ABCA1 Expression in Carotid Atherosclerotic Plaques

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Background and Purpose—The ATP-binding cassette transporter A1 (ABCA1) facilitates cholesterol efflux from cells, a key process in reverse cholesterol transport. Whereas previous investigations focused on mutations causing impaired ABCA1 function, we assessed the role of ABCA1 in human carotid atherosclerotic disease.

Methods—We compared the mRNA and protein levels of ABCA1, and one of its key regulators, the liver X receptor α (LXRα), between minimally and grossly atherosclerotic arterial tissue. We established *ABCA1* and *LXRα* gene expression by real-time quantitative polymerase chain reaction in 10 control and 18 atherosclerotic specimens. Presence of ABCA1 protein was assessed by immunoblotting. To determine whether differences observed at a local level were reflected in the systemic circulation, we measured ABCA1 mRNA in leukocytes of 10 patients undergoing carotid endarterectomy and 10 controls without phenotypic atherosclerosis.

Results—*ABCA1* and *LXRα* gene expression were significantly elevated in atherosclerotic plaques ($P<0.0001$ and 0.03, respectively). The increased mRNA levels of ABCA1 and LXRα were correlated in atherosclerotic tissue ($r=0.85$; $P<0.0001$). ABCA1 protein expression was significantly reduced in plaques compared with control tissues ($P<0.0001$). There were no differences in leukocyte ABCA1 mRNA expression ($P=0.67$).

Conclusions—ABCA1 gene and protein are expressed in minimally atherosclerotic human arteries. Despite significant upregulation of ABCA1 mRNA, possibly mediated via LXRα, ABCA1 protein is markedly reduced in advanced carotid atherosclerotic lesions. No differences in leukocyte ABCA1 expression were found, suggesting the plaque microenvironment may contribute to the differential ABCA1 expression. We propose that the decreased level of ABCA1 protein is a key factor in the development of atherosclerotic lesions. **(*Stroke*. 2004;35:2801-2806.)**

Key Words: atherosclerosis ■ ATP binding cassette transporter 1 ■ carotid artery plaque ■ carotid arteries ■ polymerase chain reaction

Cerebral atherosclerotic disease is the commonest etiological factor in stroke, a major cause of morbidity and mortality. Increasing attention is being given to the possible role of the ATP-binding cassette transporter A1 (ABCA1) in the development and behavior of atherosclerotic plaques. ABCA1 is a transmembrane protein involved in cholesterol and phospholipid transport from cells to lipid-poor apolipoproteins in the plasma. The clinical implications of ABCA1 deficiency were recognized when the *ABCA1* gene was discovered to be responsible for Tangier disease[1] and familial high-density lipoprotein (HDL) deficiency.[2] These conditions are characterized by low levels of HDL, the deposition of lipid-laden macrophages in tissues, and increased atherosclerotic disease in a proportion of patients. The pathophysiology of this process is thought to result from failure of ABCA1 to transport cholesterol and phospholipids out of cells to form complexes with apolipoproteins to generate HDL.[3] This, in turn, leads to intracellular sterol accumulation and the subsequent development of foam cells, a hallmark of the atheromatous plaque. ABCA1 mRNA is highly expressed in leukocytes and macrophages,[4] and in a wide range of human tissues, including placenta, liver, lung, and adrenal gland.[5] Attention has focused on ABCA1 because of its potential role in atherosclerosis, and the fact that therapeutic interventions could be targeted at the regulatory pathway controlling ABCA1 expression. Likely pharmacological targets include the liver X receptor-α (LXRα)[6] and peroxisome proliferator-activated receptor-γ.[7]

Insights into ABCA1 function and roles in disease have been elucidated by overexpression[8] and *ABCA1* gene inactivation studies in mice,[9] as well as observational studies in individuals deficient in ABCA1 because of gene mutations.[10] Although the systemic effects of loss of function in ABCA1-

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deficient individuals is well-characterized, less is known about the potential role of the transporter in localized atheromatous disease in individuals without reported mutations in the gene.

Apart from its role in cholesterol efflux, ABCA1 is implicated in phosphatidylserine translocation between the leaflets of cell membranes[11] and the engulfment of apoptotic cells.[11] Apoptosis, phosphatidylserine externalization and lipid dysregulation play critical roles in the development and subsequent behavior of atherosclerotic plaques. ABCA1 is implicated in these processes, making the transporter a promising candidate in the context of atherosclerosis. The key question, therefore, is whether there is a localized arterial loss of ABCA1 function in relation to atherosclerotic disease.

To investigate both ABCA1 gene and protein expression in human carotid atherosclerotic disease, we analyzed ABCA1 mRNA and protein in atheromatous plaques taken from patients undergoing carotid endarterectomy (CEA) and compared it to human arteries obtained from controls with no phenotypic atherosclerotic disease. To gain insight into regulatory mechanisms involved in *ABCA1* gene expression, we also measured mRNA expression of one of its key regulators, LXRα. As leukocytes are known to be involved in the pathogenesis of the atheromatous plaque,[12] and because deficient leukocyte ABCA1 expression is implicated in increased susceptibility to atherosclerosis in animal studies,[4] we analyzed ABCA1 mRNA expression in peripheral leukocytes.

Methods

Subjects and Specimens

Carotid plaques were collected from 18 consecutive subjects with internal carotid artery stenoses of >70% undergoing CEA. Symptomatic carotid disease was diagnosed in 16 patients with a history of transient ischemic attacks, strokes, or amaurosis fugax, whereas 2 patients were asymptomatic. Ten inferior mesenteric arteries dissected from colectomy specimens of subjects having elective operation served as controls. These patients were phenotypically free of symptomatic atherosclerotic disease by history, examination, and a normal electrocardiogram tracing. Sections of the control arteries were stained with hematoxylin and eosin and assessed by a histopathologist for evidence of age-related atheromatous change. They were found to be within histological grading (types I to III) of the initial lesions of atherosclerosis according to the American Heart Association classification.[13]

The endarterectomy specimens consisted of the atheromatous plaque, together with adjacent intima and medial layers. The inferior mesenteric artery specimens were full-thickness and included the adventitial layer. Therefore, a greater preponderance of connective tissue would be expected in the control samples. Previous work indicates that ABCA1 is expressed primarily in macrophages within the atheromatous lesion,[14] therefore ABCA1 expression levels are expected to be higher in CEA (with advanced atherosclerosis) than inferior mesenteric artery specimens (with mild atherosclerosis).

To assess leukocyte ABCA1 expression, peripheral venous blood was collected pre-operatively from a subgroup of 10 patients undergoing elective CEA for symptomatic carotid disease and 10 age- and sex-matched controls, phenotypically free of symptomatic atherosclerosis. There were no statistically significant differences between subjects and controls with respect to age and sex. The study had ethical approval from the Riverside Research Committee and informed consent was obtained from subjects. Demographic details of patient and control groups are listed in Tables 1 and 2.

TABLE 1. Demographic Details of Patients and Controls (Arterial Specimens)

Characteristics	Patients (n=18)	Controls (n=10)
Age (mean)	72	66
Males	10	4
Females	8	6
Smoking	1	2
Diabetes	4	0
Hypertension	13	2
Coronary disease	7	0
Peripheral arterial disease (other than carotid)	2	0
Drugs		
Aspirin	13	1
Clopidogrel	2	0
Warfarin	2	0
Statins	11	1

ABCA1 Gene Expression

RNA Isolation and cDNA Preparation

Plaques and control arteries were immediately snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from approximately 30 mg of pulverized frozen tissue with the RNeasy Mini or Lipid Tissue Mini kit (Qiagen) according to the manufacturer's instructions.

Leukocytes were isolated from 8 mL whole blood and total RNA was extracted using RNeasy Midi columns (Qiagen). Monocytes were isolated from whole blood of 11 individuals using magnetic beads technique (Dynal, product no. 113.09) according to the manufacturer's instructions.

For cDNA synthesis, total RNA (100 ng for plaques/control arteries, 1 μg for leukocytes) was transcribed with a first strand cDNA synthesis kit for reverse-transcription polymerase chain reaction (Roche, UK), according to the supplier's instructions.

TABLE 2. Demographic Details of Patients and Controls (Blood Specimens)

Characteristics	Patients (n=10)	Controls (n=10)
Age (mean)	71.8	60.4
Males	5	5
Females	5	5
Smoking	1	0
Diabetes	4	0
Hypertension	6	2
Coronary disease	3	0
Peripheral arterial disease (other than carotid)	2	0
Drugs		
Aspirin	8	0
Clopidogrel	3	0
Warfarin	2	0
Statins	7	0

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction

Primers and probe for Taqman analysis of ABCA1 mRNA were designed to span 2 adjacent exons with PrimerExpress software (PE Applied Biosystems). The forward primer was GGGAGGCTCCCGGAGTT (exon 3), the reverse primer was GTATAAAAGAAGCCTCCGAGCATC (exon 4), and the FAM-labeled probe, spanning exons 3 and 4, was AACTTTAACAAATCCATTGTGGCTCGCCTGT. 5′-3′-sequences for *LXRα* primers and probe were: CAAGTGTTTGCACTGCGTCT, CAGGAATGTTTGCCCTTCTC, and CACTTCTAGGAGGCAGCCAC. Single-tube Taqman analysis was performed on an ABI Prism 7700 sequence detection system with 300 nM of forward and reverse primers in the presence of 200 nM 5′FAM-3′TAMRA-tagged probe for *ABCA1*, and 900 nM of forward and reverse primers in the presence of 300 nM 5′FAM-3′TAMRA-tagged probe for *LXRα*.

The internal standard was β-actin mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were performed in duplicate and contained 5 μL of 4-fold diluted (leukocytes) or undiluted (plaques and control arteries) cDNA in a total volume of 25 μL.

Quantitation

The amount of ABCA1 mRNA in cells was calculated according to the relative standard curve method described in the PE User bulletin number 2. Target quantity was calculated from the standard curve and normalized to β-actin (arterial specimens, leukocytes) or GAPDH (leukocyte-monocyte correlation). Relative ABCA1 and LXRα levels were evaluated for at least 2 different RT reactions.

Total Membrane Preparation and Western Blotting

Samples were snap-frozen in liquid nitrogen and stored at −80°C. Total membrane fractions were prepared using a protocol based on an established method published by Rosenberg et al.[15] Approximately 100 mg of pulverised sample was homogenized in lysis buffer (50 mmol/L mannitol, 2 mmol/L EDTA, 50 mmol/L Tris HCl pH 7.6, complete mini protease inhibitors; Roche). Nuclei and debris were pelleted by centrifugation at 500 × g for 10 min, and the supernatant loaded onto 600 μl of a 300 mmol/L mannitol, 2 mmol/L EDTA, 50 mmol/L Tris HCl pH 7.6 cushion. The total membrane fraction was pelleted by centrifugation at 100 000 × g for 45 minutes. The membrane pellet was resuspended in 40 μl of lysis buffer and incubated for one hour on ice with 50 U of benzonuclease (Sigma), then SDS added to a final concentration of 1%. Protein content was measured by Biorad Dc protein assay kit. 50 μg of membrane protein was loaded on a 7% SDS-PAGE gel and transferred onto polyvinylidine difluoride membranes (Immobilon-P; Millipore). Proteins were detected with a polyclonal antibody against ABCA1 (1:1500; Abcam, Cambridge, UK) and a monoclonal antibody against Na^+/K^+-ATPase (1:1000; Research Diagnostics, Flanders, NJ) followed by anti-rabbit or anti-mouse HRP secondary antibodies (1:1000; Dako Cytomation, Cambridgeshire, UK). Signal was visualised by chemiluminescence (Amersham ECL system).

Protein Quantification

The optical density (OD) of ABCA1 and Na^+/K^+-ATPase bands was determined using Metamorph software (Universal Imaging Corporation). The average digital signal per band was measured after subtraction of the appropriate background. The OD for each ABCA1 band was normalized to the corresponding average signal of the Na^+/K^+-ATPase band, estimated for the same sample as loading control. Data are shown as average±SEM.

Statistics

For statistical evaluations, the SAS 8.1 program package was used.[16] Samples were tested for normality by using the UNIVARIATE procedure and the Shapiro–Wilk W test. To test differences between the patient and control groups and plaques and control arteries for significance, an analysis of variance based on least-square means was calculated by using the General Linear Model procedure. The model used was: Y=group+residual error. For statistical analysis of ABCA1 and LXRα mRNA expression, the logarithmic delta cycle threshold (ct) values (ct target gene−ct β-actin) were used. Sex differences between the patient and control groups were assessed using the χ^2 test (FREQ procedure). To evaluate the relationships between LXRα and ABCA1 in arterial specimens, Pearson correlations were calculated by using the CORR procedure. $P<0.05$ was considered significant.

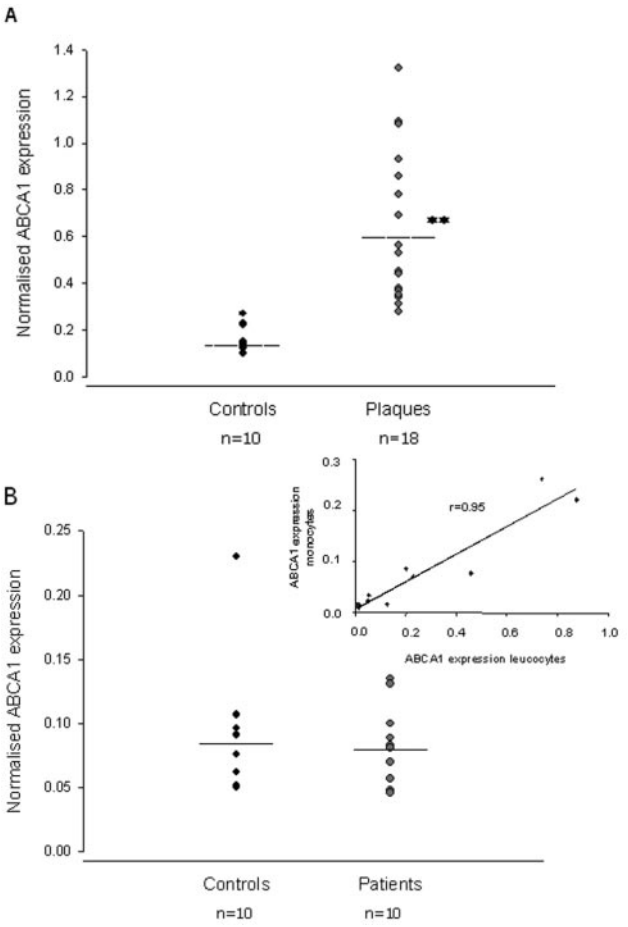

Figure 1. ABCA1 mRNA expression in plaques, control arteries, leukocytes, and monocytes. A, Quantitative, real-time polymerase chain reaction was performed on total RNA extracted from plaques (n=18) and control arteries (n=10). ABCA1 mRNA expression was normalized to β-actin as housekeeping gene. Values represent the average of 2 different measurements. Bars indicate mean values of each group; **denotes $P<0.0001$. B, Quantitative, real-time polymerase chain reaction was performed on total RNA extracted from leukocytes of patients (n=10) and control subjects (n=10). Inset: Correlation of ABCA1 mRNA expression between leukocytes and monocytes from whole blood (n=11) using magnetic beads technique. ABCA1 mRNA expression in leukocytes and monocytes was quantified with real-time polymerase chain reaction and normalised to GAPDH.

Results

ABCA1 Gene Expression

Real-time quantitative polymerase chain reaction was used to determine ABCA1 mRNA levels in plaques and control arteries. ABCA1 mRNA was significantly increased in plaques as compared with control arteries (Figure 1A, $P<0.0001$). To evaluate whether the upregulation of ABCA1

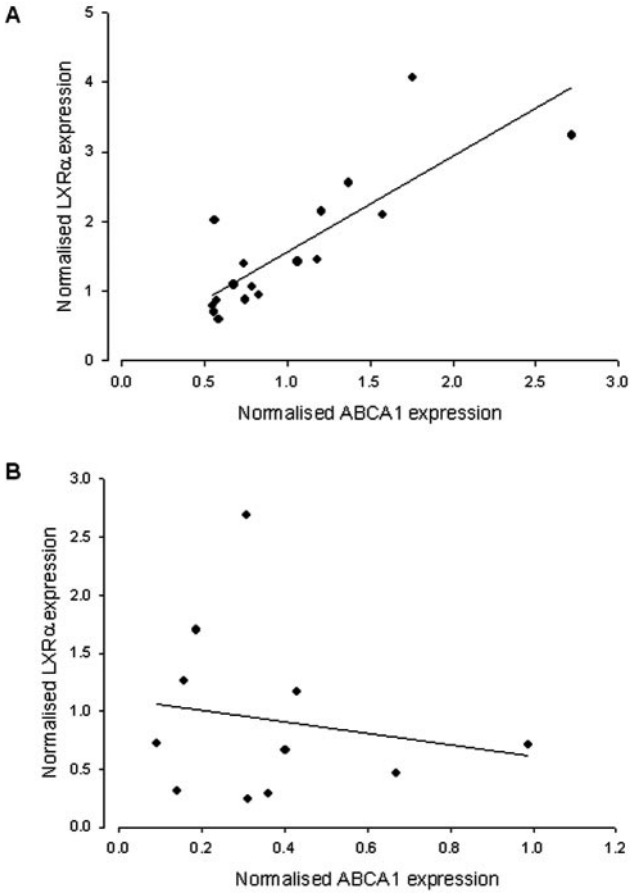

Figure 2. Association between mRNA levels of ABCA1 and LXRα in plaques and control tissue. ABCA1 and LXRα mRNA expression in plaques (A) and control arteries (B) was quantified with real-time polymerase chain reaction and normalized to β-actin.

was restricted to the diseased artery or reflected in the systemic circulation, we compared ABCA1 expression in leukocytes from patients undergoing CEA and age- and sex-matched controls. In previous experiments ABCA1 expression in leukocytes was comparable to monocytes (Figure 1B inset, $r=0.95$) and therefore seemed to reflect circulating monocyte ABCA1 expression. No difference in ABCA1 expression levels was found ($P=0.6741$; Figure 1B), indicating a localized upregulation of ABCA1 mRNA levels in the plaque tissue. To gain insight into potential underlying mechanisms, mRNA expression of LXRα was measured. The increase in ABCA1 mRNA detected in plaques was paralleled by a significant >2-fold increase in average LXRα mRNA levels ($P=0.0287$). Interestingly, in plaque tissue, a significant correlation between ABCA1 and LXRα mRNA expression levels was found (Figure 2A; $r=0.85$, $P<0.0001$), whereas in control arteries no association was detected (Figure 2B; $r=0.24$, $P=0.5070$).

ABCA1 Protein Expression

To assess protein expression, immunoblot analysis was performed. Total membrane fractions of plaques and control arteries were tested with antibodies against ABCA1 (Figure 3A; upper bands) and Na^+/K^+-ATPase (Figure 3A, lower bands), a plasma membrane protein used to ascertain equal sample loading. In contrast to mRNA levels, ABCA1 protein expression was significantly lower in plaques than in control arteries. Semiquantitative analysis using the OD of the bands confirmed the marked difference between controls and plaques ($P<0.0001$; Figure 3B) and remained highly significant after normalization of ABCA1 expression to Na^+/K^+-ATPase ($P=0.0004$; Figure 3C). No difference with regard to the Na^+/K^+-ATPase loading control was found ($P=0.8316$; Figure 3B).

Discussion

Primary and secondary prevention of stroke rely on the identification and treatment of modifiable risk factors such as hyperlipidemia, hypertension, hyperglycemia, and smoking. All of these conditions give rise to cerebral atherosclerosis, the commonest cause of stroke. Increasing interest and research in the field of the ABC transporters has raised the possibility of further new pharmacological interventions in atherosclerosis. The importance of ABCA1 in HDL formation has become apparent with the identification of mutations responsible for Tangier disease and familial HDL deficiency.[1,2] Although these investigations highlighted the importance of ABCA1 in reverse cholesterol transport and the development of early atherosclerosis, the potential role of ABCA1 in localized atherosclerosis in the absence of familial gene mutations has not been addressed.

We found both ABCA1 mRNA and protein were expressed in inferior mesenteric arteries with early asymptomatic atherosclerosis. Unsurprisingly, the histology of these age-matched control arteries showed early atheromatous changes (American Heart Association classification of atherosclerosis types I to III). These lesions ranging from foam cells to extracellular lipid pools in the intima do not result in clinically relevant sequelae, and may not progress to atheroma formation. The only previous report of ABCA1 localization in human atherosclerosis using in situ hybridization demonstrated that ABCA1 mRNA was predominantly localized to macrophages[14] in atherosclerotic lesions. However, it was not detected in normal arterial tissue, possibly because of the lower detection sensitivity of the in situ hybridization technique as compared with the quantitative real-time polymerase chain reaction (PCR) method that was used in the current study. ABCA1 protein has been detected in human endothelial and smooth muscle cell cocultures,[17] but there are no reports to date of ABCA1 protein detection in human arterial tissue in vivo. This study demonstrates the presence of ABCA1 protein in minimally atherosclerotic arteries.

The CEA specimens demonstrated significantly higher levels of ABCA1 mRNA compared with control arterial tissues. One potential source of confound might be related to the difference in drug usage between patient and control groups illustrated in Table 1. Therefore, linear regression modelling was used to assess ABCA1 expression in control and patient groups adjusted for age, sex, aspirin and statin usage. This analysis revealed that differences in ABCA1 expression between control and patient arteries remained significant once adjusted for drug usage (coef=0.646, $P=0.001$). This increase was not mirrored by an upregulation

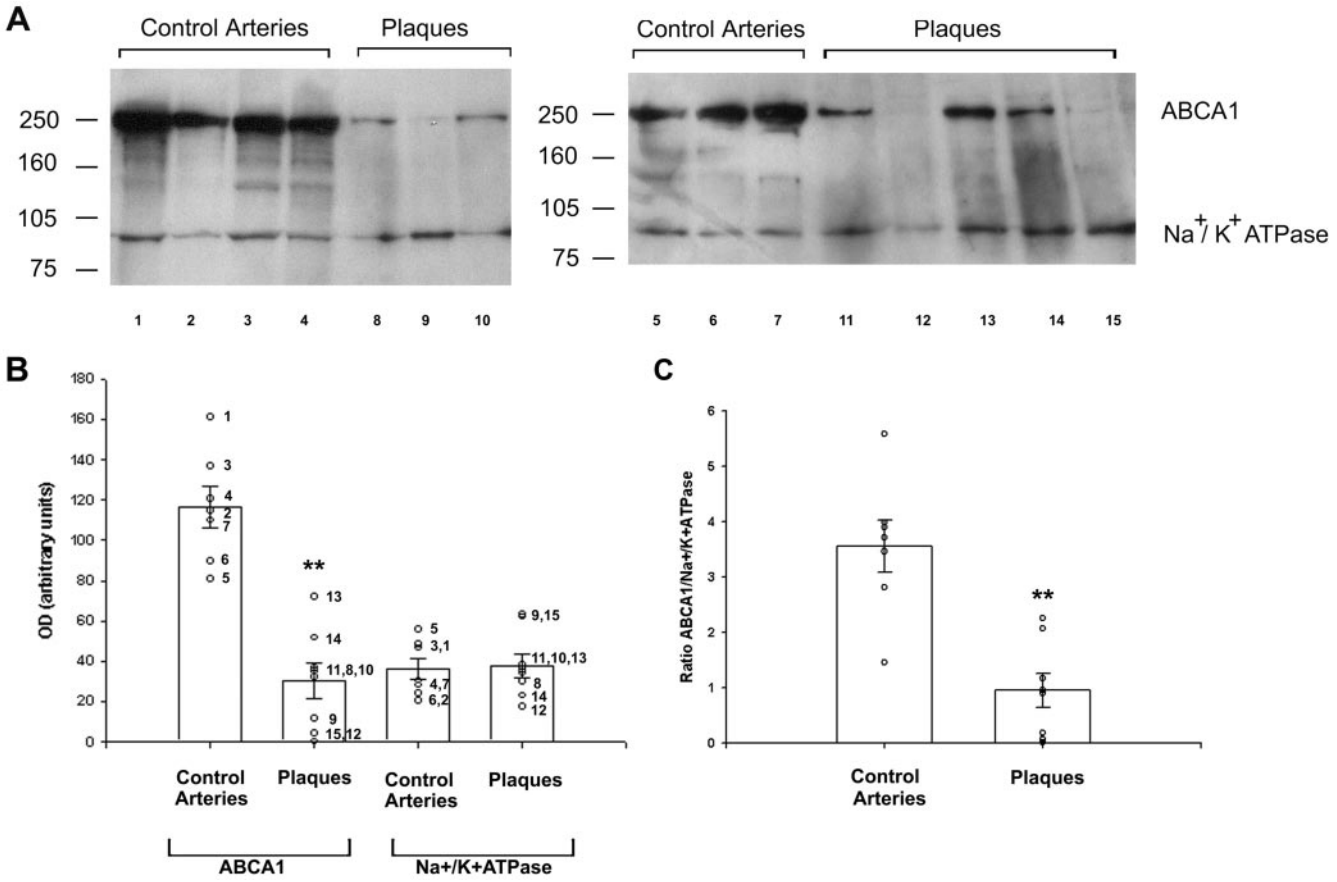

Figure 3. Western blot analysis of ABCA1 in plaques and control arteries. A, Representative immunoblots of ABCA1 protein expression are shown. Membrane proteins from plaques and control arteries were separated by reducing SDS-PAGE and immunoblotted with rabbit anti-ABCA1 and mouse anti-Na^+/K^+-ATPase antibodies. The positions of marker proteins (kDa) are indicated. Samples have been labelled with numbers 1 through 7 for controls and 8 through 15 for plaques. B, Quantification of protein expression was performed by assessing the OD of ABCA1 and Na^+/K^+-ATPase bands. Average values±SEM are shown. Individual values are also shown as open symbols with adjacent numbers representing the respective samples in panel A. **denotes $P<0.0001$. C, Ratio between ABCA1 and Na^+/K^+-ATPase protein expression according to results as presented in Figure 3B. **denotes $P=0.0004$.

of ABCA1 mRNA expression in peripheral leukocytes. This implies the observed upregulation in mRNA occurs predominantly at localized sites of disease.

The increase in *ABCA1* gene expression in diseased tissue is surprising given the fact that animal studies of *ABCA1* inactivation, as well as human *ABCA1* gene mutations, suggest loss of function is the key to lipid deposition and its sequelae. However, mRNA levels do not necessarily accurately reflect protein expression and, particularly for ABCA1, relative mRNA distribution in tissue shows significant discordance with protein expression patterns suggesting that post-transcriptional regulation may be important.[18] The relationship between ABCA1 mRNA and protein expression was assessed by analyzing the same specimen for both parameters. In control arteries, *ABCA1* gene expression was reflected by the presence of protein. Intriguingly, however, markedly lower levels of ABCA1 protein were present in advanced atherosclerotic lesions. Thus, despite increased transcription, a reduction in protein expression was observed. However, no positive or negative correlation was observed between the mRNA and protein levels in the control arteries and the plaques. This could be attributed to the low n-numbers, the semiquantitative nature of the protein data and various other influences on the intracellular sterol levels that regulate gene expression.

There is evidence that the composition and microenvironment of the atherosclerotic plaque could be associated with ABCA1 protein degradation. In advanced atherosclerotic lesions, macrophages tend to accumulate large amounts of free cholesterol.[19] Increased intracellular free cholesterol has been shown to accelerate the degradation of ABCA1 in macrophages.[20] Long chain fatty acids present in the plaque[21] can promote macrophage ABCA1 protein degradation.[22] Furthermore, it has recently been demonstrated that ABCA1 contains a PEST sequence that appears to enhance protein degradation.[23]

We have shown that in atherosclerotic tissues, both ABCA1 and LXRα mRNA levels were upregulated, and a clear correlation between mRNA levels of both genes was found. Nuclear receptors act as cholesterol sensors that respond to elevated sterol concentrations.[24] It has been previously shown in vitro that *ABCA1* transcription is stimulated by LXRα and the retinoid X receptor,[25] and the induction of *ABCA1* expression reflected that of *LXRα*.[26] Such a parallel increase in LXRα and ABCA1 mRNA was also found in our study. The observed upregulation of both LXRα and ABCA1 mRNA could be attributed to the

oxysterol-rich environment inside the plaque potentially amplified by low ABCA1 protein levels. Increased degradation of ABCA1 protein could hypothetically diminish cellular cholesterol efflux, resulting in increased free cholesterol, enhanced intracellular oxysterol loading, and stimulation of regulatory pathways involving LXRα.[6]

In conclusion, this study has shown that both the *ABCA1* gene and protein are expressed in mildly atherosclerotic arterial tissue in vivo. Advanced carotid atherosclerotic lesions are characterized by reduced ABCA1 protein levels despite significant upregulation of both ABCA1 and LXRα mRNA. This finding has potentially important clinical consequences. The observation that upregulation of ABCA1 mRNA fails to translate into ABCA1 protein implies that pharmacological targeting of the ABCA1 and LXRα pathways may not achieve the anticipated atheroprotective effect in advanced atherosclerotic lesions.

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