Genes of Cholesterol Metabolism in Human Atheroma
Overexpression of Perilipin and Genes Promoting Cholesterol Storage and Repression of ABCA1 Expression

Fabien Forcheron, Liliana Legedz, Guileta Chinetti, Patrick Feugier, Dominique Letexier, Giampierro Bricca, Michel Beylot

Objective—Accumulation of cholesterol in foam cells of atheroma plaques depends on the balance between uptake and efflux of cholesterol. It may also depend on proteins surrounding lipid droplets, adipophilin, and perilipins. They favor triglyceride storage in adipocytes and could play a similar role for cholesterol in atheroma.

Methods and Results—We measured in human atheroma and nearby macroscopically intact tissue (MIT) the expression of perilipin, adipophilin, and regulatory factors of cholesterol metabolism. We identified perilipin A in human arterial wall. Its expression was largely increased in atheroma compared with MIT, and perilipin was present in macrophages and vascular smooth muscle cells. Adipophilin, ACAT1, and CD36 were also overexpressed in atheroma. mRNA levels of low-density lipoprotein receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and SREBP-2 were unchanged. With respect to efflux of cholesterol, the mRNA levels of NCEH and ABCA-1 were unchanged, whereas CLA-1 mRNA was slightly higher in atheroma. Importantly, immunoblotting of ABCA-1 showed a dramatic decrease of ABCA1 protein, the key molecule of cholesterol efflux, in atheroma compared with MIT.

Conclusion—We show the presence and induction of perilipin in atheroma. This overexpression and the coordinated modifications of expression of key regulatory factors for cholesterol metabolism could favor cholesterol accumulation.

Key Words: atherosclerosis • genes • lipids • macrophages • smooth muscle cells

Accumulation of cholesterol esters (CEs) in macrophages and smooth muscular cells (SMCs) transforming them in foam cells is a hallmark of atherosclerosis. This accumulation depends on the balance between the uptake of cholesterol-rich lipoproteins such as oxidized low-density lipoprotein (LDL) and the efflux of free cholesterol (FC) controlled by the transporter ABCA1 and to a less extent by CLA-1 (or SR-B1). CE accumulation depends also on the intracellular metabolism of cholesterol. CE taken up by cells is hydrolyzed in lysosomes to FC that is directed to various cell membranes by the protein NPC-1 (Nieman-Pick C1 protein). FC can also be produced by endogenous synthesis. Because excessive accumulation of FC is toxic for cells, FC must be either removed through efflux to extracellular acceptors such as high-density lipoprotein or esterified. This esterification depends in macrophages on the microsomal enzyme acyl-coenzyme A (CoA) cholesterol acyl-transferase 1 (ACAT-1) and on the availability of long-chain fatty acyl CoA. CE stored in lipid droplets can be removed from cells only after hydrolysis to FC by a cholesterol ester hydrolase, whose nature is still debated. In murine macrophages, CE seems to be hydrolyzed by hormone-sensitive lipase (HSL), the enzyme controlling the hydrolysis of triglycerides (TGs) stored in adipocytes. However, HSL is not expressed in human macrophages and CE is probably hydrolyzed in these cells by the neutral cholesterol ester hydrolase (NCEH) cloned by Ghosh, also called triacylglycerol hydrolase and carboxylesterase 3.

Storage of lipids in droplets is also dependent in cells accumulating TG (mainly adipocytes and hepatocytes) or CE (steroidogenic cells) on specific proteins surrounding these droplets, particularly adipophilin (or ADRP) and the perilipins. ADRP is considered to have a role in the transport of lipids from cell membranes to lipid droplets and is present in all cells storing lipids. ADRP is expressed in foam cells of atherosclerotic plaques. Its expression increases during differentiation of monocytes into macrophages and incubation of macrophages with oxidized LDL. Its overexpression in THP-1 macrophages enhances lipid accumulation. There are 3 different perilipins, A, B, and C, resulting from alternative splicing of a common premessenger RNA. Perilipins A and B are found in adipocytes, with the A form being predominant. Perilipin C is found in steroidogenic cells. In adipocytes, in the basal state, ie, in the absence of...
stimulation of lipolysis, perilipin protects TG from hydrolysis by HSL.\textsuperscript{19} When lipolysis is stimulated by β-adrenergic agents, perilipins are phosphorylated on specific serine sites\textsuperscript{20,21} and allow phosphorylated HSL to hydrolyze TG. However, this role of perilipin in the control of lipid storage has been demonstrated only for perilipin A and in adipocytes.\textsuperscript{22} The expression of perilipins was considered to be limited to adipocytes and steroidogenic cells.\textsuperscript{13} Recently, evidence for the presence of perilipin in atheroma plaques has been presented.\textsuperscript{23} Perilipin could therefore be implicated in the development of atherosclerosis by controlling as in adipocytes the hydrolysis of stored lipids. However, the nature (A, B, or C) of the perilipin expressed in atheroma plaques was not determined, an important point because the role of perilipin in the control of lipolysis in adipocytes has been demonstrated only for perilipin A.\textsuperscript{22}

We determined in the present report the expression level of perilipin, ADRP, and main genes controlling cholesterol metabolism in human atherosclerotic plaques compared with nearby macroscopically intact tissue (MIT). We show that perilipin is overexpressed, as ADRP, in human atheroma. The perilipin form is perilipin A and it is expressed in macrophages and SMCs. Lastly, these modifications of ADRP and perilipin expression in atheroma appear associated with coordinated modifications of the expression of other genes controlling cholesterol metabolism in a way promoting the synthesis and storage of CE.

**Methods**

**Human Subjects**

The procedure was approved by local ethical committee. Human atheroma plaques were removed during carotid endarterectomy from 10 subjects (5 males, 5 females, aged 68±3 years) who gave their informed consent. Five subjects had overt type 2 diabetes. The others had evidence of insulin resistance with moderate increase in basal level of plasma glucose and/or insulin concentrations. Four subjects (among whom 3 had type 2 diabetes) were treated with statins. Samples collected directly in the surgery room were divided in 2 parts, atherosclerotic plaque and MIT situated in the vicinity of the plaque, and were frozen in liquid nitrogen (LN2) or fixed for immunohistology. A fragment of MIT was spared for SMC culture.

**Studies in Rats**

Male Sprague-Dawley rats, obese Zucker rats, and their lean littermates (Charles River, L’Arbresle, France) were anesthetized with pentobarbital in the fed state. After blood removal for determination of plasma total cholesterol and TG levels (enzymatic methods), aorta was removed, carefully cleaned from adjacent fibrous and adipose tissue, and quickly frozen in LN2. In lean and obese Zucker rats, half of the aorta was used for culture of vascular SMC.

**Cells Culture**

Mononuclear cells isolated from blood of healthy donors (Ficoll gradient) were suspended in RPMI 1640 medium containing gentamicin (40 mg/mL), glutamine (0.05%), and 10% pooled human serum. Monocytes differentiation into macrophages occurs spontaneously by adhesion into the culture dishes. After 10 days of culture, mature monocytes-derived macrophages were in a first experiment incubated with AcLDL (100 μg/mL) during 12, 24, 48, and 72 hours. In a second experiment, monocytes-derived macrophages were incubated 5 or 8 days with 100 or 200 μg/mL of AcLDL. THP-1 cells (ATCC, Rockville, Md) were suspended in RPMI 1640 medium containing calf serum (10%), D-glucose (0.45%), 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, penicillin (100 U/mL), streptomycin (100 mg/mL), 2 mmol/L glutamine, and bicarbonates (7.5%). Human SMC were obtained from MIT. Briefly, in the surgery room, MIT was placed in culture medium (Promocell SMC growth medium) and immediately transported to the laboratory. Tissue was minced in culture dishes and cells grown in the same medium. Cells used for measurements were at passage P3 and P4 and maintained 24 hours without serum before mRNA measurements. SMC from rat aortas were obtained after the same general protocol.

**mRNA**

Purified total RNA samples (Trizol; Gibco-Brl, Cergy Pontoise, France) were quantified by spectrophotometry and their integrity checked by agarose gel electrophoresis. ABCA1 and SREBP-1c mRNA levels were measured by competitive reverse-transcription (RT) polymerase chain reaction (PCR).\textsuperscript{24} CD36 mRNA concentrations were determined by RT-PCR using cyclophilin as reference. Other mRNA, LDL receptor (LDL-r), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, SREBP-2, NPC1, ACAT1, NCEH, CLA-1, LXRs, and stearil-CoA desaturase (SCD) were measured by RT-PCR using β-actin as internal standard. β-actin mRNA levels were measured by competitive RT-PCR with a specific competitor to detect and take in account possible variations of abundance between MIT and atherosclerotic plaques. mRNA concentrations were expressed as ratio versus a normalized amount (100 attomoles/μg of total RNA) of β-actin mRNA level. The presence of some mRNA (HSL, resistin) was checked by using in RT-PCR only primers specific for these targets. Primers are shown in Table 1.

**Immunoblot**

Samples were homogenized in PBS with proteases inhibitors and protein concentrations measured with the BCA kit (Pierce). Proteins (100 μg) were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were probed with a polyclonal guinea pig anti-human perilipin antibody (Progen, Heidelberg, Germany), a polyclonal rabbit anti-human ABCA1 antibody (Novus Biologicals, Littleton, Colo), or a polyclonal goat anti-human SCD antibody (Santa Cruz Tech), followed with incubation of anti-IgG conjugated with peroxidase (Sigma, St Louis, Mo; 1/1000) and revelation with ECL. The intensity of the bands was determined by densitometry (Image Quant, Molecular Dynamics, Calif). Samples form the plaque and MIT of each subject were run in the same immunoblot.

**Immunohistology**

Fixed samples were sectioned (4 μm), deparaffined (xylene), and hydrated. Sections were separated in 2 parts. One was incubated with the polyclonal guinea pig anti-human perilipin antibody (Progen, Heidelberg, Germany), a polyclonal rabbit anti-human ABCA1 antibody (Novus Biologicals, Littleton, Colo), or a polyclonal goat anti-human SCD antibody (Santa Cruz Tech), followed with incubation of anti-IgG conjugated with peroxidase (Sigma, St Louis, Mo; 1/1000) and revelation with ECL. The intensity of the bands was determined by densitometry (Image Quant, Molecular Dynamics, Calif). Samples form the plaque and MIT of each subject were run in the same immunoblot.

**Statistics**

Results are shown as mean and SEM. Comparisons between MIT and atheroma plaques were performed by Student t test for paired values. Comparisons between diabetic and nondiabetic subjects and between subjects treated or not with statins were performed by Mann-Whitney test.

**Results**

**Perilipin A Is Expressed in Arterial Wall and Is Overexpressed in Atherosclerotic Plaques**

Perilipin mRNA was present in MIT and in plaques with concentrations clearly increased in plaques (Figure 1).
Rats (1.21 ± 0.27 vs MIT). mRNA levels were mildly higher in aorta of obese Zucker rats and of their lean littermates; comparable results were observed in samples from diabetic and nondiabetic subjects, as well as from subjects treated or not with statins. Neither resistin nor HSL mRNA could be detected in any sample running out contamination of samples by adipose tissue. Immunoblot was using an antibody raised against the common (N-terminal) domain of perilipin A, B, and C detected a protein with an apparent molecular weight ≈ 55 kDa. This protein was also more abundant in plaque than in the MIT of the same subject (Figure 1). The presence of perilipin was confirmed by immunohistology. Again, the staining was more abundant in plaque than in MIT and perilipin was present in macrophages and SMCs loaded with cholesterol as shown by colocalization studies with CD68 (marker of macrophages). Right, Staining for perilipin. Middle, Staining for actin. Left, Merging of the 2 stainings. Lower panel, Colocalization of perilipin and CD68. Left, Merging of the 2 stainings. 

Perilipin +CD68

Perilipin

CD68

Perilipin

α-actin

Figure 1. Left panel, mRNA concentrations of perilipin in atherosclerotic plaques (ATH) and in adjacent macroscopically intact tissue (MIT). "P < 0.05 vs MIT (n = 10). Right panel, Representative immunoblot and expression level of perilipin protein in plaques and in MIT. "P < 0.05 vs MIT (n = 6).

Figure 2. Immunohistological studies of perilipin localization in atheroma. The upper panel shows the colocalization of perilipin and CD68 (marker of macrophages). Right, Staining for perilipin. Middle, Staining for CD68. Left, Merging of the 2 stainings. Lower panel, Colocalization of perilipin and α-actin (marker of SMCs). Right, Staining for perilipin. Middle, Staining for α-actin. Left, Merging of the 2 stainings.

TABLE 1. Primers Used for RT-PCR and Size of the Fragments Obtained for Each Target

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>5’caggaaggtqagtcttgctgacc3’</td>
<td>5’tgggtgttcctcatgaaggtgc3’</td>
<td>449</td>
</tr>
<tr>
<td>CLA1</td>
<td>5’tgctctcataacaggaggag3’</td>
<td>5’gcccaagtcgagggttg3’</td>
<td>553</td>
</tr>
<tr>
<td>LXRα</td>
<td>5’gagcgcgtcaagggatctt3’</td>
<td>5’gttacgtgtgcctggcag3’</td>
<td>332</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’gacpaggccagaagaagag3’</td>
<td>5’gggttgtgaagctcaaaa3’</td>
<td>225</td>
</tr>
<tr>
<td>LDL-r</td>
<td>5’caattgtctcctcagaacgtc3’</td>
<td>5’ctcgtctcaggggtaagct3’</td>
<td>258</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>5’taccatgctcgggtaacgt3’</td>
<td>5’cagactagacagatatactc3’</td>
<td>246</td>
</tr>
<tr>
<td>NCEH</td>
<td>5’ggaacacattgcgcttgg3’</td>
<td>5’cacatctgtcaacgcaag3’</td>
<td>667</td>
</tr>
<tr>
<td>Acat1</td>
<td>5’agagacactccacaaaggg3’</td>
<td>5’gcctctgttgatattccg3’</td>
<td>731</td>
</tr>
<tr>
<td>ADRP</td>
<td>5’aggggctgacctgacgagag3’</td>
<td>5’agcggagggagctttgagac3’</td>
<td>437</td>
</tr>
<tr>
<td>NPC-1</td>
<td>5’agacactctctttgtatgccga3’</td>
<td>5’aatgtagctgaagacaccca3’</td>
<td>534</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>5’atcccaagccgcttg3’</td>
<td>5’gatgctgggccagaaagtg3’</td>
<td>377</td>
</tr>
<tr>
<td>SCD</td>
<td>5’tgtctctcaaggtggtt3’</td>
<td>5’gaaaccagataatttcgg3’</td>
<td>335</td>
</tr>
<tr>
<td>HSL</td>
<td>5’gacqagccccacctcaaggtc3’</td>
<td>5’ctctttctcagacccg3’</td>
<td>311</td>
</tr>
<tr>
<td>h-Resistin</td>
<td>5’tcgacagatpaaagctct3’</td>
<td>5’ctgtgcapatctggtgc3’</td>
<td>285</td>
</tr>
<tr>
<td>r-Resistin</td>
<td>5’gatgtaagactgcaacgtc3’</td>
<td>5’ctgatttccaaagatca3’</td>
<td>268</td>
</tr>
<tr>
<td>Perilipin</td>
<td>5’cagaaacagcatcaggtc3’</td>
<td>5’ctggctctcatagttc3’</td>
<td>520</td>
</tr>
</tbody>
</table>

actin mRNA; n = 5; P = 0.07), suggesting that insulin resistance and/or the presence of raised lipid levels (total cholesterol 2.20 ± 0.27 versus 1.13 ± 0.07 mmol/L in lean rats, P < 0.01; TG 2.22 ± 0.27 versus 0.56 ± 0.11 mmol/L,
introns resulting in the presence of a codon stop and the synthesis of Perilipin B results from the lack of splicing of intron 8. Fragment amplified with the mRNA of perilipin A is 367 bp. No such fragment was obtained. Therefore, the perilipin containing intron 8 of perilipin is present, a fragment of 368 bp. Lastly, we performed RT-PCR amplification by PCR of a fragment of 1360 bp. We observed an increase of mRNA levels of LXRα. SCD mRNA concentrations were very low, and may even be overexpressed (CD36).

**Expression of Other Genes of Cholesterol Metabolism**

NPC1, ADRP, and ACAT1 were expressed in MIT and this expression was increased inside the plaques (Table 2) for ADRP and ACAT1. NCEH was clearly expressed in MIT and atheroma without difference in mRNA levels between these 2 tissues. On the contrary, we could not detect any HSL mRNA in MIT or in plaques. Results obtained were comparable in diabetic and nondiabetic subjects and in patients treated or not with statins.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>MIT</th>
<th>ATH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRP</td>
<td>1.07±0.34</td>
<td>2.39±0.71*</td>
</tr>
<tr>
<td>ACAT1</td>
<td>0.68±0.38</td>
<td>1.16±0.42*</td>
</tr>
<tr>
<td>NCEH</td>
<td>0.65±0.29</td>
<td>0.80±0.26</td>
</tr>
<tr>
<td>NPC-1</td>
<td>1.58±0.84</td>
<td>2.14±0.91</td>
</tr>
<tr>
<td>LDL-r</td>
<td>0.50±0.32</td>
<td>0.70±0.27</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>0.76±0.52</td>
<td>0.92±0.48</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>0.88±0.71</td>
<td>0.84±0.65</td>
</tr>
<tr>
<td>CD36</td>
<td>1.10±0.38</td>
<td>9.70±2.40*</td>
</tr>
<tr>
<td>CLA-1</td>
<td>1.38±0.34</td>
<td>1.81±0.41</td>
</tr>
<tr>
<td>LXRα</td>
<td>0.88±0.30</td>
<td>1.43±0.29*</td>
</tr>
<tr>
<td>ABCA1</td>
<td>5.60±2.40</td>
<td>5.09±1.50</td>
</tr>
</tbody>
</table>

* P<0.05 vs the corresponding mRNA value in MIT.
N=10 except for NPC-1 (n=6).

lower level, in cultured SMC from aorta of obese Zucker rats. ADRP was also clearly expressed in these cells.
The mechanisms regulating perilipin expression are still unclear. Its expression in adipocytes requires the presence and the intracellular metabolism of fatty acids. Therefore, its expression in macrophages and/or SMCs could be stimulated by the fatty acids released during the hydrolysis of CE taken up by these cells, or taken up from circulating NEFA or TGs. This would be consistent with the increased perilipin mRNA level found in the aortas of hyperlipidemic Zucker rats. This action of fatty acids could be mediated through the nuclear receptors PPARγ because it has been shown that the promoter of the perilipin gene contains functional PPAR-responsive elements and that PPARγ agonists stimulate in adipocytes the expression of perilipin.

Perilipin opposes in adipocytes in the basal state the hydrolysis of TGs by HSL. On stimulation of lipolysis by β-adrenergic agonists, perilipin is phosphorylated by the protein kinase PKA and allows phosphorylated HSL to access lipid droplets and hydrolyze TGs. This mechanism has been demonstrated for perilipin A but not for perilipin B. We have evidence that the perilipin expressed in arterial wall is indeed perilipin A. Perilipin could thus have in foam cells a role comparable to the one played in adipocytes, ie, protect in the basal state CE stored in lipid droplets from hydrolysis, not by HSL as in adipocytes because HSL was not found expressed in human arterial wall, but by NCEH and allow this hydrolysis after phosphorylation. NCEH activity is stimulated by dibutyryl cAMP and PKA and PKC in rat aortae. The simultaneous hydrolysis of CE by NCEH and esterification of FC by ACAT1 result in a permanent intracellular cycling between CE and FC. ACAT1 and NCEH mRNA were more abundant in atherosclerotic plaques, but this increase was significant only for ACAT1. If these modifications of mRNA concentrations are also present at the level of enzyme activities, they would favor storage of CE. The overexpression in plaques of perilipin opposing the action of NCEH would further shift the balance of this CE–FC cycle toward cholesterol esterification and storage.

We also observed differences in the expression of other important regulatory genes of cholesterol metabolism in plaques compared with MIT. The mRNA concentrations of CD36 were increased, whereas those of LDL-r, contributing also to the cellular uptake of cholesterol, and of HMG-CoA reductase, the key regulatory enzyme of cholesterol synthesis, were unchanged, suggesting that the pathways for both cholesterol synthesis and uptake were not repressed in foam cells of the plaques compared with MIT. Overexpression of CD36 agrees with previous studies showing that its expression is stimulated in cholesterol-loaded macrophages. The trend for higher expression of LDL-r and HMG-CoA reductase is surprising because the expression of these genes is repressed by cellular accumulation of cholesterol and stimulated when cells are depleted of cholesterol. However, it is also possible that the expression of these genes was already near maximally suppressed in MIT.

Cellular cholesterol efflux is controlled by ABCA1 and, to a lesser degree, by CLA-1 that can promote either cholesterol efflux or uptake. There was in plaques a dramatic decrease in the protein amount of ABCA1, suggesting that cholesterol...
efflux was largely decreased. This decrease in ABCA1 protein was not caused by a diminished transcription because mRNA levels were unchanged. Moreover, mRNA concentrations of LXRα,35,36 the main nuclear factor stimulating the transcription of ABCA1, were increased. ABCA1 is also regulated at the post-translational level via changes in the intracellular accumulation of FC38 accelerate the degradation of ABCA1 protein, and both could therefore be responsible of the decrease in ABCA1 protein observed in plaques. The mRNA concentration of SCD, an enzyme synthesizing unsaturated fatty acids and whose expression is also stimulated by LXRα,40 was increased in plaques, but we detected no increase in the amount of protein and it is therefore uncertain whether the activity if the enzyme was increased. The effect of FC is dependent of cholesterol concentration of ABCA1 and decreased cholesterol efflux could also help to prevent an excessive accumulation of FC. Excess FC has toxic effects on cells and could play a key role in the evolution of atheroma plaques toward instability.7,41–43 Perilipin could therefore have on the contrary a favorable and protective role by shifting the balance between FC and CE toward CE.

Acknowledgments

This study was supported by grants from the Fondation de France, the Société Française d’Athérosclérose, and the Société Française de Nutrition

References


Giampierro Bricca and Michel Beylot

Fabien Forcheron, Liliana Legedz, Guilett Chinetti, Patrick Feugier, Dominque Letexier, Giampierro Bricca and Michel Beylot

Arterioscler Thromb Vasc Biol. 2005;25:1711-1717; originally published online June 16, 2005; doi: 10.1161/01.ATV.0000174123.19103.52

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/8/1711

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/