Effects of Hypoxia on Sterol Synthesis, Acyl-CoA: Cholesterol Acyltransferase Activity, and Efflux of Cholesterol in Cultured Rabbit Skin Fibroblasts

Junichi Mukodani, Yuichi Ishikawa, and Hisashi Fukuzaki

To elucidate the mechanism of cholesterol accumulation in cells under hypoxic conditions, we tested the effects of hypoxia on sterol synthesis, on the activity of acyl-CoA: cholesterol acyltransferase (ACAT), and on the efflux of cholesterol in cultured rabbit skin fibroblasts. Sterol synthesis was measured by the incorporation of \(^{14}\)C-acetate into sterol, and ACAT activity, by the incorporation of \(^{14}\)C-oleate into cholesteryl ester. Hypoxia suppressed both sterol synthesis and the efflux of cholesterol but increased ACAT activity. These results suggest that hypoxia disturbs the balance of cholesterol metabolism in cells and induces intracellular lipid accumulation. (Arteriosclerosis 10:106–110, January/February 1990)

The inner half of the arterial wall has few vasa vasorum, and the oxygen supply to this area depends on direct diffusion from the arterial lumen. Therefore, hypoxia in the arterial wall could easily be induced by a deterioration of this diffusion process. For example, Heughan et al.\(^1\) reported that the oxygen tension in the arterial walls of normal rabbits was 30 to 40 mm Hg, while that of cholesterol-fed rabbits was 10 mm Hg. These results suggest that atherosclerosis induced by cholesterol feeding affects the oxygen supply to the arterial walls.

Arterial tissue hypoxia could be induced by systemic hypoxia. Kjeldsen et al.\(^2\) reported that systemic hypoxia promoted atherosclerosis in cholesterol-fed rabbits, and we also reported similar results with Watanabe heritable hyperlipidemic (WHHL) rabbits.\(^3\) Hypoxia reportedly affects lipid metabolism in aortic tissue cultures\(^4\) and in cultured aortic smooth muscle cells.\(^5\) Hypoxia may, therefore, play a role in the initiation or promotion of atherosclerosis.

We recently reported that, under hypoxic conditions, there was an increased cholesterol accumulation in cultured rabbit aortic smooth muscle cells.\(^6\) The present study was designed to further elucidate the mechanisms of cholesterol accumulation in cells under hypoxic conditions. We tested the effects of hypoxia on the following: de novo synthesis of sterol, acyl-CoA: cholesterol acyltransferase (ACAT) activity, and the efflux of cholesterol in cultured rabbit skin fibroblasts.

Methods

Materials

\(^{14}\)C-acetate and \(^{14}\)C-oleate were obtained from New England Nuclear (Boston, MA). Unlabeled cholesteryl olate, free cholesterol, and oleate were purchased from Sigma (St. Louis, MO). Dulbecco’s modified Eagle’s minimum essential medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). Culture flasks (25 cm\(^2\) and 75 cm\(^2\)) were purchased from Corning (Iwaki Glass, Tokyo, Japan). All other chemicals were of commercially available reagent grade.

Serum

Normolipemic rabbit serum (NRS) was obtained from male Japanese White rabbits fed a normal rabbit chow diet (ORC-4 Oriental Yeast, Tokyo, Japan). Hyperlipemic rabbit serum (HRS) was obtained from rabbits fed a rabbit chow diet supplemented with 1% cholesterol for at least 1 month. Blood was collected from unanesthetized rabbits by venous puncture of the marginal ear following overnight fasting. Serum was obtained by centrifugation and was sterilized by filtration through 0.45 \(\mu\)m filters (Millipore Japan, Tokyo, Japan). Serum cholesterol was measured by an enzymatic technique with Determiner TC \(^5\) (Kyowa Hakko Kogyo, Tokyo, Japan).

Preparation of Lipoprotein-deficient Serum

Lipoprotein-deficient serum (LPDS) (d>1.21 g/ml) from NRS was isolated by ultracentrifugation, as described by Havel et al.\(^8\) The fraction was dialyzed against 0.15 M NaCl containing 0.01% ethylenediaminetetraacetic acid (EDTA), pH 7.4, and sterilized by passage through 0.45 \(\mu\)m filters.

Cells

Skin fibroblasts were cultured from tissue explants derived from male Japanese White rabbits as described previously.\(^9\) We used Dulbecco's modified Eagle's minimal essential medium, supplemented with penicillin (100 IU/ml), streptomycin (100 \(\mu\)g/ml), and NaHCO\(_3\) at pH 7.4 as the “basal medium.” Cells were grown in 75 cm\(^2\) flasks with the basal medium containing 10% fetal bovine serum. The medium was changed every third day. Cells in the stationary phase of growth were subcultured with a 3:1 split ratio by washing with Dulbecco's phosphate-
buffered saline (PBS) and then incubating with 0.05% trypsin-0.02% EDTA solution for 10 minutes at 37°C. All cells used in the experiments had been grown in a monolayer for seven to nine generations.

For the experiments, the cells were dissociated from the flasks with trypsin-EDTA, transferred to 25 cm² flasks at a density of 4.5×10⁶ cells/flask, and grown to confluence. The cells were then washed with PBS and incubated in the basal medium containing 2.5 mg/ml LPDS for 48 hours (pre-incubation).

**Hypoxia**

Next, 3 ml of medium was infused into each flask, and the flasks were divided into two groups, a control group and a hypoxic group. The control group was incubated under the usual conditions (95% air and 5% CO₂); the flasks of the hypoxic group were incubated with 95% N₂ plus 5% CO₂ for 1 minute and were packed tightly to induce hypoxic conditions. Both groups were incubated for 1 and 4 hours, and the oxygen tension of each medium was measured by gas analyzer (model 1312-Btood Gas Manager, Instrumentation Laboratory, Milano, Italy).

**Sterol Synthesis**

Sterol synthesis was determined by measuring the amount of [¹⁴C]-acetate incorporated into the cholesterol. After the pre-incubation period, the medium was exchanged for 3 ml of the basal medium containing 10% NRS or HRS. To this medium, [¹⁴C]-acetate (2 μCi/ml) was added. The flasks were then divided into two groups, a control group and a hypoxic group. Each group was incubated as described above. Both groups were incubated for 48 hours at 37°C, then the medium was distributed into glass tubes. The cells were washed three times with PBS, were removed from the flasks with trypsin-EDTA, and were collected into glass tubes. The tubes were then centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded, and the cell precipitate was washed three times with PBS. The lipid portion of the medium and the cells were extracted by the method of Folch et al. ¹¹ Cholesteryl esters and free cholesterol were separated by thin-layer chromatography, and cholesteryl esters were saponified by KOH to distinguish the cholesterol from fatty acids. The radioactivity of each cholesterol component was measured and expressed as cpm/mg of cell protein. We also separated triglyceride free fatty acid and phospholipid to evaluate the uptake of [¹⁴C]-acetate into total fatty acid. The protein content of the cells was determined by the method of Lowry et al. ¹² by using bovine serum albumin as the standard.

**Acyl-CoA: Cholesterol Acyltransferase Activity**

ACAT activity was determined by measuring the incorporation of [¹⁴C]-oleate into the cholesteryl ester of cells. After the pre-incubation period, the medium was exchanged for the basal medium containing 10% HRS, and the flasks were divided into a control and a hypoxic group as previously described. The cells were incubated for the indicated times and pulse-labeled for 2 hours with [¹⁴C]-oleate (0.8 μCi/ml of medium). The radioactivity of the cellular cholesterol esters was measured as described above. We also measured the incorporation of [¹⁴C]-oleate into triglyceride in a control and a hypoxic group.

**Efflux of Cholesterol**

After the pre-incubation period, the cells were loaded with cholesterol by a 48-hour incubation in the basal medium containing 10% HRS. This period was designated as the "load phase." After the load phase, a group of cells was harvested and analyzed for total and free cholesterol contents, according to the method of Heider and Boyett. ¹⁴ The remaining cells were divided into control and hypoxic groups and were incubated for 24 hours with the medium containing 10% NRS or 2.5 mg/ml LPDS. This period was designated the "efflux phase." The cells were then harvested, and the cholesterol contents were analyzed.

**Statistical Analysis**

For statistical analysis, the nonpaired Student's t test was used.

**Results**

The total cholesterol content of the HRS was 2000 to 3000 mg/dl, that of the NRS was 30 to 60 mg/dl, and that of the LPDS was undetectable.

**Oxygen Tension**

The oxygen tension of the medium in the hypoxic group was 42.5±6.0 mm Hg at 1 hour and 44.8±5.6 mm Hg at 4 hours and was about one-fourth of the oxygen tension of the control group (Table 1).

**Sterol Synthesis**

Table 2 shows the incorporation of [¹⁴C]-acetate into the sterol of the cells and medium. In cells incubated with
Table 2. Effect of Hypoxia on $^{14}$C-acetate Incorporation Into Cholesterol of Cultured Rabbit Skin Fibroblasts

<table>
<thead>
<tr>
<th>Serum</th>
<th>FC Cell</th>
<th>EC Cell</th>
<th>FC Medium</th>
<th>EC Medium</th>
<th>Total</th>
<th>Total fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS</td>
<td>47.9</td>
<td>*</td>
<td>13.7</td>
<td>23.6</td>
<td>85.2</td>
<td>61.2</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>15.0</td>
<td>*</td>
<td>4.0</td>
<td>10.4</td>
<td>29.4</td>
<td>45.7</td>
</tr>
<tr>
<td>HRS</td>
<td>1.9</td>
<td>†</td>
<td>0.4</td>
<td>6.9</td>
<td>9.2</td>
<td>69.6</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1.2</td>
<td>†</td>
<td>0.4</td>
<td>4.9</td>
<td>6.5</td>
<td>50.5</td>
</tr>
</tbody>
</table>

All values are expressed as $\times 10^3$ cpm/mg of cell protein. Each value represents the mean of two flasks.

After the 48-hour pre-incubation in the basal medium containing LPDS (2.5 mg/ml), the medium was exchanged for the basal medium containing 10% NRS or HRS. To this medium, $^{14}$C-acetate (2 $\mu$Ci/ml) was added. Flasks were divided into a control group and a hypoxic group as described in the Methods section. Both groups were incubated for 48 hours.

*We could not saponify the cholesteryl ester in cells incubated with NRS because the radioactivity was too low. †After saponification, almost all radioactivity in the cholesteryl esters of cells incubated with HRS was detected in the fatty acid component.

FC=free cholesterol, EC=esterified cholesterol, NRS=normolipemic rabbit serum, HRS=hyperlipemic rabbit serum, LPDS=lipoprotein-deficient serum.

NRS, the cholesteryl ester radioactivity was so low that we did not saponify the cholesteryl ester. The radioactivity of the cholesteryl ester from cells incubated with HRS was $49.4 \times 10^3$ cpm/mg cell protein in the control group and $47.7 \times 10^3$ cpm/mg cell protein in the hypoxic group. In both groups, almost all the radioactivity was detected in the fatty acids after saponification.

When the cells were incubated in the NRS-containing medium, acetate-derived synthesis in the hypoxic group was only about one-third that of the control group. Sterol synthesis was markedly suppressed when cells were incubated in the HRS-containing medium, compared to when the cells were incubated in the NRS-containing medium. Sterol synthesis in the hypoxic group was about two-thirds that of the control group. Table 2 also shows the incorporation of $^{14}$C-acetate into fatty acid. Fatty acid synthesis in the hypoxic group was about three-fourths of the control group in both the NRS- and the HRS-containing mediums. We performed another experiment using cells from another rabbit. In that experiment, the sterol synthesis of the hypoxic group was also smaller than that of the control group (data not shown). These results suggest that, under hypoxic conditions, sterol synthesis is decreased.

**Acyl-CoA: Cholesterol Acyltransferase Activity**

As shown in Figure 1, the incorporation of $^{14}$C-oleate into cellular cholesteryl ester was increased in the hypoxic group relative to that in the control group. On the other hand, the incorporation of $^{14}$C-oleate into triglyceride in the hypoxic group was almost equal to that in the control group (Table 3). These results suggested that, under hypoxic conditions, ACAT activity was increased.

**Cholesterol Efflux**

Figure 2 shows the cholesterol contents of cells before and after the efflux phase with NRS. During the efflux phase, the levels of free cholesterol were similar in both the control and the hypoxic groups. However, during the efflux phase, esterified cholesterol decreased in the control group but not in the hypoxic group, and after this phase, the cells of the hypoxic group contained significantly more esterified cholesterol than did the cells of the control group ($p<0.01$).
HYPOXIA AND LIPID METABOLISM

Table 3. Incorporation of 14C-oleate into Triglyceride of Rabbit Skin Fibroblasts under Control and Hypoxic Conditions

<table>
<thead>
<tr>
<th>Period of pulse labeling</th>
<th>Control</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0—2</td>
<td>67.3±3.8</td>
<td>76.7±4.2</td>
</tr>
<tr>
<td>12—14</td>
<td>460.1±68.4</td>
<td>423.4±47.6</td>
</tr>
<tr>
<td>24—26</td>
<td>485.0±33.1</td>
<td>415.7±83.0</td>
</tr>
</tbody>
</table>

All values are expressed as x10 cpm/mg of cell protein, and each value is the mean ± SD of four flasks.

Figure 3. The cholesterol content of lipid-loaded fibroblasts before and after incubation in the medium containing lipoprotein-deficient serum (LPDS) under control and hypoxic conditions. After a 48-hour pre-incubation in the basal medium containing LPDS (2.5 mg/ml), esterified cholesterol content was 4.4±1.5 nmol/mg of cell protein, and free cholesterol was 33.2±1.8 nmol/mg of cell protein. Then cells were loaded with cholesterol by incubation in the basal medium containing 10% hyperlipemic rabbit serum (HRS) for 48 hours. A group of cells was harvested, and the remaining cells were washed three times with phosphate-buffered saline. The medium containing LPDS (2.5 mg/ml of medium) was then added to each flask, and the flasks were divided into a control group and a hypoxic group as described in the Methods section. After a 24-hour incubation period, cells of both groups were harvested. All values are expressed as nmol/mg of cell protein. Each bar represents the mean ± SD of four flasks. *p<0.01 compared to control group. A. Free cholesterol. B. Esterified cholesterol.

When cells are incubated with NRS, extracellular cholesterol can be internalized and may accumulate during the efflux phase. To prevent this occurrence, we used LPDS instead of NRS in the efflux phase. As shown in Figure 3, in cells of the hypoxic group, the esterified cholesterol content was still significantly larger (p<0.01) than that of the control group after the efflux phase in which LPDS was used. These results suggest that, under hypoxic conditions, the efflux of cholesterol is suppressed.

Discussion

Lipid accumulation in arterial wall cells is one of the main features of atherosclerosis. To elucidate atherogenesis, it is important to clarify the mechanism of lipid accumulation.

We recently found that more esterified cholesterol accumulated in rabbit aortic smooth muscle cells cultured in HRS-supplemented medium under hypoxic conditions (5% O2) than under control conditions (20% O2).7 Cholesterol accumulation in cells could depend on the balance of three factors: the uptake of cholesterol by cells, cholesterol synthesis within cells, and cholesteryl efflux from cells. Hypoxia may disturb this balance and promote cholesterol accumulation in cells.

Albers and Bierman6 reported that, under hypoxic conditions, the uptake of 125I-low density lipoprotein (LDL) by cultured smooth muscle cells did not change. We reported that hypoxia promoted cholesterol accumulation in skin fibroblasts cultured from WHHL rabbits that were deficient in LDL receptors.9 These results indicate that, under hypoxic conditions, lipoprotein uptake by LDL receptors did not play an important role in cellular cholesterol accumulation. It is necessary to study the effects of hypoxia on lipoprotein uptake by other pathways that have not yet been studied.

Table 2 illustrates the hypoxia-induced suppression of sterol synthesis in cells. Robertson15 reported that in a suspension culture of human arterial intimal cells, low oxygen levels caused a reduction in cholesterol synthesis. Filipovic and Ruttenmuller4 reported that hypoxia suppressed cholesterol synthesis in human aortic tissue segments, and
Howard reported similar results with rabbit aortic tissue fragments. Our data are consistent with these results.

The effect of hypoxia on ACAT activity had not been studied previously. Figure 1 shows that ACAT activity was increased under hypoxic conditions. ACAT activity could be regulated by two independent mechanisms: Adenosine triphosphate (ATP) dependent phosphorylation of ACAT and the pool size of the substrate, i.e., free cholesterol. It has not been clear which mechanism influences ACAT activity under hypoxic conditions, but presumably the increase of ACAT activity under hypoxic conditions may be due to an increase in the pool size of cholesterol in cells rather than to promotion of ATP-dependent phosphorylation, because Aw and Jones reported that in hepatic cells under hypoxic conditions, ATP was decreased and ATP-dependent enzyme activity was suppressed.

The efflux of cholesterol from cells is important for preventing the accumulation of cholesterol in cells, although the effect of hypoxia on this process has not been well studied. As shown in Figure 2, in hypoxic cells the efflux of cholesterol was suppressed. When the efflux medium contained NRS, cholesterol uptake during the efflux phase may affect cholesterol content in cells. We therefore tested the effect of hypoxia on cholesterol efflux using an efflux medium containing LPDS instead of NRS. Cholesterol was not detected in the LPDS. So in the experiment using LPDS-containing medium, the influence of cholesterol uptake was negligible, and in hypoxic cells the efflux of cholesterol was suppressed, as shown in Figure 3.

We previously reported that hypoxia promoted cholesterol accumulation in cells cultured in a medium containing HRS but had little effect on cholesterol accumulation in cells cultured in a medium containing NRS. De novo cellular synthesis of cholesterol was suppressed by hypoxia. These results suggested that the cholesterol that accumulated in cells under hypoxic conditions was derived from lipoprotein in the medium rather than from de novo cellular synthesis.

Albers and Bierman reported that the degradation of LDL was inhibited by hypoxia, but since only the LDL proteins were labeled, the flux of cholesterol could not be elucidated. Under hypoxic conditions, the cholesterol may accumulate not in the lysosomes but in the cytosol, because ACAT activity is increased and cholesterol is accumulated in the esterified form. Hypoxia may have a direct stimulatory effect on ACAT activity, which leads to a decrease in cholesterol efflux.

In our experiments, the oxygen tension of the medium in the hypoxic group was about 40 mm Hg and was similar to the oxygen tension of normal aorta as reported by Niilikoski et al. This suggests that even in intact arterial walls, cells are probably exposed to hypoxic conditions, the efflux of cholesterol may be suppressed, and the accumulation of cholesterol in cells could easily occur. Further studies are needed to clarify the effects of hypoxia on lipid metabolism within the arterial wall.

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References


Index Terms: tissue hypoxia • hypercholesterolemia • sterol synthesis • acyl-CoA: cholesterol acyltransferase • cholesterol efflux • cell culture • fibroblasts
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