Hypoxia Increases LDL Oxidation and Expression of 15-Lipoxygenase-2 in Human Macrophages

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Objective—Macrophage-mediated oxidation of low-density lipoprotein (LDL) by enzymes, such as the lipoxygenases, is considered of major importance for the formation of oxidized LDL during atherogenesis. Macrophages have been identified in hypoxic areas in atherosclerotic plaques.

Methods and Results—To investigate the role of hypoxia in macrophage-mediated LDL oxidation, we incubated human monocyte-derived macrophages with LDL under normoxic (21% O_2) or hypoxic (0% O_2) conditions. The results showed that hypoxic macrophages oxidized LDL to a significantly higher extent than normoxic cells. Interestingly, the mRNA and protein expression of 15-lipoxygenase-2 (15-LOX-2) as well as the activity of this enzyme are elevated in macrophages incubated at hypoxia. Both the unspliced 15-LOX-2 and the spliced variant 15-LOX-2sv-a are found in macrophages. In addition, 15-LOX-2 was identified in carotid plaques in some macrophage-rich areas but was only expressed at low levels in nondiseased arteries.

Conclusions—In summary, these observations show for the first time that 15-LOX-2 is expressed in hypoxic macrophages and in atherosclerotic plaques and suggest that 15-LOX-2 may be one of the factors involved in macrophage-mediated LDL oxidation at hypoxia. **(*Arterioscler Thromb Vasc Biol*. 2004;24:2040-2045.)**

Key Words: atherosclerosis ■ macrophages ■ hypoxia ■ oxidized-LDL ■ 15-lipoxygenase-2

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An early phenomenon in atherosclerosis is the retention, oxidation, and accumulation of low-density lipoprotein (LDL) in the vessel wall.[1,2] Oxidized LDL (oxLDL), one of the key players in atherogenesis, attracts monocytes to the vessel wall where they differentiate into macrophages.[3,4] Oxidation of LDL mediated by macrophages is considered to be of major importance for the formation of oxLDL within the atherosclerotic plaque. Enzymes involved in this process are 15-lipoxygenase (15-LOX),[5,6] myeloperoxidase (MPO),[7] and NADPH oxidase.[8] Macrophages in the arterial wall take up oxLDL through scavenger receptors and accumulate oxLDL as cholesterol esters, which results in foam cell formation.

The thickness of the arterial wall increases as the atherosclerotic plaque develops. This leads to an impaired diffusion, which results in oxygen and nutrient deficiency in the deeper portion of the arterial intima and in atherosclerotic plaques. Simultaneously, oxygen consumption by cells within the plaque rises,[9,10] which could be because of the increased number of energy-consuming foam cells.[10] In healthy tissues, oxygen tension is 20 to 70 mm Hg (2.5% to 9% O_2). However, in diseased tissue, eg, in atherosclerotic plaques, inadequate perfusion may reduce O_2 tension to below 10 mm Hg (<1% O_2) in some regions.[11] Results from our laboratory have previously shown that areas of hypoxia occur within atherosclerotic plaques in cholesterol-fed rabbits.[12] Hypoxia may lead to retention of macrophages in these areas, because it has been shown that macrophage migration is reduced by hypoxia.[13]

The role of hypoxia in the development of atherosclerotic plaques is not known. In this study, we have explored the effect of hypoxia on macrophage-mediated LDL oxidation and the expression of enzymes, which could be involved in this process. We found that hypoxia increases macrophage-mediated LDL oxidation but also the mRNA, protein expression, and activity of 15-LOX-2 in macrophages. This enzyme was also identified in atherosclerotic plaques. These findings suggest that 15-LOX-2 could be an enzyme involved in hypoxia-induced LDL oxidation in atherosclerotic plaques.

Materials and Methods

Macrophage Preparation

Human mononuclear cells were isolated from buffy coats, obtained from the blood bank at Sahlgrenska University Hospital, Hospital, Göteborg, Sweden, and isolated using Ficoll-Paque discontinuous

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gradient centrifugation (Amersham Pharmacia Biotech).[14] Cells were seeded at a density of 1.25×10^6 cells per mL and cultured as previously described.[15] Macrophages in 6-well plates (1.5 mL per well) were used to study mRNA expression and LDL oxidation. Cells plated on Petri dishes with a diameter of 10 cm (8 mL per dish) were used for Western blot analyses.

Macrophage Experiments

Macrophages were incubated with or without 50 μg/mL LDL under normoxic (21% O_2) or hypoxic (0% O_2) conditions. For details on LDL preparation, please refer to the online Methods, available at http://atvb.ahajournals.org. For hypoxia, the medium was equilibrated to 0% O_2 with 5% CO_2 and 95% N_2, and macrophages were incubated in a humid incubator at 37°C with a constant flow of 5% CO_2 and 95% N_2. After incubation, the cells were immediately harvested in a hypoxic chamber and collected in the different lysis buffers. Normoxic cells were incubated under normal cell culture conditions at 37°C with 21% O_2, 5% CO_2, and 74% N_2. Total cell protein extracts were harvested in 0.2 mol/L NaOH, and protein concentrations were determined using the Bradford assay.[16] Potential cytotoxic effects of the different culture conditions were measured as lactate dehydrogenase leakage in a Cobas-BIO autoanalyzer. Lactate dehydrogenase leakage from cells was <13%, indicating that the cells were viable under the culture conditions used.

LDL Oxidation

LDL from media incubated with macrophages for 24 hours at normoxia or at hypoxia was reisolated by sequential centrifugation (density=1.019 to 1.063 g/mL), and 20 μg of the LDL was characterized after electrophoresis on a 0.5% agarose gel. The protein was visualized after staining with Coomassie brilliant blue. Further characterization of LDL was done as described[17] and expressed as thiobarbituric acid reactive substances (TBARS; nmol malondialdehyde [MDA] equivalents per mg LDL). LDL oxidation was also analyzed by the formation of conjugated dienes measured as absorbance at 234 nm in the medium.[18]

DNA Microarray Analysis and Real-Time RT-PCR

Total RNA was isolated with the RNeasy kit (Qiagen) from macrophages incubated at normoxia or hypoxia for 24 hours. RNA was analyzed as described in the online Methods.

Splice Variants of 15-LOX-2

15-LOX-2 exists as 3 splice variants (15-LOX-2sv-a/b/c). Compared with the unspliced form, 15-LOX-2sv-a and 15-LOX-2sv-b are shorter variants caused by deletions of exons, whereas the 15-LOX-2sv-c contains an additional 80-bp segment.[19] The Taqman reverse transcriptase reaction kit, with random hexamer primers (Applied Biosystems), was used to synthesize cDNA from RNA of both normoxic and hypoxic macrophages. To identify the splice variants, the polymerase chain reaction (PCR) was performed and analyzed as described in the online Methods.

Tissue Samples

Fresh surgical specimens of human carotid atherosclerotic plaques and nondiseased internal mammary arteries were obtained from surgery, according to protocols approved by the Ethical Research Committee at Sahlgrenska University Hospital.

Western Blot

Macrophages incubated under hypoxic or normoxic conditions for 8, 24, or 48 hours were harvested in lysis buffer (0.15 mol/L NaCl, 10 mmol/L Tris·HCl, pH 7.2, 2 mmol/L EDTA, and 1% Triton X-100) with protease inhibitors (Complete Mini; Roche Diagnostics). Tissue extracts of surgical specimens were prepared as previously described.[20] 15-LOX-2 positive control tissue extracts from prostate glands were a generous gift from Dr Scott B. Shappell at the Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tenn. Protein concentrations were determined with the BC Assay (Optima, Biosite), and 50 μg protein (cell lysates) or 40 μg protein (tissue extracts) were separated on an 8% SDS-PAGE under nonreducing conditions and transferred to polyvinylidene fluoride membranes (BioRad) as described.[21] Immunoreactive bands were visualized with rabbit anti–15-LOX-2 (1:1000; Oxford Biomedical Research, Oxford, Mich) and peroxidase-conjugated swine anti-rabbit immunoglobulin (IgG; 1:3000; DAKO, Carpinteria, Calif) using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Bands were densitometrically analyzed with ImageQuant 5.0 Software (Academic Computing Health Sciences).

Immunohistochemistry

Serial formalin-fixed and paraffin-embedded sections of human carotid atherosclerotic plaques and nondiseased internal mammary artery were analyzed by immunohistochemistry after high temperature antigen unmasking. Sections were stained with rabbit polyclonal anti–15-LOX-2 (1:150; Oxford Biomedical Research, Oxford, Mich), mouse monoclonal anti-human CD68 (Ki-M6; 1:100; BMA Biomedical AG, Augst, Switzerland), and mouse monoclonal anti-human α-actin (1:2000; Cedarlane Laboratories Ltd, Ontario, Canada). Proteins were visualized with the ABC (avidin-biotin-peroxidase complex) method (Vector Laboratories, Petersborough, UK) using donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, Penn) and donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, Penn) as secondary antibodies. Hematoxylin was used for nucleus staining. As negative controls for the 15-LOX-2 stainings, 2 plaque sections were either stained with PBS or rabbit IgG instead of primary antibody.

15-LOX-2 Enzyme Activity

Macrophages treated with normoxia or hypoxia for 24 hours were collected in PBS supplemented with protease inhibitor (Complete Mini; Roche Diagnostics). The cells were lysed by freezing (−80°C) and thawing 5× before 100 μmol/L arachidonic acid (AA; Cayman Chemical) was added and incubated for 30 minutes at room temperature. The cell reaction mixture was stored at −80°C until liquid chromatography/mass spectrometry (LC/MS) analysis. AA was also incubated in PBS for 30 minutes. Five mL of methyl-tert-butyl ether/hexan (50:50, v/v) were added to the cell homogenate (250 μL). The extraction was performed at pH=3 for 15 minutes at room temperature. The organic phase was separated (1500*g*, 5 minutes) and evaporated under nitrogen, the residue was reconstituted in 100 μL of the mobile phase consisting of methanol/water (50:50, v/v) with acetic acid (0.1%), and 10 μL was injected into an LC/MS system. The chromatography was performed on a 5 μm Thermo column (HyPURITY C18, 50×2.1 mm) at a flow rate of 0.3 mL/min. Single ion monitoring was performed on a Platform LCZ (Micromass) in electrospray ionization negative mode using ion mass-to-charge ratio (m/z) 319.2. Quantification was performed against external standard (15-HETE) and deuterated internal standard (15-HETE-d8) from Cayman Chemicals.

Statistical Analysis

Results are shown as mean±SD. Differences between groups were assessed with Student 2-tailed paired *t* test. $P<0.05$ was considered statistically significant.

Results

Hypoxia Increases Macrophage-Mediated LDL Oxidation

LDL oxidation was studied by incubating LDL with macrophages under normoxic or hypoxic conditions. LDL in cell culture media incubated with hypoxic macrophages for 24 hours had increased electrophoretic mobility compared with LDL in media from normoxic cells (Figure IA, available online at http://atvb.ahajournals.org). Furthermore, TBARS

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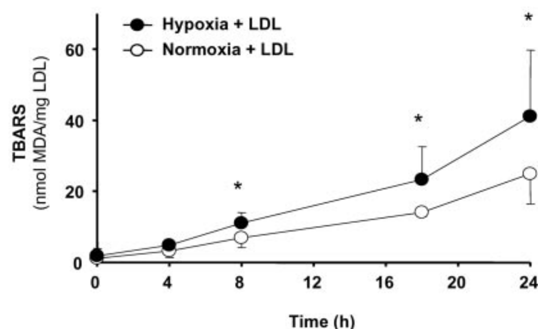

Figure 1. Oxidation of LDL by macrophages incubated at normoxia or at hypoxia. LDL oxidation by macrophages (n=6) is illustrated as TBARS. Values are mean±SD. *$P<0.05$ by Student 2-tailed paired *t* test.

from LDL incubated with hypoxic macrophages was higher than TBARS obtained from LDL incubated at normoxia (from 2 to 40 nmol MDA/mg LDL versus 2 to 24 nmol MDA/mg LDL, $P<0.05$; Figure 1). In line with these results, the diene formation was also higher in LDL incubated with hypoxic macrophages compared with normoxic cells (Figure IB). In contrast, no LDL oxidation was found in media without macrophages (data not shown), suggesting that the LDL oxidation observed is mediated by the macrophages. Together these results, obtained with 3 different methods, suggest that hypoxic macrophages oxidize LDL more under hypoxic conditions than at normoxia.

Hypoxia Induces Expression of 15-LOX-2 in Macrophages

Oxidation of LDL by macrophages is suggested to be mediated by 15-LOX, MPO, and NADPH oxidase.[5–8] These enzymes could therefore be involved in the LDL oxidation mediated by hypoxic macrophages. DNA microarray analysis showed that the 15-LOX-2 mRNA expression was increased 4-fold at hypoxia, whereas the expression of other enzymes, suggested to be involved in LDL oxidation, was not significantly affected (Figure II, available online at http://atvb.ahajournals.org). RT-PCR confirmed that the 15-LOX-2 mRNA expression was increased over time when macrophages were incubated at hypoxia. 15-LOX-2 mRNA was 2-fold higher after 8 hours of hypoxia and almost 6-fold higher after 24 hours ($P<0.001$) but was unchanged at normoxia (Figure 2A). In contrast, only a low constitutive expression of 15-LOX-1 and MPO mRNA was found in these cells. Western blot showed that the protein expression of 15-LOX-2 increased over time and was 5-fold higher in hypoxic compared with normoxic macrophages at 48 hours ($P<0.05$; Figure 2B). A tendency of increased 15-LOX-2-protein expression was seen at 24 hours, although this result was not significant. These observations show that hypoxia increases both the mRNA and protein expressions of 15-LOX-2 in macrophages, whereas the mRNA expressions of NADPH oxidase, 15-LOX-1, and MPO are low and unaffected by hypoxia.

Macrophages Express Splice Variants of 15-LOX-2

Both normoxic and hypoxic macrophages express unspliced 15-LOX-2 and the spliced variant 15-LOX-2sv-a. The results obtained with primers A and C (Figure 3A) showed a PCR product of 368 bp which corresponds to either the unspliced

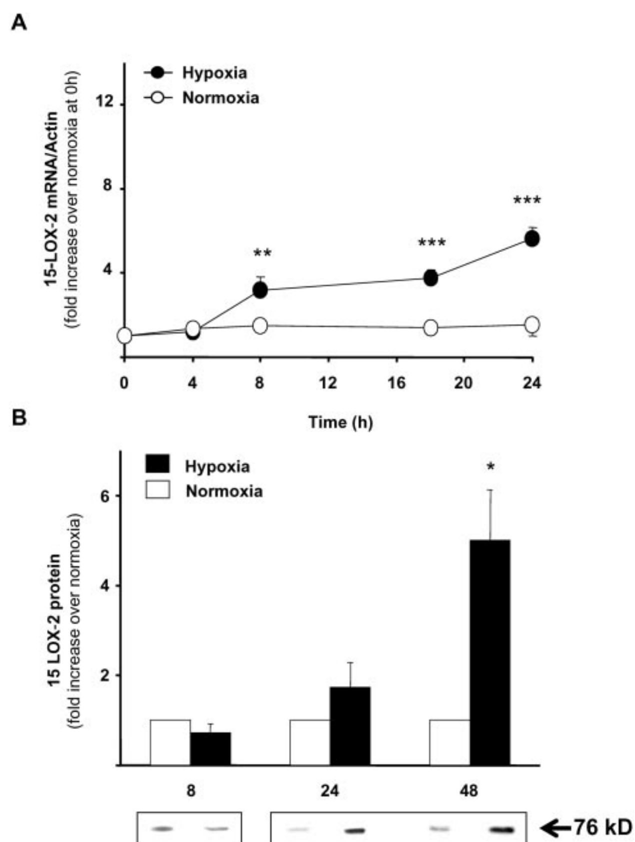

Figure 2. Effects of hypoxia on 15-LOX-2 mRNA and protein expression. A, Total cellular RNA from macrophages (n=4) incubated at normoxia or at hypoxia for 24 hours was extracted for RT-PCR analysis. The amount of 15-LOX-2 mRNA was normalized to actin mRNA expression. Values are mean±SD. *$P<0.05$, **$P<0.01$ by Student 2-tailed paired *t* test. B, The protein expression of 15-LOX-2 in macrophages (n=6) incubated at normoxia or at hypoxia for 8, 24, or 48 hours was determined by Western blot using a specific antibody against 15-LOX-2, and the bands were densitometrically analyzed. Values are mean±SD. *$P<0.05$ by Student 2-tailed paired *t* test. Below the graph, a representative Western blot is shown.

form of 15-LOX-2, the spliced variant of 15-LOX-2sv-a, or both. No PCR products corresponding to the splice variants 15-LOX-2sv-b (233 bp) and 15-LOX-2sv-c (448 bp) were found, indicating that these two splice variants were not expressed in macrophages. To separate unspliced 15-LOX-2 from the spliced variant 15-LOX-2sv-a, primers B and C were used and two PCR products were obtained (Figure 3B). The 619-bp product corresponds to the unspliced form of 15-LOX-2, whereas the 532-bp product corresponds to the spliced variant 15-LOX-2sv-a. No further PCR products were found, which confirmed the results obtained with primers A and C. Together these observations show that the unspliced form of 15-LOX-2 and the spliced variant 15-LOX-2sv-a were expressed in macrophages incubated at both hypoxia and normoxia.

Macrophages Express Active 15-LOX-2

15-HETE was formed when cell lysates from macrophages treated with normoxia or hypoxia for 24 hours were incubated with AA and analyzed by LC/MS. 12-HETE, which appeared

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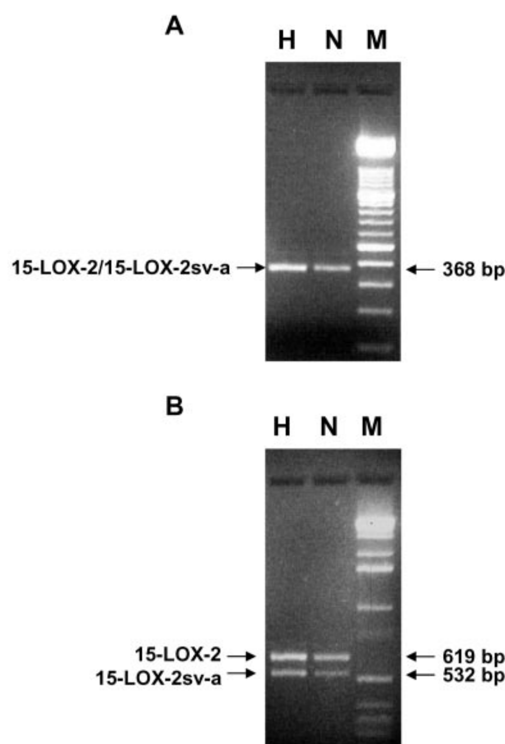

Figure 3. The mRNA expression of 15-LOX-2 variants in macrophages. cDNA was synthesized with RT-PCR from total RNA in macrophages incubated at hypoxia (H) or normoxia (N) for 24 hours. A, With the PCR primers A and C (see online Methods), one product was synthesized, corresponding to the unspliced 15-LOX-2 or the spliced variant 15-LOX-2sv-a or both. The XIV DNA ladder from Roche was used as marker (M). B, With the PCR primers B and C, two products were synthesized, corresponding to the unspliced 15-LOX-2 and the spliced variant 15-LOX-2sv-a. The 1-kb DNA Ladder from Invitrogen was used as marker (M).

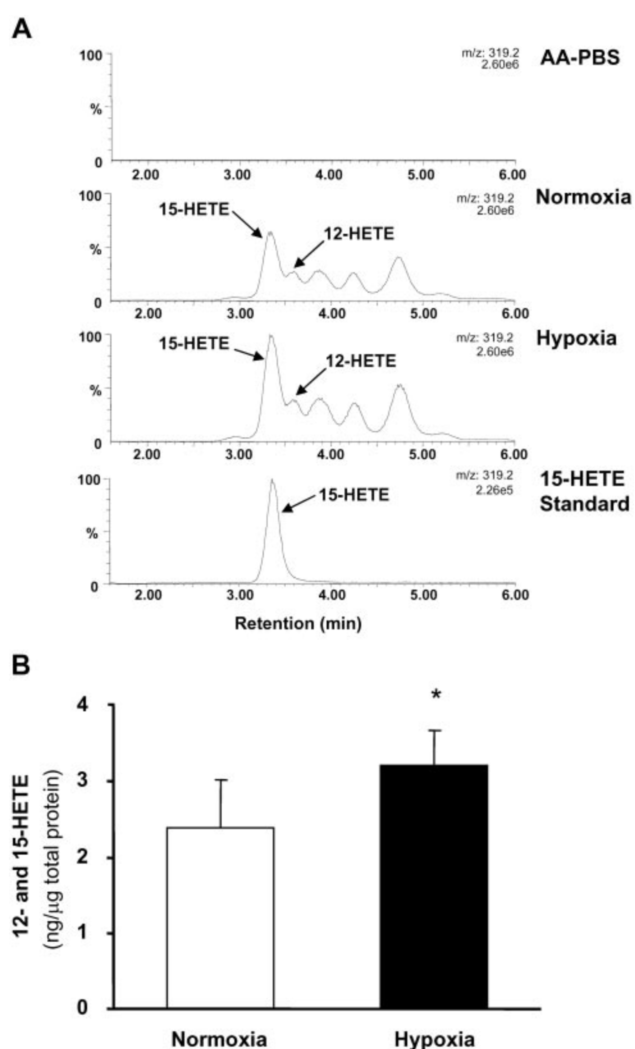

Figure 4. Enzyme activity of 15-LOX-2 in macrophages incubated at normoxia or hypoxia for 24 hours. The cell lysates (n=4) were incubated with arachidonic acid (AA) for 30 minutes at room temperature. After extractions, the enzyme product 15-HETE was separated and detected on an LC/MS system. A, A typical chromatogram obtained after a separation in an LC/MS. AA was incubated with PBS, and with cell lysates from normoxic and hypoxic macrophages. In the last chromatogram only the external standard, 15-HETE, was separated. B, The amount of 12- and 15-HETE formed by cell lysates from macrophages incubated at normoxia or hypoxia after incubation with AA. Quantification of 15-HETE was performed against an external standard. Values are mean±SD. **P*<0.05 by Student 2-tailed paired *t* test.

immediately after 15-HETE, could not be efficiently separated from 15-HETE under the conditions used in this study. Nevertheless, these results show that substantially more 15-HETE than 12-HETE was formed (Figure 4A). Because these compounds were not sufficiently separated, the production of both 15-HETE and 12-HETE was considered when the activities in the lysates from macrophages were estimated. (Figure 4B). These results confirm that the 15-LOX-2 in hypoxic macrophages is an active enzyme.

15-LOX-2 Protein Is Elevated in Carotid Plaques

The presence of 15-LOX-2 was studied in tissue extracts from carotid atherosclerotic plaques and from normal arterial tissue from the internal mammary artery. All atherosclerotic tissues had an increased 15-LOX-2 expression, although the expression in nondiseased mammary arteries was low but detectable (Figure 5A). Immunohistochemical analysis of tissue sections confirmed that 15-LOX-2 is expressed in atherosclerotic plaques (Figure 5B, bottom left), in contrast to nondiseased mammary arteries where no staining for 15-LOX-2 was found (Figure 5B, bottom right). The macrophage CD68 staining suggests that 15-LOX-2 is expressed in some but not all macrophage-rich areas of the plaque (Figure 5B, middle and upper, respectively). At least some of the immunoreactive 15-LOX-2 material was found extracellulary in the macrophage-rich areas. No staining was found in the negative controls (Figure III, available online at http://atvb.ahajournals.org).

Discussion

The results in this study suggest that hypoxia increases macrophage-mediated LDL oxidation, because LDL incubated with hypoxic macrophages have increased electrophoretic mobility, TBARS, and diene formation compared with the LDL in normoxic macrophages. Several studies suggest that 15-LOX may participate in LDL oxidation in

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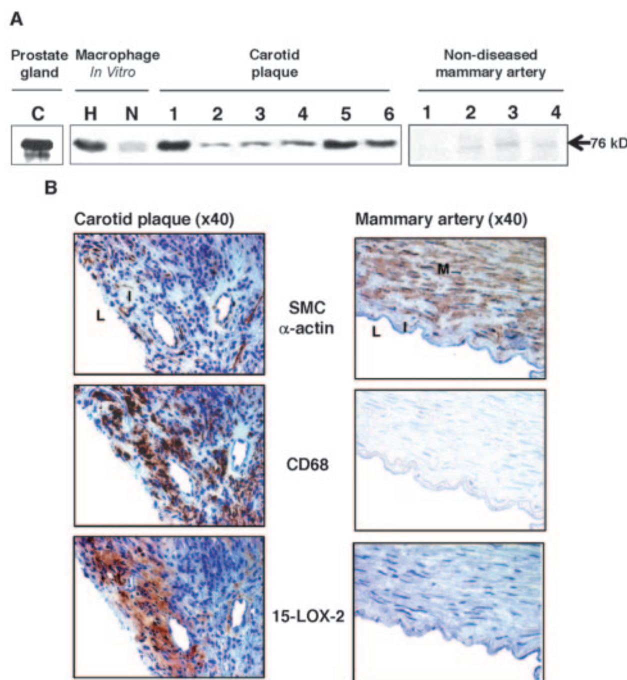

Figure 5. The 15-LOX-2 protein expression in carotid atherosclerotic plaque and internal mammary artery. A, Human carotid atherosclerotic plaques (n=6) and nondiseased internal mammary artery (n=4) from surgery were homogenized and the protein expression of 15-LOX-2 was analyzed by Western blot using rabbit anti–15-LOX-2. For comparison, 15-LOX-2 expression in prostate glands (C) or hypoxic (H) or normoxic (N) human monocyte-derived macrophages are shown. The position corresponding to the molecular size of 15-LOX-2 is indicated. B, Immunohistochemical analysis of sections from a carotid atherosclerotic plaque and an internal mammary artery. The SMCs are identified with mouse anti–α-actin (SMC α-actin) and the macrophages with mouse anti–CD68, and 15-LOX-2 was identified with rabbit anti–15-LOX-2. The localization of lumen (L), intima (I), and media (M) is shown in the sections.

vivo.[5,6,22] 15-LOX exists as two isoforms, type 1 and type 2.[23] In our study, both the mRNA and protein expression of 15-LOX-2 were increased in macrophages at hypoxia compared with normoxia. In contrast, no increased expression of 15-LOX-1 was seen in hypoxic macrophages, suggesting that 15-LOX-2 may be one of the enzymes involved in the hypoxia-induced macrophage-mediated LDL oxidation. Furthermore, the presence of 15-LOX-2 protein was confirmed with both Western blot and immunohistochemical techniques in carotid plaques.

The DNA homology of the 15-LOX isoforms is 40%,[23] and the differences between them are reflected in their enzymatic activities and substrate specificities. Both isoforms convert polyunsaturated fatty acids, although the type 2 isoform prefers AA as a substrate and forms 15-HETE, whereas the type 1 isoform prefers linoleic acid, which is converted to 13-hydroxyoctadecadienenoic acid. In atherosclerotic plaques, large amounts of linoleic acid oxidation products have been detected in the forms of 9- and 13-hydroxyoctadecadienenoic acid but also considerable levels of the AA oxidation products of 15- and 11-HETE, the main product of 15-LOX-2 activity. [24] The importance of 15-LOX-1 in atherogenesis has been elucidated in several studies which suggest that either 15-LOX-1 generates minimally modified LDL and enhances atherogenesis[25–28] or that 15-LOX-1 protects against atherosclerosis. [29,30]

Originally identified in hair follicles,[23] 15-LOX-2 has been found in skin, prostate, lung, and cornea.[23,31] This study shows for the first time the expression of 15-LOX-2 in human macrophages and in atherosclerotic plaques. The biological function of 15-LOX-2 in different cell types is still unclear. Until now, 15-LOX-2 has mainly been studied in relation to cancer. Shappell et al suggested that this enzyme regulates cell proliferation and differentiation in the prostate, and that reduced expression is associated with a malignant phenotype.[32] In our study, hypoxia is a strong activator of 15-LOX-2 expression in macrophages.

The gene encoding 15-LOX-2 consists of 14 exons.[33] Three splice variants of 15-LOX-2 (15-LOX-2-sv-a/b/c) have thus far been identified, where some of these forms lack enzymatic activity.[19] In human macrophages, we found two forms of 15-LOX-2: both the unspliced 15-LOX-2 and the splice variant 15-LOX-2sv-a were expressed in these cells. This splice variant lacks exon 9, which encodes the substrate-binding pocket of the enzyme.[34] This could explain the low biological activity of this splice variant compared with the unspliced form.[19] The splice variants are generally expressed at much lower levels than unspliced 15-LOX-2.[35] The biological function of these splice variants is still unclear. Like other alternatively spliced gene products,[35] 15-LOX-2sv-a could be involved in regulating the enzymatic effect of unspliced 15-LOX-2.[33]

Hypoxia increases both macrophage-mediated LDL oxidation and the expression of 15-LOX-2. However, the relation between 15-LOX-2 expression and LDL oxidation over time may suggest that this enzyme is not involved in the initiation of the oxidation process. After 8 hours, there is a significant increase of LDL oxidation as well as of mRNA expression for 15-LOX-2, but at this time point no significant increase in protein expression could be detected. However, the enzyme activity analysis showing increased 15-HETE production suggests that 15-LOX-2 is already enzymatically active at 24 hours of hypoxia, despite no significant increased protein expression of 15-LOX-2 at this time point. An increased enzyme activity of 15-LOX-2 in hypoxic macrophages could therefore occur before 24 hours, because 15-LOX-2 activity at that time is significantly higher in hypoxic macrophages than in normoxic cells. On the other hand, the LDL oxidation process is complex, and several different enzymes and mechanisms may be involved. 15-LOX-1, known to be expressed in some normoxic cells, such as macrophages, increases its transmigration to the membrane during hypoxia in smooth muscle cells (SMCs) and endothelial cells.[36] This increased membrane binding due to hypoxia may explain the enhanced LDL oxidation in macrophages seen at the beginning of hypoxia where 15-LOX-1 protein may still be present. Prolonged treatment with hypoxia increases 15-LOX-2 expression, which may override 15-LOX-1 during these conditions. This is in agreement with Kuhn et al who suggested that 15-LOX-1 may be involved in the early stages of atherogenesis.[5,37] The identification of 15-LOX-2 in atherosclerotic tissue in association with some macrophage-rich areas may support its role in vivo and suggest that 15-LOX-2 is involved in a later stage of atherogenesis where we find more advanced and hypoxic lesions.

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Hypoxic areas are found in atherosclerotic lesions, but the role of hypoxia in the development of atherosclerotic plaques is not known. This study shows that macrophage-mediated LDL oxidation is significantly higher at hypoxia than at normoxia and that hypoxia significantly increases the levels of both mRNA and protein of the active form of 15-LOX-2. Interestingly, this is the first report of 15-LOX-2 expression in atherosclerotic plaques. These findings suggest that hypoxia, by increasing macrophage-mediated LDL oxidation, may contribute to an enhanced development of atherosclerosis, and that 15-LOX-2 may be one of the factors involved in this hypoxia-induced LDL oxidation.

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Hypoxia Increases LDL Oxidation and Expression of 15-Lipoxygenase-2 in Human Macrophages

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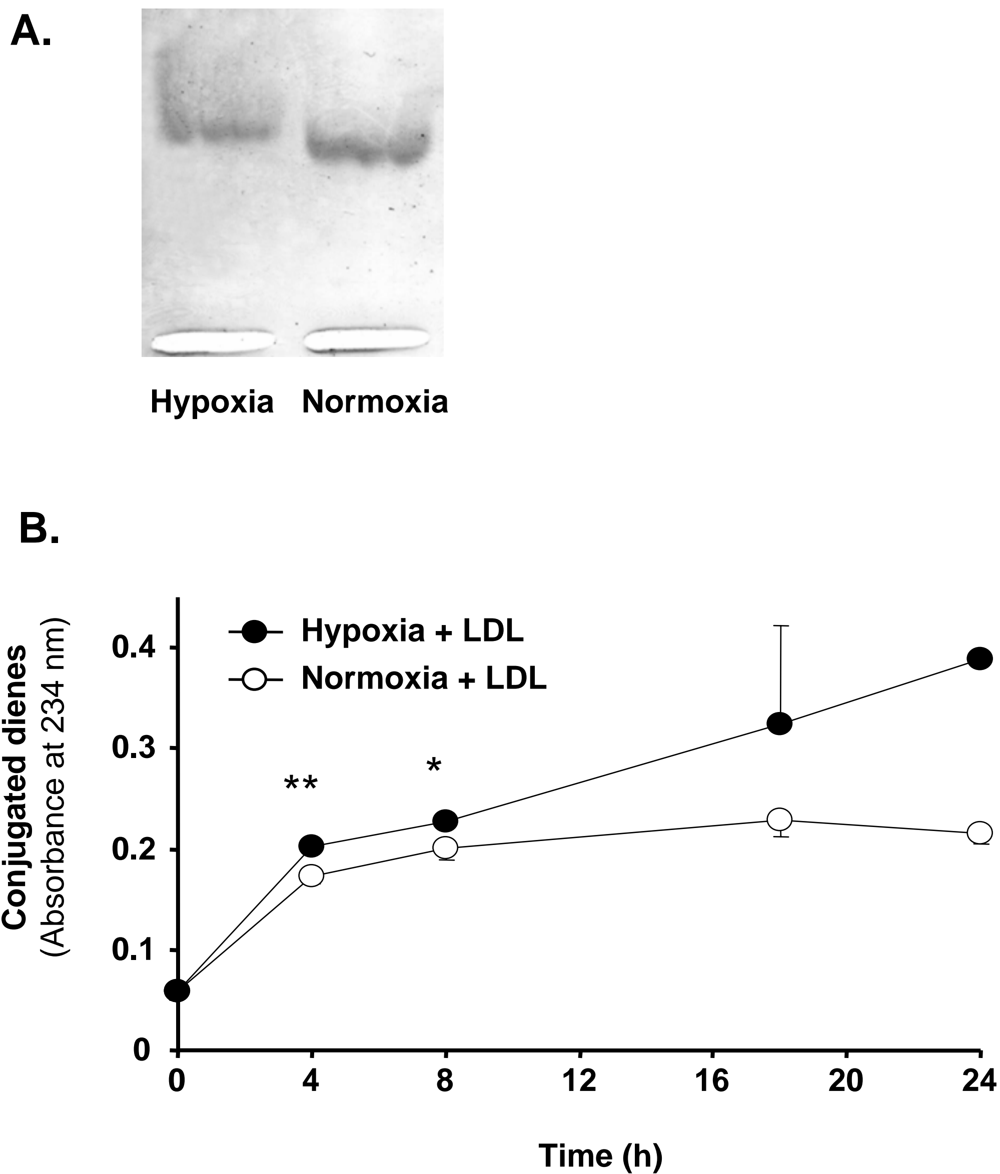


Figure I. Oxidation of LDL by macrophages incubated at normoxia or at hypoxia.

A. Agarose gel electrophoresis of LDL incubated with macrophages for 24 h. **B**. LDL oxidation by macrophages (n=3) measured as formation of conjugated dienes. Values are mean ± SD. **P*<0.05, ** *P*<0.01 by Student´s two-tailed paired *t* test.

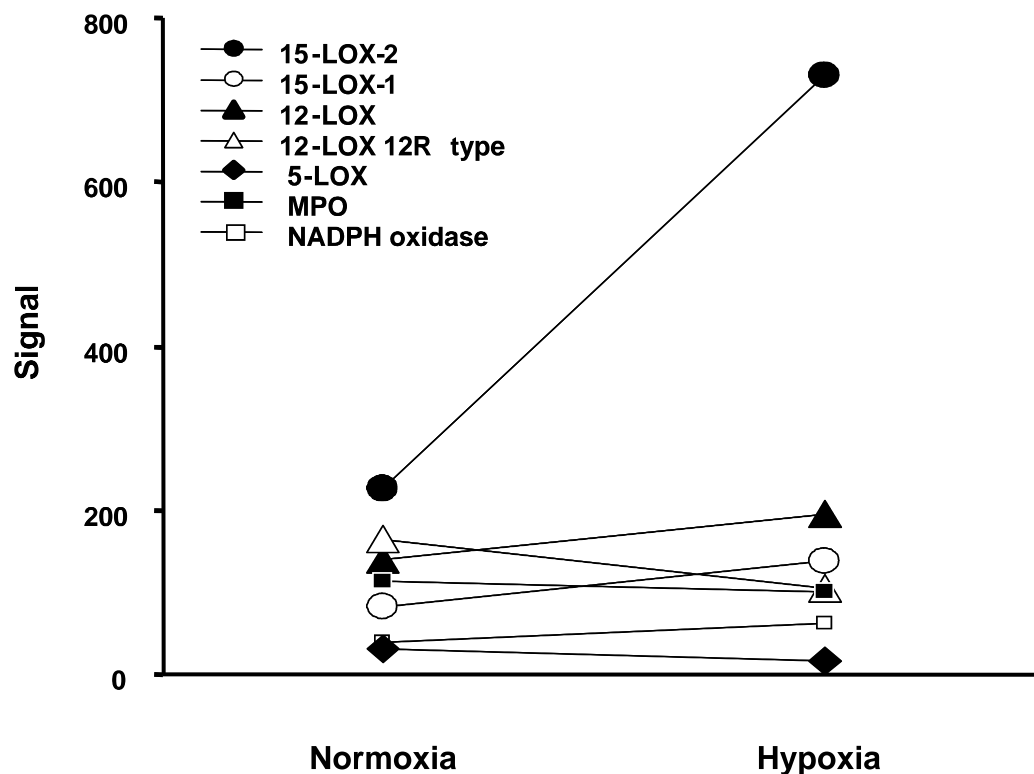
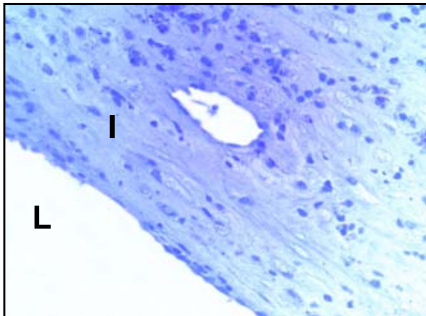


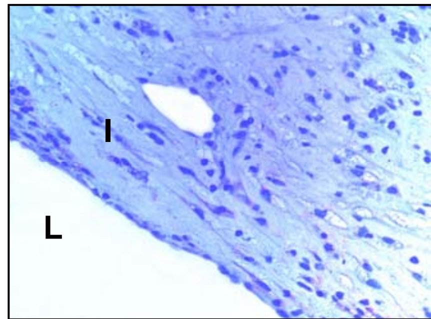
Figure II. DNA microarray analysis of macrophages incubated at normoxia or at hypoxia for 24 h.

Total RNA from macrophages (n=4) was collected, 2 μg RNA from each donor pooled, and analyzed by DNA microarray analysis. The expression of 7 genes involved in LDL oxidation was investigated. Results are shown as mean signal (mean average difference) of two DNA microarrays in normoxic and hypoxic groups.

Carotid plaque (x40)



Negative control
without primary Ab



Negative control
With rabbit IgG as primary Ab

Figure III. Negative control staining for 15-LOX-2 protein expression. Sections from a carotid atherosclerotic plaque were stained without primary antibody (right panel) or with rabbit IgG as primary antibody (left panel). Positions of lumen (L) and intima (I) are indicated.

LDL Preparation

Human LDL (*d* = 1.019 to 1.063 g/mL) was prepared by sequential centrifugation of plasma from healthy fasted male donors in the presence of EDTA (0.2% w/v) at 4°C. For each experiment LDL from 2 donors were pooled, filtered through 0.22-µm sterile filters, stored at 4°C, and used within 1 week. Before use, LDL was separated from EDTA over two consecutive PD-10 columns (Amersham Pharmacia Biotech).

DNA Microarray Analysis

Total RNA was isolated with the RNeasy kit (Qiagen) from macrophages incubated at normoxia or hypoxia for 24 h. Two µg RNA from 4 donors incubated at normoxia and 4 donors at hypoxia was pooled respectively and these 2 pools were used for the target preparation and analyzed on duplicate DNA microarrays (Hu95A; Affymetrix). Target preparation, DNA microarray hybridization, and scanning were performed as described [1]. Scanned output files were analyzed with GeneChip 3.1 software (Affymetrix) and globally scaled to an average intensity of 500. Potential candidate genes encoding proteins involved in LDL oxidation and lipoxygenase-related genes were selected based on information from current literature [2-6]. The probe sets corresponding to these genes were designed using the Netaffix database (www.affymetrix.com). The mean average signal difference was compared between normoxic and hypoxic groups.

Real-Time RT-PCR

Real-time RT-PCR was performed with a TaqMan RT-PCR kit (Applied Biosystems) to detect mRNA expression of 15-lipoxygenase type 1 (15-LOX-1), 15-LOX-2, and MPO. The

expression was normalized to actin mRNA expression using a pre-developed TaqMan assay reagent kit, with primers and the probe purchased from Applied Biosystems. The actin probe was 5′-labeled with VIC® and 3′-labeled with tetramethylrhodamine (TAMRA). Oligonucleotide primers and probes for the different genes of interest were designed with the Primer Express 1.5 software and purchased from Applied Biosystems. These probes were 5′-labeled with 5-carboxyfluorescein (FAM) and 3′-labeled with TAMRA. The following primers and probes were used. Human 15-LOX-2 (GenBank No. U78294): forward primer, 5′-GGCCTCATTGTTGGGTCCT-3′; reverse primer, 5′-TGCCGTGATCCACCA AGAA-3′; probe, FAM-5′-AGCCCTTCTCTAGCTCAGCCTGCAAGC-3′-TAMRA. Human 15-LOX-1 (GenBank No. M23892): forward primer, 5′-TGAGCGATTTCTGGAAGACA AGA-3′; reverse primer, 5′-ATTTAGAGAGTCTTTGTATAGCGAGGTC-3′; probe, FAM-5′-CCTTGGCCAGCGAAACCTCAAAGTC-3′-TAMRA. Human MPO (GenBank No. M19507): forward primer, 5′-CAGGACAAATACCGCACCATC-3′; reverse primer, 5′-CACAAAGGC ACGGTTGGAG-3′; probe, FAM-5′-ACCGGGATGTGCAACAACAGACGC-3′-TAMRA. The RT reaction was performed with a Gene Amp PCR system 9700 (Applied Biosystems). PCR amplification for all genes was performed for 40 cycles on an ABI PRISM 7700 sequence detection system (Applied Biosystems).

Splice Variants of 15-Lipoxygenase-2

The Taqman reverse transcriptase reaction kit, with random hexamer primers (Applied Biosystems), was used to synthesize cDNA from RNA of normoxic or hypoxic treated macrophages. To identify the splice variants 15-LOX-2sv-b (233 bp) and 15-LOX-2sv-c (448 bp), the PCR was performed using the forward primer A (5′-CAGGCTACTACTACCGTGATG-3′) corresponding to position 1477-1498 and the reverse

primer C (5′-TATGAAGCCCTCGCATGTTG-3′) corresponding to position 1844-1825 of the 15-LOX-2 cDNA sequence (GenBank No.U78294). Using these primers, the unspliced form of 15-LOX-2 and the splice variant 15-LOX-2sv-a result in two PCR products with equal sizes (368 bp). Therefore, another PCR was performed to separate the unspliced 15-LOX-2 from the splice variant 15-LOX-2sv-a by using the forward primer B (5′-CCTGGCTACCCTGCGTCA-3′) corresponding to position 1226-1243 together with the reverse primer C. In this reaction, the unspliced 15-LOX-2 and the splice variant 15-LOX-2sv-a, 15-LOX-2sv-b and 15-LOX-2sv-c would correspond to PCR products of 619 bp, 532 bp, 397 bp and 699 bp, respectively. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

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