with 20% HRS were much higher than those of control cells by 2.3-fold in normal cells and 1.9-fold in WHHL cells. On the other hand, in the medium containing 20% NRS, hypoxia did not promote the accumulation of either free or esterified cholesterol in the cells. The PL levels of cells incubated with 20% HRS were a little higher than those of the

Fig. 1. The ratio of lipid content in hypoxic cells (2% or 5% O₂) to control cells (20% O₂) incubated in medium containing 20% HRS for 48 h. Normal fibroblasts were grown to subconfluence in culture medium containing 10% newborn calf serum, rinsed once with PBS, and incubated in medium containing LPDS (2.5 mg protein/ml) for 24 h. Then the latter medium was removed and that containing 20% HRS was added to each dish. The dishes were transferred either to the control (20% O₂) or the hypoxic (2% O₂ or 5% O₂) incubator and the cells were incubated for 48 h. The total cholesterol, triglyceride and phospholipid values of HRS were 1,765, 193, and 451 mg/dl, respectively. The 2% O₂ and 5% O₂ series were carried out separately. The ratios were obtained from each experiment. Each bar represents the value of the mean of triplicate cultures under hypoxic conditions (2% O₂ or 5% O₂)/control conditions (20% O₂), respectively. FFA, Free fatty acid; TG, triglyceride; FC, free cholesterol; EC, esterified cholesterol; PL, phospholipid.

cells incubated with 20% NRS, but there was no significant difference between control and hypoxic cells. Thus, after a 48 h incubation of normal fibroblasts in the medium with 20% NRS, the FFA level increased slightly and TG level increased markedly in hypoxic cells. On the other hand, in the medium with 20% HRS, the free cholesterol level increased slightly and the esterified cholesterol level increased markedly in addition to the increased FFA and TG levels in the hypoxic cells. These lipid increases in normal fibroblasts were almost identical with those in the WHHL fibroblasts.

Another experiment was carried out using normal fibroblasts. Figure 1 shows the ratio of lipid content in hypoxic cells (2% O₂ or 5% O₂) to control cells (20% O₂) incubated in the medium containing 20% HRS for 48 h. The ratios of FFA and free cholesterol increased slightly and those of TG and esterified cholesterol increased markedly, while the ratio of PL did not increase. These increases correlated inversely with O₂ concentrations.

DISCUSSION

We previously reported that hypoxia (2% O₂) promoted cholesterol accumulation, especially that of the esterified form, in cultured rabbit aortic smooth muscle

cells in the presence of hyperlipemic serum [2]. In the present study, not only cholesterol but also other lipids (FFA, TG, and PL) were measured in normal fibroblasts and WHHL fibroblasts incubated under hypoxic conditions for 48 h. Pietila *et al.* reported that the cellular content of cholesterol in smooth muscle cells cultured from rabbit aorta in medium containing 10% fetal calf serum showed no difference between control cells and those kept under hypoxic conditions [12]. Moreover, Gordon reported that hypoxic cultured mouse fibroblasts grown in a suspension culture system with 10% horse serum showed no significant changes in cellular cholesterol or phospholipid content, although increased FFA and TG levels were noted [13]. In the present study, after a 48 h incubation in medium with 20% NRS, the FFA level was increased slightly, and the TG level, markedly, in hypoxic cells. On the other hand, in medium with 20% HRS, in addition to the increased FFA and TG levels, a marked accumulation of esterified cholesterol and a lesser extent of free cholesterol accumulation were noted in hypoxic cells (Table 1 and Fig. 1).

Filipovic *et al.* reported that hypoxia, inducing a rise of NADH/NAD ratio, increased FFA synthesis in arterial tissue [14, 15]. Spector *et al.* reported that FFA oxidation took place mainly in the mitochondria and required energy from oxidative phosphorylation [16]. The increased fatty acid synthesis and the impairment of utilization of FFA in hypoxia will result in increased esterification, since the intracellular level of FFA is closely regulated [17]. Excessive FFA, which is stored as TG under normolipemic conditions, seems to be stored not only as TG but also as cholesterol esters under hyperlipemic conditions (Table 1 and Fig. 1). It is thought that both excessive FFA and excessive cholesterol are key factors in cholesterol ester accumulation in hypoxic cells.

Cultured skin fibroblasts possess specific high affinity receptors that bind LDL on their cell surface. It is believed that the receptor-mediated uptake of LDL particles is the major process by which most extrahepatic cells take up cholesterol [1]. Hyperlipemic serum from cholesterol-fed animals induces accumulation of esterified cholesterol in tissue culture cells [18–20]. And in our study, when cultured rabbit fibroblasts were incubated with 20% HRS for 48 h, lipids including free and esterified cholesterol accumulated in WHHL fibroblasts almost as much as in normal fibroblasts. This indicates that LDL receptor-independent uptake of lipoproteins plays a major role in cellular lipid accumulation under hyperlipemic conditions. Hypoxic effects on lipid accumulation in cultured cells were not mediated by LDL receptors, since there were no marked differences in cellular lipid accumulation under hypoxic conditions between normal fibroblasts and WHHL fibroblasts.

In hypoxic cells under hyperlipemic conditions, the ratio of PL to cholesterol content ratio fell, since the PL content did not increase although the cholesterol content did. Adams reported that the PL-cholesterol ratio fell in the later stage of atheroma and synthesis of PL was an early defense mechanism of the arterial wall against the infiltration of cholesterol [21]. Kjeldsen *et al.* showed that systemic

hypoxia promoted aortic atherosclerosis with increases in plasma cholesterol and TG levels in cholesterol-fed rabbits and also that the aortic content of total cholesterol and TG were higher in the rabbit under hypoxia, while no changes were seen in aortic PL [22]. The profile of lipid accumulation in hypoxic cells with HRS in our study is similar to that in aortic atheroma under hypoxic conditions in Kjeldsen's report. We previously reported that in the WHHL rabbit systemic hypoxia aggravated aortic atherosclerosis without causing a further increase in the plasma cholesterol level [23]. Our previous *in vivo* study and the present *in vitro* study suggested that enhanced lipid accumulation in the arterial wall by systemic hypoxia is produced not through the increased plasma lipid levels but through the direct effect of hypoxia on the tissue.

The present study indicates that under hyperlipemic conditions, non-LDL receptor mediated uptake of lipoproteins plays an important role in cellular lipid deposition and that tissue hypoxia promotes the lipid accumulation in peripheral cells by a LDL receptor-independent mechanism.

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