Lipid Accumulation in Smooth Muscle Cells Under LDL Loading Is Independent of LDL Receptor Pathway and Enhanced by Hypoxic Conditions


Objective—The effect of a variety of hypoxic conditions on lipid accumulation in smooth muscle cells (SMCs) was studied in an arterial wall coculture and monoculture model.

Methods and Results—Low density lipoprotein (LDL) was loaded under various levels of oxygen tension. Oil red O staining of rabbit and human SMCs revealed that lipid accumulation was greater under lower oxygen tension. Cholesterol esters were shown to accumulate in an oxygen tension–dependent manner by high-performance liquid chromatographic analysis. Autoradiograms using radiolabeled LDL indicated that LDL uptake was more pronounced under hypoxia. This result holds in the case of LDL receptor–deficient rabbit SMCs. However, cholesterol biosynthesis and cellular cholesterol release were unaffected by oxygen tension.

Conclusions—Hypoxia significantly increases LDL uptake and enhances lipid accumulation in arterial SMCs, exclusive of LDL receptor activity. Although the molecular mechanism is not clear, the model is useful for studying lipid accumulation in arterial wall cells and the difficult-to-elucidate events in the initial stage of atherogenesis. (Arterioscler Thromb Vasc Biol. 2002;22:1712-1719.)

Key Words: smooth muscle cells ■ LDL ■ hypoxia ■ cholesteryl ester

The accumulation of cholesteryl esters in macrophages and smooth muscle cells (SMCs) in the arterial intimal layer is a characteristic of early atherosclerotic lesions. Many studies have been performed in an effort to elucidate the mechanisms of atherogenesis, including studies in animal models such as the Watanabe heritable hyperlipidemic rabbit, apoE-deficient mice, and various diet-induced hyperlipoproteinemic animal models as well as studies in various human subjects with primary and secondary hyperlipoproteinemia. These studies have revealed a transfer of LDL into the arterial wall, the recruitment of monocytes, and the proliferation of SMCs as critical steps in lesion progression. However, direct in vivo evidence for the uptake of LDL or “modified LDL” leading to cellular cholesteryl ester accumulation is still awaited. A good in vitro model system of atherogenesis would enable investigation of the accumulation process, including the mechanisms of LDL modification and uptake by monocyte-derived macrophages. Such a system would provide a powerful tool for the screening of potential compounds to inhibit certain crucial atherogenic steps.

The accumulation of cholesteryl esters leads to the formation of the foam cells that are abundantly found in atheromatous lesions, the cytoplasm of which is filled with cholesteryl esters and tests positively for lipid staining, such as with oil red O. Foam cells also exhibit positive liquid crystal birefringence of cholesteryl esters and observably contain homogeneous electron-dense spheres on electron microscopy. To study the in vivo mechanism of foam cell formation, we developed a system that effectively mimics the arterial cell wall structure by cultivating rabbit arterial SMCs (RASMCs) and rabbit arterial endothelial cells (RAECs) in layers, to which human peripheral monocytes are added and cocultivated for an additional 1 to 2 weeks. On the addition of copper-oxidized or acetylated LDL at a concentration of 30

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From the Department of Molecular Biology and Medicine (Y.W., A.S., M.K., A.I., T. Kohro, T. T. Kodama) and the Department of Genome Science (N.N., H.T.), Research Center for Advanced Science and Technology, University of Tokyo; the Division of Cellular and Molecular Pathology (T.Y., M.N.), Niigata University Graduate School of Medical and Dental Sciences, Niigata; the Department of Biochemistry, Cell Biology and Metabolism (S.Y., M.T.), Nagoya City University Graduate School of Medical Sciences, Nagoya; Research Center (T.T.), Research and Development, Grelan Pharmaceuticals Co., Ltd, Tokyo; Human Stress Signal Center (E.N.) and the Department of Metabolism, Endocrinology, and Molecular Medicine (K.H., S.Y., Y.M.), Osaka University Graduate School of Medicine, Osaka; and Chugai Pharmaceutical Co., Ltd (Y.K.), Shizuoka; Japan.
Correspondence to Tatsuhiko Kodama, Department of Molecular Biology and Medicine, Research Center for Advanced Science and Technology (335), University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-0061, Japan. E-mail kodama@med.rcast.u-tokyo.ac.jp

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μg/mL, foam cells were formed from macrophages. However, when a high concentration of LDL was applied to this coculture system in an attempt to directly observe the specific LDL modifications in the arterial wall, no foam cells developed, even in the presence of LDL at a concentration of 5 mg/mL.

Arterial wall cells are exposed to various types of stimuli, including the known examples of low oxygen tension, growth factors, cytokines, and rheological stress factors, but the specific contribution of each of these stimuli to LDL modification in the arterial wall has not proven to be easy to elucidate. In the course of normal aging, intimal thickening occurs in the human arterial wall, leading to an impaired oxygen diffusion capacity. Thus, a progressively worsening imbalance between the demand and supply of oxygen in the arterial wall has been widely reported to be a key factor in atherogenesis. Previous studies have suggested that the actual oxygen tension in the arterial wall is ~2% to 5%. To study the role of low oxygen tension in cellular lipid uptake in the arterial wall, we undertook examination of the effect of hypoxic conditions in our static coculture system. Oil red O–positive cells were observed under 2%, but not 21%, oxygen tension and were mostly composed of SMCs. This same phenomenon appeared in a monocultivation of human coronary arterial SMCs (CASMCs). In the present study, we present results that identify a critical step of lipid accumulation affected by oxygen tension, and we show that enhanced lipid accumulation is likely due to the increase of LDL-lipid uptake under hypoxic conditions. Furthermore, we help to elucidate the contribution of LDL receptors (LDLRs) by using the RASMCs of an LDLR-deficient strain.

Methods

Reagents

The reagents and their suppliers are as follows: DMEM, medium 199, trypsin–EDTA, penicillin-streptomycin, and other tissue culture materials were from GIBCO-BRL, Life Technologies, Inc; PBS was from Takara Co Ltd. EGM-2, the culture medium for endothelial cells, and SmGM-2, the medium for SMCs, both including cell growth supplements, were from Clonetics Corp. Collagenase D was from Roche Diagnostics GmbH. The 24-well tissue culture plates and inserts, including the known examples of low oxygen tension, growth factors, cytokines, and rheological stress factors, but the specific contribution of each of these stimuli to LDL modification in the arterial wall has not proven to be easy to elucidate. In the course of normal aging, intimal thickening occurs in the human arterial wall, leading to an impaired oxygen diffusion capacity. Thus, a progressively worsening imbalance between the demand and supply of oxygen in the arterial wall has been widely reported to be a key factor in atherogenesis. Previous studies have suggested that the actual oxygen tension in the arterial wall is ~2% to 5%. To study the role of low oxygen tension in cellular lipid uptake in the arterial wall, we undertook examination of the effect of hypoxic conditions in our static coculture system. Oil red O–positive cells were observed under 2%, but not 21%, oxygen tension and were mostly composed of SMCs. This same phenomenon appeared in a monocultivation of human coronary arterial SMCs (CASMCs). In the present study, we present results that identify a critical step of lipid accumulation affected by oxygen tension, and we show that enhanced lipid accumulation is likely due to the increase of LDL-lipid uptake under hypoxic conditions. Furthermore, we help to elucidate the contribution of LDL receptors (LDLRs) by using the RASMCs of an LDLR-deficient strain.

Lipoproteins

Lipoproteins were separated from the plasma of a normal healthy volunteer and were used within a few days of isolation.

Construction of Arterial Wall Model and Coculture With Monocytes

The arterial wall model coculture was constructed as reported previously. Human peripheral monocytes were added on day 0, floating monocytes were washed away with PBS, and fresh EGM-2 (2% rabbit serum) was added on day 1. On day 3, the medium was changed to fresh EGM-2 (2% rabbit serum) with or without LDL. On day 6, the medium was changed, and then the chamber was made hypoxic by using a hypoxic cultivation incubator supplied by Juji Field Co Ltd. After 72-hour hypoxic cultivation, coculture samples were prepared for various observations.

Cell Culture

CASMCs were grown in SmGM-2 medium in a 75-cm² flask. On the next day of passage in various cultivation apparatuses for each experiment and on day 4, the culture medium was changed to EGM-2 with 2% rabbit serum. On day 7, the culture medium was changed to fresh EGM-2 (2% rabbit serum) with or without LDL at a final concentration of 3 mg/mL. Then the cells were moved to normoxic or hypoxic incubators, cultivated for various time periods (including a maximal 72 hours), and fixed or recollected for analysis.

Histopathologic and Semiquantitative Analysis

After the experiments described above, the cells were recollected from the coculture and monoculture by means of collagenase D solution (2 mg/mL), attached to the glass slide by a cytospin apparatus (Shandon, Inc), and stained with oil red O as previously described. Semiquantitative analysis of lipid staining was performed after a grading of the lipid accumulation in each cell. The classification of stained cells was based on the measurable area of the lipid droplets in the cytoplasm. Cells without any lipid were designated −; cells with a lipid droplet area equal to the width the nucleus were designated +; and cells with lipid droplets twice that of the nucleus were designated ++. The number of cells classified as either + or ++ were counted as ORO + cells and compared in terms of the 2 levels of partial oxygen tension.

Double Staining Using Lipid Staining and Immunostaining

After inhibition of endogenous peroxidase activity by the method of Brown et al., we performed immunohistochemistry with the use of monoclonal antibodies against smooth muscle actin (SMA) or human macrophages. Monoclonal mouse anti-human SMA (mouse IgG2a–κ, Dako), monoclonal mouse anti-human macrophages, CD68 (mouse IgG1–κ, Dako), and anti-human von Willebrand factor antibody (Dako) were used as the primary antibodies. As a secondary antibody, we used a Histofine SAB-PO (M) kit (Nichirei) as described by the manufacturer. After visualization with 3,3'-diaminobenzidine (Dojin Chemical Co), sections were stained with oil red O and mounted with resin.

Lipid Extraction and HPLC Analysis

To determine the cellular cholesteryl ester content, the lipid was extracted and analyzed by a method modified from the literature. The cells were recollected and homogenized by freezing and thawing, followed by sonication with a microsonicator. The cell suspension aliquot was extracted with 2 vol of chloroform/methanol (2:1 [vol/vol]). The chloroform phase was removed under N₂ and redissolved in 50 μL acetoni trile/isopropanol (1:1 [vol/vol]). Free cholesterol and cholesteryl esters were separated by high-performance liquid chromatography (HPLC) on an LC18 column (Supelec, 25 cm×0.46 cm, 5-μm particle size), eluted with acetoni trile/isopropanol (45:55 [vol/vol]) at 1 mL/min, and then UV-quantified at 210 nm. The peak areas were compared with those of the corresponding authentic standards.
view fields, and the first 100 cells were counted for each view. Note that 15 times more oil red O–positive cells were observable at 2% than at 21% O₂. *P<0.01 vs 21% O₂ (n=5). d and e, Double staining of coculture cells by oil red O and anti-SMA antibody. Arrows indicate positive staining with anti-SMA antibody, and arrowheads indicate negatively stained cells. Asterisks indicate positive staining with oil red O. Note that the SMA-positive cells and oil red O–positive cells are identical at 2% O₂. Original magnification of microphotographs ×400. Bar=10 μm.

**Protein Assay**
Protein concentration was determined with a BCA protein assay reagent (Pierce), with serum albumin used as the standard.

**Preparation of Radiolabeled LDL and Autoradiography**
Incorporation of the radiolabeled cholesterol ester into LDL was performed as described previously.²⁴,²⁵ Radiolabeled LDL (relative radioactivity 5.3 Bq/μg protein) was applied to CASMCs or RASMCs grown in a 24-well plate at a concentration of 3 mg protein/mL. After cultivation, recollected cells were applied to a cytospin apparatus, stained by oil Red O, dipped in NTB-2 autoradiography emulsion (Kodak), and exposed at 4°C for 3 weeks. Autoradiograms were developed in Kodak D-19 solution (diluted 1:1 with water) for 1 minute at room temperature, fixed, rinsed in water, and then back-stained with Mayer’s hemalum solution (Merck).

**Incorporation of [14C]Acetate Into [14C]Cholesterol**
Cholesterol synthesis assay was assayed as described previously.²⁶ After 70 hours, hypoxic or normoxic cultivations of CASMCs at 9×10⁵ Bq per well of [2-¹⁴C]sodium acetate (NEN Life Science Products, Inc) were applied to each 6-well culture plate. After 2 hours of cultivation, cells were washed and solubilized with NaOH. After saponification, [¹⁴C]cholesterol was isolated and quantified by high-performance thin-layer liquid chromatography (Merck).

**Release of Cellular Cholesterol**
Assays were performed as described previously.²⁷ Briefly, cells were prelabeled for 24 hours with medium containing 148 kBq [4-¹⁴C]cholesterol/mL. Subsequently, cells were incubated for 8 hours in a medium with only 0.2% ovalbumin (Sigma) to allow for equilibration of the radioactive isotope in the various cellular cholesterol pools. CL277082 (Grelan Pharmaceuticals Co Ltd), an acyl coenzyme A:cholesterol acyltransferase inhibitor, was added during labeling and equilibration (1.5 μmol/L). Then cells were rinsed 3 times with PBS and fresh medium (EGM-2, 2% rabbit serum) containing CL277082, with or without LDL (final concentration 3 mg/mL). The cholesterol release assay was initiated by incubating cells at 37°C under 21% or 2% oxygen tension. After 2-, 6-, 12-, and 72-hour incubation, culture medium was removed, and radioactivity was determined. A value of 5×10⁻⁴ mmol/Bq was used to calculate the amount of cholesterol.

**Northern Blotting**
Northern blot analysis was performed as described elsewhere.²⁸ The nitrocellulose membranes were hybridized with a cDNA probe spanning 1218 to 1515 of the LDLR cDNA, a scavenger receptor class A type I–specific probe amplified by reverse transcription–polymerase chain reaction with use of the primers macrophage scavenger receptor I (MSRI) forward (5’ TCAATGACAGCTTTGCTTCCCG) and MSRI reverse (5’ TCTATTGGCTCCCCATGTCCTG),²⁹ a CD36 probe,³⁰ and a tubulin probe.

**Statistical Analysis**
Results are given as mean±SD. Statistical comparisons were performed with the 2-tailed Student paired t test. Results were considered significant at P<0.01.

**Results**
**Hypoxic Cultivation of the Coculture**
To evaluate the effect of hypoxia on foam cell formation, we performed a coculture of arterial wall cells and macrophages with LDL loading under hypoxic conditions. Online Figure I (please see http://atvb.ahajournals.org) provides a diagram of the hypoxic coculture cultivation. For the final 72 hours, arterial wall cells and macrophages were cultivated in the medium along with LDL at a concentration of 3 mg/mL at either 21% O₂ or 2% O₂. Panels a and b of Figure I are photomicrographs of cells that were collected from the extracellular matrix layer of the coculture and applied to the arterial wall cells and macrophages were cultivated in the coculture system. a and b, Photomicrographs of cytospin samples stained by oil red O. Back-staining using hematoxylin eosin was performed. Representative images are shown at 21% O₂ (a) and 2% O₂ (b). The large columns were originally at ×100 magnification, and the small columns were at ×400 magnification. c, Numbers of cells stained positively with oil red O per 100 cells. Cytospin samples were observed at ×100 magnification, and cells were classified on the basis of the area of lipid droplets in their cytoplasm. Cells without lipid droplets were designated –; cells with a lipid droplet area the width of the nucleus, +; and cells with lipid droplets twice the width of the nucleus, ++. Cells classified as + and ++ were counted as ORO + cells, and their number was compared for 2 levels of oxygen tension. In each sample, the average number was obtained from 5 separate
SMCs, even in the cultivated state. Therefore, these results suggest that it is SMCs that actually have accumulated lipid in the coculture.

**Hypoxic Monoculture of RASMCs and CASMCs**

To observe whether lipid accumulation in SMCs under hypoxic conditions holds in the case of monocultivation as well as in the coculture, RASMCs and human CASMCs were loaded with LDL at 2% O2. The lipid staining results are shown in online Figure II (please see http://atvb.ahajournals.org) and Figure 2. Online Figure Iia through IId illustrates the results of oil red O staining of RASMCs cultivated in the same medium as the coculture along with EGM-2 and 2% rabbit serum. In the absence of LDL, no cell stained positively with oil red O; i.e., there was no staining at either 21% O2 (online Figure Iia) or 2% O2 (online Figure IIc). With LDL loading at a concentration of 3 mg/mL, positively stained cells were observed only at 2% O2 (online Figure IId) but not at 21% O2 (online Figure IIb), strongly suggesting that only with 2% O2 were cells accumulating lipids in their cytoplasm.

Figure 2a through 2h shows the lipid staining in CASMCs. Not only is the lipid stain in fact observable in cells subjected to LDL loading compared with the cells not subjected to LDL loading (Figure 2a), the intensity of the oil red O stain is also quite clearly inversely correlated with oxygen tension (Figure 2b through 2d). The most significant lipid accumulation was observed at 2% O2.

To confirm the role of LDL, CASMCs were cultivated at 2% O2 with various concentrations of LDLs. Figure 2d through 2g illustrates that a greater lipid accumulation was readily obvious at higher concentrations. To our knowledge, the best evidence indicates that arterial SMCs can just barely accumulate lipids under conditions of a loading of native LDL under normal oxygen pressure, ostensibly because of the positive-feedback regulation of LDLs. However, as is shown in Figure 2h, lipid droplets in the cytoplasm were detectable on cultivation of these cells under lipid loading and at 2% O2. The evidence indicates that RASMCs and human CASMCs are able to more efficiently accumulate lipids under hypoxic conditions.

**Lipid Profiles of CASMCs**

To confirm that the lipid accumulation indicated by the oil red O staining had indeed occurred, as well as to directly quantify the amount of cholesteryl esters collected in the cells, we determined the precise amount of lipids extractable from CASMCs. Figure 3 presents the result of HPLC analysis. The total cholesteryl ester amount increased in a manner inversely correlated to the oxygen tension. This was especially the case at 2% O2 tension, where twice the total cholesteryl ester amount was observed compared with that obtained at 21% O2. At the same time, the cellular free cholesterol was measured, but no significant difference was found. Cholesteryl esters have been reported to accumulate in atherosclerotic lesions characterized by foam cells or fatty streak formation, which suggests that this in vitro lipid accumulation shares common features with the in vivo lesion. Thus, we conclude that the increase of cholesteryl ester may explain, at least in part, the lipid accumulation detected by oil red O staining.

**LDL Uptake Activity of CASMCs**

Because lipid accumulation in CASMCs depends on LDL loading, enhanced LDL uptake is thought to play a part in the accumulation process. To estimate the LDL uptake that took place under relatively high concentration conditions (maxi-
mally 3 mg/mL), we used a novel autoradiographic method. Previous studies of LDL uptake have been performed by means of degradation assay, but at a concentration of 3 mg/mL, obtaining data with a satisfactory signal-to-noise ratio and performing inhibition assays with nonlabeled LDLs are impractically difficult. By combining the radiolabeling of lipoprotein with autoradiography, a new method has been brought into play by which it is possible to quantitatively estimate the lipoprotein uptake even at high concentrations.

Figure 4a through 4c shows photomicrographs of autoradiograms taken of typical cells cultivated at 21% or 2% O_2 with or without LDL loading. More grains are observable at 2% O_2 (Figure 4c) than at 21% O_2 (Figure 4b), suggesting that a greater amount of LDL has been taken up under the lower oxygen tension. Figure 4d illustrates the number of grains observed in the cytoplasm. Under LDL loading, ~2.4 times more LDL was taken up by CASMCs at 2% O_2 than at 21% O_2. In addition, the grains found inside the cell usually were located in the cytoplasm rather than the nucleus. This location supports the contention that these grains reflect the presence of LDL, which has been taken up. LDL uptake is generally thought to be downregulated by LDL loading under each level of oxygen tension.

Cholesterol Synthesis Activity of CASMCs
In an effort to elucidate the specific mechanisms of lipid accumulation, cholesterol synthesis and cholesterol efflux activity were also assayed. Online Figure IIIa (please see http://atvb.ahajournals.org) represents the HPTLC of the lipid prepared from CASMCs, and each band represents the cholesterol that was synthesized from incorporated radiolabeled acetic acid. This band disappeared on the addition of LDL at a concentration of 3 mg/mL under both levels of oxygen tension. Quantification of synthesized cholesterol by the radioactivity in cholesterol corrected for cell protein (online Figure IIIb) further confirmed the suppression of cholesterol synthesis. In the absence of LDL loading, ~6 times more labeled cholesterol was detected at 2% O_2 than at 21% O_2. However, the mechanism of such enhanced synthesis has been obscure. From these data, de novo synthesis can be seen to be negatively regulated by LDL loading under each level of oxygen tension.

Cholesterol Release Efficiency of CASMCs
Online Figure IV (please see http://atvb.ahajournals.org) shows the radioactivity recollected from the culture medium of CASMCs that had been radiolabeled by cholesterol. With LDL loading, efficient release was observed after 72 hours of cultivation and reached 10.6 nmol cholesterol/mg cell protein at 21% O_2 and 11.0 nmol cholesterol/mg cell protein at 2% O_2. There was no significant difference in release efficiency between the 2 levels of the oxygen tension. In the absence of LDL loading, cholesterol release efficiency was restricted to 2.60 nmol cholesterol/mg cell protein at 21% O_2 and to 2.57 nmol cholesterol/mg cell protein at 2% O_2. Cholesterol release usually increases when the cytoplasm is filled with lipid, and a greater release is observed with more acceptors present in the culture medium. In the present study, this previously reported tendency was confirmed and was not measurably affected by the oxygen tension.

Northern Blot of Native and Modified LDLRs
As shown in online Figure V (please see http://atvb.ahajournals.org), LDL loading suppresses LDLR expression at 21% O_2.
At 2% O₂, the expression level of the LDLR was the same as that of the reduced level at 21%, and there was no difference between the 2 levels of lipoprotein concentration. Thus, the positive feedback of LDL uptake is partly achieved by a downregulation of the expression level of the LDLR, and this was observed at 21% O₂. However, at 2% O₂, this regulatory response was not observed. CD36 and scavenger receptor class A type I/II were studied as possibly modified versions of the LDLR, but their expression was lower than the detection level. In advanced atheromatous lesions, CD36 and scavenger receptor class A type I/II are expressed by SMCs, but in the present study, they were not found to have any detectable involvement.

**Lipid Accumulation in LDLR-Deficient Rabbit SMCs**

Although a decrease in the transcriptional level of the LDLR was observed, enhanced turnover is conceivably responsible for the lipid accumulation in SMCs. Figure 5a through 5d illustrates oil red O staining in the wild-type Japanese white rabbit and in the KHC rabbit, a strain lacking LDLR. In both of these strains, SMCs were not stained by oil red O in the absence of LDL loading (data not shown) or even with LDL loading (Figure 5a and 5c). However, under the condition of 2% O₂, LDL loading caused significant lipid staining of SMCs, and this was true in both strains (Figure 5b and 5d). Figure 5e though 5h shows the autoradiogram of RASMCs loaded with radiolabeled LDLs under normoxia or hypoxia. In the case of the wild-type and the KHC rabbit, more grains were observable in the cytoplasm under hypoxia than under normoxia. Although more LDL was taken up by LDLR-deficient SMCs than by wild-type SMCs, almost twice the number of grains was determinable under hypoxia in both types of SMC (Figure 5i). Because KHC rabbits are characterized by a loss of functional LDLR resulting from a 12-bp deletion in the N-terminus, these data suggest that the LDLR does not play an important role in the lipid accumulation that occurs under hypoxic conditions.

**Discussion**

Proliferation of arterial SMCs in the medial layer during aging begins in human beings in their 20s,12 eventually resulting in an increase of the thickness of the arterial wall and leading to an impaired oxygen diffusion capacity. Yet despite this widely known phenomenon having been known for some time, to date, the role played by low oxygen tension in atherogenesis is still not well understood. In the present study, we have demonstrated that SMCs accumulate more lipid under low oxygen tension than under normoxic conditions.

The presence of oxidized lipid in atherosclerotic lesions is well documented,13 and we have reported foam cell formation in an arterial wall model system6 in which copper-oxidized LDL was used. To elucidate the precise process from lipid peroxidation to its accumulation in arterial wall cells, native LDL was used as a source of the lipid in the experiments reported in the present study. In these coculture experiments, loading of native LDL failed to induce foam cells in vitro, even at a final concentration of 5 mg/mL LDL. However,
among the applied physiological stimuli, hypoxia was able to induce 20 times more lipid accumulation in the arterial wall cells (Figure 1c). This is the first evidence to suggest that at least 1 arterial wall cell can accumulate lipid as a consequence of LDL loading under hypoxic conditions.

The second finding that supports a role for hypoxia in lipid accumulation was obtained from the monocultivation of SMCs. To make foam cells in vitro, SMCs were incubated with LDL, but only a small amount of cholesteryl esters accumulated. This failure is best explained by the positive feedback of LDL uptake. Moreover, SMCs of 2 different species accumulated lipid more efficiently at 2% O₂ than at 21% O₂ (online Figure II and Figure 2). This observation corresponds with previous reports showing that hypoxic stress stimulates lipid accumulation by SMCs in vivo and in vitro. On the basis of lipid extracted from SMCs, esterified cholesterol, the most prominent component accumulated in the atheromatous lesion, has been shown to accumulate under hypoxia (Figure 3). Considering these reports together with the findings of the present study, we conclude that hypoxia is essential for enhancing lipid accumulation in arterial SMCs.

The third line of evidence that argues for the role of hypoxia in lipid accumulation is the enhancement of LDL uptake under low oxygen tension. CASMCs were able to take up twice the LDL at 2% O₂ than at 21% O₂ (Figure 4d), but inhibition of cholesterol synthesis by LDL (online Figure IIIa and IIIb) and the efficiency of cholesterol release (online Figure IV) were unaffected by oxygen tension. This observation is consistent with earlier reports on the metabolism of SMCs under hypoxia. Taking these results together, lipid accumulation in CASMCs under hypoxia is brought about, for the most part, by an increased uptake of LDL, and neither cholesterol synthesis nor cholesterol release from CASMC contributes to this phenomenon.

Although these results demonstrate enhanced uptake of LDL by SMCs under hypoxia, the precise mechanism of lipid transfer remains to be worked out. Because the intensity of lipid staining increased in proportion to the LDL concentration (Figures 2d through 2g), the lipids accumulated in SMCs may be derived from LDL. The expression level of the LDLR was repressed by LDL loading at 21% O₂ (online Figure V), which may be regulated at the level of transcription by sterol regulatory element binding protein-2 (SREBP-2). At 2% O₂, the level was the same as the repressed level with LDL loading under normoxia, and no further suppression was found with further LDL loading. To closely analyze the functional turnover of the LDLR, we used LDLR-deficient rabbit SMCs. The data (Figure 5) obtained clearly show that the LDLR pathway was not involved in this phenomenon.

Another possible pathway of lipid uptake is the scavenger pathway of modified lipoproteins, including complexes formed with other proteins. However, no such aggregation was detected in the culture medium with LDL. The extent of oxidative modification, as estimated from the amount of the lipid peroxidation products of LDL, such as hydroperoxide and hydroxide from cholesterol esters (CEOOH and CEOH), was lower under hypoxia. The amount of CEOOH and CEOH was 0.339±0.069 mol/mol LDL at 21% O₂ and 0.112±0.031 mol/mol LDL at 2% O₂, respectively (mean±SD, n=4; P<0.005; unpublished data, by Y.W. and N.N., 2002). Because CD36 and scavenger receptor class A type I/II are known to be modified LDLRs and are expressed by SMCs in advanced atheromatous lesions, their expression levels were also studied. However, their expression level was lower than the detection level (online Figure V). These findings suggest that neither oxidative modification of lipoprotein nor the scavenger receptor pathway contributed to lipid accumulation under hypoxia. Although the precise mechanisms remain to be elucidated, massive lipid accumulation in CASMCs by LDL loading under hypoxic conditions can be taken to be a representative model of the atherosclerotic lesion SMCs observed in vivo, where LDL accumulation in the extracellular matrix takes place in the arterial wall under endogenous hypoxic conditions.

In the present study, an endothelium growth medium supplemented with 2% rabbit serum was used to maintain a functional monolayer of RAECs in coculture and to reproducibly induce lipid accumulation even in monocultivation of SMCs. This medium contains several supplements: epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, and hydrocortisone. These supplements have all been reported to have an influence on lipid metabolism. Although these effects might be construed as bringing the data in this report into question, this point is probably best understood as being dependent on the difference in the time window of the highest LDL concentrations, differences in cultivation conditions, including those in the culture medium, and the concentration of growth factors. Therefore, further studies are necessary to determine whether enhanced lipid accumulation in SMCs can be brought about under other conditions and, if so, exactly what those conditions are.

In conclusion, we have shown enhanced cholesteryl ester accumulation in CASMCs at 2% O₂. This accumulation was chiefly mediated by an enhanced uptake of lipid from LDL particles. Although the molecular mechanism of lipid uptake has at present not been determined, this culture model should nevertheless prove useful in helping to elucidate the specific steps of foam cell formation from SMCs, as has been observed in the hypoxic medial layer of the arterial wall under physiological conditions. Furthermore, hydrophobic antioxidants may be delivered to SMCs by using LDL as a shuttle vehicle without the use of organic solvents, such as dimethyl sulfoxide, and their antiatherogenic effects may be elucidated by means of this model system. To address these questions, further studies will be undertaken characterizing LDL in the culture medium as well as identifying the specific genes involved.

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