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Atorvastatin reduces CD68, FABP4, and HBP expression in oxLDL-treated human macrophages

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Abstract

With the aim of identifying new target genes that could contribute to limit foam cell formation, we analyzed changes in the pattern of gene expression in human THP-1 macrophages treated with atorvastatin and oxidized-LDL (oxLDL). To this end, we used a human cDNA array containing 588 cardiovascular-related cDNAs. Exposure to oxLDL resulted in differential expression of 26 genes, while coincubation with atorvastatin modified the expression of 29 genes, compared to treatment with oxLDL alone. Changes in the expression of candidate genes, potentially connected to the atherosclerotic process, were confirmed by quantitative RT-PCR and Western blot. We show that atorvastatin prevents the increase in the expression of scavenger receptor CD68 and that of fatty acid binding protein 4 caused by oxLDL. In addition, atorvastatin reduces the expression of HDL-binding protein, apolipoprotein E, and matrix metalloproteinase 9. These findings are relevant to understand the direct antiatherogenic effects of statins on macrophages.

Keywords: Atorvastatin; oxLDL; THP-1; FABP4; CD68; HBP

Atherosclerosis and its consequences (coronary heart disease and stroke) remain the leading cause of death in most industrialized countries [1]. The identification of lipoprotein abnormalities as a major risk factor for cardiovascular disease, specifically high levels of low density lipoprotein (LDL)-cholesterol, led to consider atherosclerosis primarily as a lipid disorder. However, atherogenesis involves also an inflammatory response in the arterial wall [2] and, nowadays, it is regarded as an inflammatory disease that develops in the context of hypercholesterolemia [3].

The disruption of the normal physiology of the vascular endothelium, also known as endothelial dysfunction, is one of the earliest events in the atherosclerotic process [4]. The upregulation of cell-adhesion molecules [5] greatly increases the adherence of blood monocytes to the endothelium. After adhesion, monocytes migrate into the subendothelial space, where they differentiate into macrophages, a process that is accompanied by an increase in the surface expression of scavenger receptors. The uptake of oxidized-LDL (oxLDL) in a non-regulated manner leads to the accumulation of lipid droplets in the cytosol of macrophages, which are then transformed into foam cells.

Today, the main pharmacological approach to reduce the risk for coronary heart disease is the correction of abnormally high plasma lipid levels [6]. The most effective drugs to reduce plasma LDL-cholesterol are statins, inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, which significantly reduce coronary and total mortality in clinical trials [7]. However, the clinical benefit of these drugs is manifested early in the course of lipid-lowering therapy, before plaque regression could occur. Moreover, quantitative angiographic studies have shown that reversal of arterial narrowing occurs slowly and only to a small extent, despite a substantial decrease in cardiovascular events [8]. These findings led to the concept that statins may

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influence vascular biology by mechanisms other than the lowering of plasma cholesterol [7]. These so-called pleiotropic effects include antiinflammatory [9], immunomodulatory [10], and direct antiatherosclerotic effects. In this sense, statins reduce monocyte recruitment by inhibiting MCP-1 [11], limit cholesteryl ester deposition, and diminish proliferation of macrophages exposed to oxLDL [12–14], key events in the atherogenic cascade. Moreover, statins also inhibit the secretion of matrix metalloproteinases (MMP), enzymes that digest and weaken the plaque cap, contributing to plaque unstability [15]. Most of these pleiotropic effects are attributed to a decreased synthesis of mevalonate, precursor of cholesterol and isoprenoids [16] such as farnesyl pyrophosphate and geranyl-geranyl pyrophosphate, that participate in the post-translational modification of small G proteins, e.g., Ras and Rho [17]. G proteins are involved in several cellular processes, including the regulation of gene expression. However, given the complexity of atherogenesis, the pattern of gene expression underlying this process, and how it can be modified by antiatherosclerotic drugs, is not well known.

The use of cDNA arrays allows the simultaneous analysis of the expression of hundreds of genes, in contrast to the traditional approach aimed to study an individual gene or protein. By using this technique, several authors examined the changes in gene expression profile in atherosclerotic lesions [18–20] or in macrophages [21,22], a cell type known to be intimately involved in all the phases of the atherosclerotic process [23]. The objective of the present work was to study the role of statins in regulating macrophage gene expression during exposure to a pro-atherogenic environment. To this end, we used the human monocyte/macrophage cell line THP-1, a well-known model of foam cell formation [24]. PMA-differentiated THP-1 cells were exposed to oxLDL in the absence or in the presence of atorvastatin, analyzing subsequently the relative expression of 588 human genes disposed in the Atlas Human cardiovascular cDNA Array (Clontech). We report here that atorvastatin attenuates the increase in gene expression of fatty acid binding protein 4 (FABP4) and CD68 caused by oxLDL.

Materials and methods

Pure atorvastatin was provided by Parke-Davis (Ann Arbor, MI), now Pfizer. Cell culture reagents were from Gibco, Invitrogen (Paisley, UK), with the exception of fetal bovine serum and 4-β-phorbol 12-β-myristate 13-α acetate (PMA), both from Sigma–Aldrich (St. Louis, MO). Atlas Pure Total RNA Labeling System and Atlas Human Cardiovascular cDNA Array were from Clontech (BD Biosciences). RT-PCR reagents were from Invitrogen, except for the random hexamers and the specific primers, which were obtained from Roche Diagnostics (Mannheim, Germany), and [α-^{32}P]dATP came from Amersham Biosciences (Freiburg, Germany). Specific antibodies against CD68 and matrix metalloproteinase 9 (MMP-9) were from Santa Cruz Biotechnology (Heidelberg, Germany); apolipoprotein E (apo E) antibody was from Biogenesis (England, UK) and the antibody against FABP4 came from R&D Systems (Minneapolis, USA).

Preparation of LDL and oxidized-LDL. Low density lipoproteins (LDL, d 1.019–1.063) were prepared from the plasma of healthy human donors by sequential ultracentrifugation [25]. Lipoproteins were dialyzed against phosphate saline buffer, and their protein concentration was assessed by the method of Bradford [26], using bovine serum albumin as a standard. Oxidized-LDLs (oxLDL) were prepared by exposing freshly isolated native LDL to 5 μM $CuSO_4$ for 5 h at 37 °C. The thiobarbituric acid-reactive substance (TBARS) content of oxLDL was approximately 20 nmol MDA/mg LDL protein. Batches of oxLDL were kept at 4 °C in the dark and used within a week.

Cell culture. THP-1 cells were maintained in RPMI 1640 medium containing 25 mM Hepes buffer (supplemented with 10% fetal bovine serum, 1% L-glutamine 200 mM, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in 5% CO_2. Monocytes were differentiated with 0.1 μM PMA during 24 h to macrophages, which then were incubated with vehicle or 100 μg/ml oxLDL in the absence or in the presence of 5 μM atorvastatin for 24 h. Cell viability, determined by MTT [27], was not decreased by any of the treatments.

cDNA arrays. Gene expression was analyzed by hybridization to cDNA arrays (Atlas Human Cardiovascular Array). Cells were harvested and total RNA was prepared following the procedure recommended by Clontech. RNA was then incubated with RNAse-free DNAse (10 U/50 μg of RNA) and its integrity was assessed after agarose gel electrophoresis in the presence of formaldehyde. Radiolabeled cDNA probes were prepared from 5 μg of total RNA. Briefly, the RNA was hybridized for 2 min at 70 °C followed by 2 min at 50 °C with 1 μl of the primer mix, containing the 588 primers for the genes present in the array. The RT reaction was carried out using MMLV RT and 30 μCi [α-^{32}P]dATP for 25 min at 50 °C. After filtering the un-incorporated nucleotide through Sephadex G-50 columns, the ^{32}P-labeled cDNAs were hybridized to the array membranes using ExpressHyb (Clontech) in an oven at 68 °C overnight. On the following day the membranes were washed lowering the astringency progressively to 0.5× SSC, 0.5% SDS, and placed in contact with europium screens for 14 days. The screens were scanned with a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Array data analysis. Image analysis and quantitation were carried out with the Atlas Image 2.01 software (Clontech). After grid assignment, the adjusted intensity for each gene was calculated after subtracting the background. This value was used as the input for the GeneSpring 6.1 program (Silicon Genetics) that allows normalization of data analysis from different experiments, the generation of restriction lists, and the functional classification of the differentially expressed genes. Normalization was applied in two steps: (i) per chip normalization, where each data-point was divided by the 50th percentile of all measurements in its array and (ii) normalization of each sample against the median of the control samples. The expression of each gene is reported as the ratio of the value obtained after each treatment relative to control after normalization of the data. Lists of differentially expressed genes (upregulated: ratio >2; downregulated: ratio <0.5) were generated using data from three independent experiments for each condition. The genes in these lists were further classified according to their function.

Quantitative RT-PCR. Levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) under quantitative conditions. Complementary DNA was synthesized, in a total volume of 20 μl, from RNA samples by mixing 0.5 μg of total RNA, 125 ng of random hexamers in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 U Moloney murine leukemia virus reverse transcriptase, 20 U RNAsin, 0.5 mM of each dNTP (Sigma), and 50 mM Tris–HCl buffer (pH 8.3). Samples were incubated at 37 °C for 60 min. A 5 μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers. Each 50 μl PCR

contained 5 μl of the RT reaction, 1.2 mM $MgCl_2$, 200 μM dNTPs, 0.25 μCi [α-^{32}P]dATP (3000 Ci/mmol), 1 U of *Taq* DNA polymerase, 0.5 μg of each primer, and 20 mM Tris–HCl, pH 8.5. To avoid unspecific annealing, cDNA and *Taq* DNA polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60 °C). The sequences of the forward and reverse primers used for PCR amplification, the length of the PCR product, the number of cycles used in the PCR, and the linear range for the amplification are given below.

PCR was performed in an MJ Research Thermocycler equipped with peltier system and temperature probe. Preliminary experiments were carried out using different number of cycles to determine the linear conditions of PCR amplification for all the genes studied, as shown in Table 1.

[^{32}P]dATP was used in the PCR to produce a radioactive product that could be detected with great sensitivity during the exponential phase of the reaction. After an initial denaturation for 1 min at 94 °C, PCR was performed for the indicated number of cycles. Each cycle consisted of denaturation at 92 °C for 1 min, primer annealing at 60 °C, and primer extension at 72 °C for 1 min and 50 s. A final 5-min extension step at 72 °C was performed. Five microliters of each PCR sample was electrophoresed on a 1-mm-thick 5% polyacrylamide gel. The gels were dried and placed on contact with europium screens that were scanned using phosphorimaging. The expression of specific mRNAs is reported upon normalization using the tubulin α1 (TUBA1) gene as internal control.

Western blotting analysis. Whole protein extracts (20–50 μg) from treated cells were subjected to 10–15% SDS–polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA). For CD68, MMP9, and ApoE immunodetection, membranes were blocked 1 h at room temperature in PBS containing 0.5% Tween 20 and 5% of nonfat dried milk and incubated with a rabbit polyclonal antibody against CD68 (1:200 in blocking buffer, overnight at 4 °C), mouse monoclonal antibody raised against MMP9 (1:200 in blocking buffer, 1 h at room temperature), and a mouse monoclonal antibody raised against ApoE (1:1000 in blocking buffer, 1 h at room temperature). Immunological detection of FABP4 was performed by incubation of the membranes in blocking solution (5% nonfat milk in PBS) overnight at 4 °C and in a goat antibody against FABP4 (1:100 in 1% nonfat milk in PBS, 1 h at room temperature). Appropriate secondary antibodies were incubated with membranes (1 h at room temperature) and detection was achieved using the enhanced chemiluminescence (ECL) detection system (Biological Industries, Israel). Blots were also incubated with a monoclonal antibody raised against β-actin, used as a control of equal abundance of protein between the samples. Size of detected proteins was estimated using Kaleidoscope Prestained Standards (Bio-Rad).

Results

Differential gene expression analysis using cDNA arrays

The differential expression of the genes included in the Atlas Human Cardiovascular Array (Clontech) was analyzed in human THP-1 macrophages untreated (CT) or exposed to oxLDL in the absence (oxLDL) or in the presence of 5 μM atorvastatin (ATV).

Table 2 lists the genes that were overexpressed more than 2-fold or underexpressed to less than 0.5-fold in oxLDL-treated cells relative to control cell values. The differentially expressed genes were classified according to their function. Out of the 588 genes included in the array, 26 genes, which correspond to 4.4% of the total, were differentially regulated in oxLDL-treated cells: 21 were downregulated and 5 upregulated. The most upregulated gene was fatty acid binding protein 4 (FABP4, also termed aP2), whose expression was increased 17-fold in cells exposed to oxLDL.

Next, we compared the gene expression profile in macrophages treated with oxLDL plus atorvastatin with that of cells exposed to oxLDL alone. Our results show that 7 genes were overexpressed more than 2-fold, while 22 genes were underexpressed to less than 0.5-fold (Table 3). It is noteworthy that FABP4 expression, which was highly upregulated by oxLDL, was reduced when atorvastatin was added to the macrophages concomitantly with oxLDL (ATV/oxLDL ratio of 0.3).

Candidate genes were selected from the cDNA array data for their potential connection to the atherosclerotic process. CD68 is a scavenger receptor expressed in the

Table 1

Gene sequence of primers (5′ to 3′)	Product length (bp)	Number of cycles	Linear cycle range
CD68			
Forward: GGGACCCTCAACTGCCACTCA	343	22	18–25
Reverse: AGAGAAGCAGGTGGGGATGGG			
FABP4			
Forward: CTGGGCCAGGAATTTGACGAA	162	22	18–25
Reverse: CATGACGCATTCCACCACCAG			
MMP9			
Forward: GCGCTGCTGCTTCTCCAGAAG	217	21	18–23
Reverse: GCAAAGGCGTCGTCAATCACC			
HBP			
Forward: GGCCTCAGCAACTGTTGCCAT	170	21	18–23
Reverse: TCTCGATGCCCTCTTTGGTGC			
ApoE			
Forward: ATGAAGGTTCTGTGGGCTGCG	209	22	18–25
Reverse: GAGCAGCTCCTCCTGCACCTG			
TUBA1			
Forward: CTTCGCCTCCTGAATCCCTAG	273	21–22	18–25
Reverse: GTAGGTGCCAGTGCGAACTTC			

Table 2
Differentially expressed genes in oxLDL-treated THP-1 macrophages

	GenBank	SwissProt	Ratio	Functional classification
(A) Overexpressed genes (ratio >2)				
Fatty acid binding protein 4, adipocyte (FABP4)	J02874	P15090	17	Intracellular communication
Annexin A1	X05908	P34810	7.35	Metabolism
GRO3 oncogene	M36821	Q16455	3.73	Extracellular secreted proteins
P450 (cytochrome) oxidoreductase	S90469	P04083	3.11	Metabolism
CD68 antigen	S57235	P19876	2.46	Cell surface antigens
(B) Underexpressed genes (ratio <0.5)				
Insulin	X70508	P01308	0.07	Extracellular secreted proteins
Cytochrome P450, subfamily I	K03191	P04798	0.09	Metabolism
Cytochrome P450, subfamily VIIA	X56088	P22680	0.20	Metabolism
Prostaglandin I2 (prostacyclin) synthase	D38145	Q16647	0.26	Metabolism
Matrix metalloproteinase 9 (MMP9)	J05070	P14780	0.27	Extracellular matrix proteins
Matrix metalloproteinase 7 (MMP7)	X07819	P09237	0.28	Extracellular matrix proteins
Cytochrome P450, subfamily XVII	M14564	P05093	0.35	Metabolism
Villin 2 (ezrin)	X51521	P15311	0.39	Intracellular transductors
Endoglin (Osler–Rendu–Weber syndrome 1)	J05481	P17813	0.39	Cell adhesion proteins/cell surface antigens
Intercellular adhesion molecule 1 (CD54)	J03132	P05362	0.40	Cell adhesion proteins/cell surface antigens
Serine (or cysteine) proteinase inhibitor	M18082	P05120	0.42	Extracellular secreted proteins
Catenin (cadherin-associated protein), α2	M94151	P26232	0.43	Cell adhesions proteins
Apolipoprotein C-II	X00568	P02655	0.44	Extracellular transport proteins
Iduronate 2-sulfatase (Hunter syndrome)	M58342	P22304	0.44	Metabolism
Vascular endothelial growth factor B (VEGF)	U48801	P49765	0.45	Extracellular secreted proteins
Adrenergic, β2-, receptor, surface	Y00106	P07550	0.45	Membrane receptors
Small inducible cytokine A5 (RANTES)	M21121	P13501	0.47	Extracellular secreted proteins
Junction plakoglobin	M23410	P14923	0.47	Intracellular transductors
Adducin 1 (α)	X58141	P35611	0.47	Intracellular transductors
Profilin 1	J03191	P07737	0.49	Cytoskeleton
Tissue inhibitor of metalloproteinase 3 (TIMP3)	U14394	P35625	0.49	Extracellular matrix proteins

The table shows the names, the GenBank and SwissProt accession numbers of the genes that were upregulated (ratio >2) (A) or downregulated (ratio <0.5) (B) in cells exposed to 100 μg/ml oxLDL in comparison with control cells. The ratio column corresponds to the expression of each gene relative to the control. The last column indicates the functional categories to which the genes belong. Results are means of three independent experiments performed per each condition.

surface of mature macrophages. The uptake of oxidatively modified LDL via scavenger receptors leads to an unregulated increase in intracellular cholesterol levels. FABP4 is a protein that binds and transports fatty acids, which are esterified to form cholesteryl esters, the preferred intracellular storage form of cholesterol. Therefore, both CD68 and FABP4 may participate in the conversion of macrophages into foam cells. Conversely, macrophage-secreted apo E is involved in the efflux of free cholesterol from these lipid-loaded cells. It is overexpressed in human foam cells and co-localized with HDL binding protein (HBP), which is probably involved in macrophage sterol metabolism. Finally, MMP-9 is a proteolytic enzyme produced by macrophages that may influence the composition and stability of atherosclerotic plaques.

Validation of selected targets by RT-PCR

To validate the array results, we performed quantitative RT-PCR analysis of these five selected genes. Using this methodology it was determined that mRNA levels for CD68 and FABP4 were increased by oxLDL 2.3- and 8.4-fold, respectively, compared to control cells (Figs. 1A and 2A). It was also demonstrated that atorvastatin decreased CD68 and FABP4 expression by 26% and 41%, respectively, in comparison with cells treated with oxLDL (Figs. 1B and 2B). In addition, we also validated by QRT-PCR the underexpression of MMP-9 (21%), apo E (26%), and HBP (38%) by atorvastatin treatment (Figs. 3A–C). These results confirmed the RNA data obtained in the screening performed using the cDNA arrays.

Western blot assays

Once the expression to mRNA had been validated for selected genes, we proceeded to perform Western blot analyses with commercially available antibodies to investigate whether the changes at the RNA level were translated into protein. Total extracts were obtained from cells treated in the same conditions as in the array experiments. CD68 protein levels were increased 2.7-fold in cells treated with oxLDL alone, while

Table 3
Differentially expressed genes in atorvastatin-treated THP-1 macrophages

	GenBank	SwissProt	Ratio	Functional classification
(A) Overexpressed genes (ratio >2)				
Homo sapiens H105e3 (H105e3)	U47105	Q15738	3	Cholesterol biosynthesis
Human lysosomal acid lipase/cholesteryl esterase (LIPA)	M74775	P38571	2.9	Lipoprotein lipase
Mitochondrial 3-ketoacyl-CoA thiolase β-subunit	D16481	P55084	2.8	Fatty acid oxidation
Endoglin precursor	J05481	P17813	2.7	Circulation/histogenesis and organogenesis
Homo sapiens delta3, delta2-CoA-isomerase	L24774	P42126	2.6	Metabolism
Protein C precursor	X02750	Q16001	2.1	Coagulation
Homo sapiens mRNA for rab 13	X75593	P51153	2	Cell adhesion/vesicle transport
(B) Underexpressed genes (ratio <0.5)				
Tissue inhibitor of metalloproteinase 1 (TIMP1)	X03124	P01033	0.23	Extracellular matrix proteins
Cystathionine-β-synthase (CBS)	L19501	P35520	0.24	Metabolism
Matrix metalloproteinase 9 (MMP9)	J05070	P14780	0.26	Extracellular matrix proteins
Atrial natriuretic peptide clearance receptor	X52282	P17342	0.26	Hormone receptors
Apolipoprotein E receptor 2	Z75190	Q99876	0.29	Lipid metabolism
Human von Willebrand factor prepropeptide	M10321	P04275	0.29	Coagulation
Fatty acid binding protein 4 (FABP4)	J02874	P15090	0.30	Intracellular communication
Plasminogen activator, tissue	M15518	P00750	0.36	Coagulation
Apolipoprotein E	M12529	P02649	0.36	Extracellular transport proteins
α-Catenin (CTNNA1)	D13866	P35221	0.36	Cell adhesion
Diaphorase (NADH) (cytochrome *b*-5 reductase)	Y09501	P00387	0.36	Metabolism
SREBP cleavage-activating protein (SCAP)	D83782	Q12770	0.37	Intracellular communication
Cadherin-associated protein-related (CTNNA2)	M94151	P26232	0.37	Cell adhesion
Fibrinogen, B β-polypeptide	J00129	P02675	0.38	Coagulation
High-density lipoprotein-binding protein (HBP)	M83789	Q00341	0.40	Extracellular transport proteins
Symplekin (SPK)	U49240	Q92797	0.4	Cell adhesion proteins
CD68 antigen	S57235	P34810	0.42	Cell adhesion proteins/cell surface antigens
3-Oxoacid CoA transferase	U62961	P55809	0.44	Metabolism
Profilin 1	J03191	P07737	0.44	Cytoskeleton/motility proteins
CD18 antigen	M15395	P05107	0.46	Cell adhesion proteins/cell surface antigens
Tenascin C	X78565	P24821	0,46	Extracellular matrix proteins
Thrombospondin 2	L12350	P35442	0.47	Cell adhesion proteins/cell surface antigens

The table shows the names, the GenBank, and the SwissProt accession numbers of the genes that were upregulated (ratio >2) (A) or downregulated (ratio <0.5) (B), in cells exposed to oxLDL plus ATV in comparison with cells treated with oxLDL alone. The ratio column corresponds to the expression of each gene relative to the control. The last column indicates the functional categories to which the genes belong. Results are means of three independent experiments performed per each condition.

cotreatment with atorvastatin led to a 53% reduction of the effect produced by oxLDL (Figs. 1C and D). Likewise, the expression of FABP4 protein was induced 2.2-fold by oxLDL and this increase was reduced by the presence of atorvastatin by 40% (Figs. 2C and D). In addition, atorvastatin decreased the levels of MMP-9 and apo E by 25% and 67%, respectively, (Figs. 3D and E). Thus, in all cases the Western results confirmed the changes observed for RNA.

Discussion

In the present work, cDNA arrays have been used to detect changes in gene expression elicited by atorvastatin in human macrophages exposed to oxLDL. Among the differentially expressed genes we focused our attention on some of the genes whose expression can modulate the conversion of macrophages to a foam cell phenotype. This approach allowed us to identify novel effects of statins that could contribute to limit atherosclerosis progression. The genomic analyses were performed using specific cardiovascular cDNA Arrays containing 588 cDNAs. Even if this number might seem limited given the total number of coding genes in the human genome, it has to be mentioned that this array is specifically designed to contain cDNAs related to the cardiovascular system.

The study revealed two genes whose upregulation by oxLDL is attenuated by atorvastatin, namely CD68 and FABP4. CD68, or macrosialin, is a class D scavenger receptor that binds minimally and highly oxidized forms of LDL [28]. The specific role of CD68 in atherosclerosis has not been totally elucidated; a direct involvement in foam cell formation has been demonstrated only for scavenger receptor class A (SRA) and CD36. On the other hand, it has been shown that a high-fat diet induces macrosialin expression in mouse liver Kupffer cells, and that treatment of RAW264.7, J774 and mouse peritoneal macrophages with oxLDL increases the

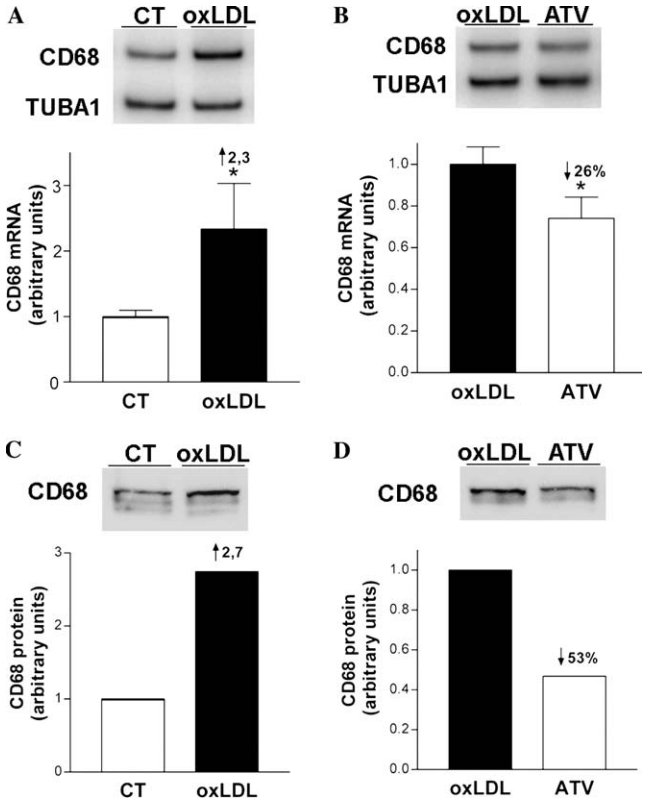

Fig. 1. Validation of CD68 gene expression by RT-PCR and Western blot. Analysis of CD68 mRNA levels (A) and protein expression (C) in PMA-treated THP-1 cells incubated with 100 μg/ml oxLDL during 24 h. Analysis of CD68 mRNA levels (B) and protein expression (D) in PMA-differentiated THP-1 cells incubated with oxLDL (100 μg/ml) + ATV (5 μM) versus oxLDL-treated cells. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of mRNA levels, normalized using TUBA-1 as reference, are shown. Data represent means ± standard deviation (SD) of three independent experiments. $^{*}p < 0.05$ compared with the corresponding control situation. Whole protein extracts (50 μg) were subjected to a 10% SDS–polyacrylamide gel electrophoresis. The blots were analyzed with an anti-CD68 antibody that detects a ~80 kDa protein. Representative autoradiograms and quantifications of two independent experiments are shown.

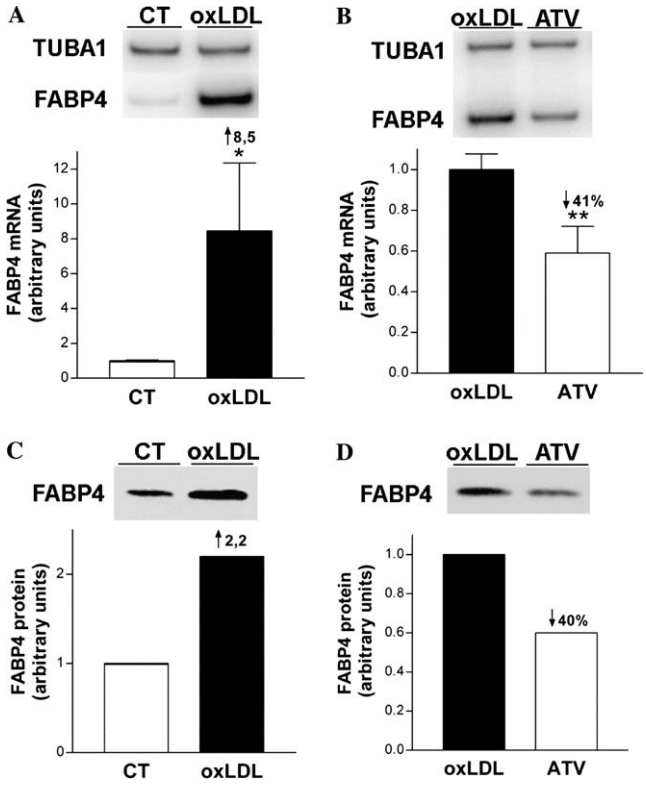

Fig. 2. Validation of FABP4 gene expression by RT-PCR and Western blot. Analysis of FABP4 mRNA levels (A) and protein expression (C) in PMA-treated THP-1 cells incubated with 100 μg/ml oxLDL during 24 h. Analysis of FABP4 mRNA levels (B) and protein expression (D) in PMA-differentiated THP-1 cells incubated with oxLDL (100 μg/ml) + ATV (5 μM) versus oxLDL-treated cells. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of the mRNA levels, normalized using TUBA-1 as reference, are shown. Data represent means ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the corresponding control situation. Whole protein extracts (20 μg) were subjected to a 15% SDS–polyacrylamide gel electrophoresis. The blots were analyzed with an anti-FABP4 antibody that detects a ~15 kDa protein. Representative autoradiograms and quantifications of two independent experiments are shown.

mRNA levels of macrosialin [29]. These data, together with the finding that the macrosialin gene is localized in a region of chromosome 11 involved in the control of HDL levels [30], suggest that this gene product plays a role in lipoprotein metabolism and atherosclerosis development. Our results (array data, PCR, and Western-blot experiments) demonstrate that oxLDL increase the expression of CD68, in accordance with previous published results [29,31]. Although it has not been demonstrated that oxLDL bound to CD68 are effectively internalized and catabolized, it is tempting to speculate that, similar to other scavenger receptors, the increase in CD68 after oxLDL stimulus could serve to remove these modified lipoproteins from the environment, leading finally to foam cell formation. In fact, it has been described that CD68 is highly expressed in macrophages from atherosclerotic lesions of apo E-deficient mice [32]. Our results also show that when ATV is included in the incubation medium, the inductive response of CD68 to oxLDL is significantly reduced. Previously, it was shown that statins reduced the binding and internalization of modified LDL in macrophages, an effect that was mainly attributed to a decrease in CD36 or SRA expression [33–35]. However, the reduction of lipoprotein-derived cholesterol accumulation in macrophages could also be attributed, at least in part, to the effects of statins on CD68 expression.

Intracellular fatty acid binding proteins (FABP) are a family of 14–15 kDa proteins that are expressed in a tissue-specific manner and bind to fatty acids with high affinity [36]. The adipocyte FABP, also known as FABP4, ALBP or aP2, was first detected in adipocytes, but it is also expressed in THP-1 and U-937 human

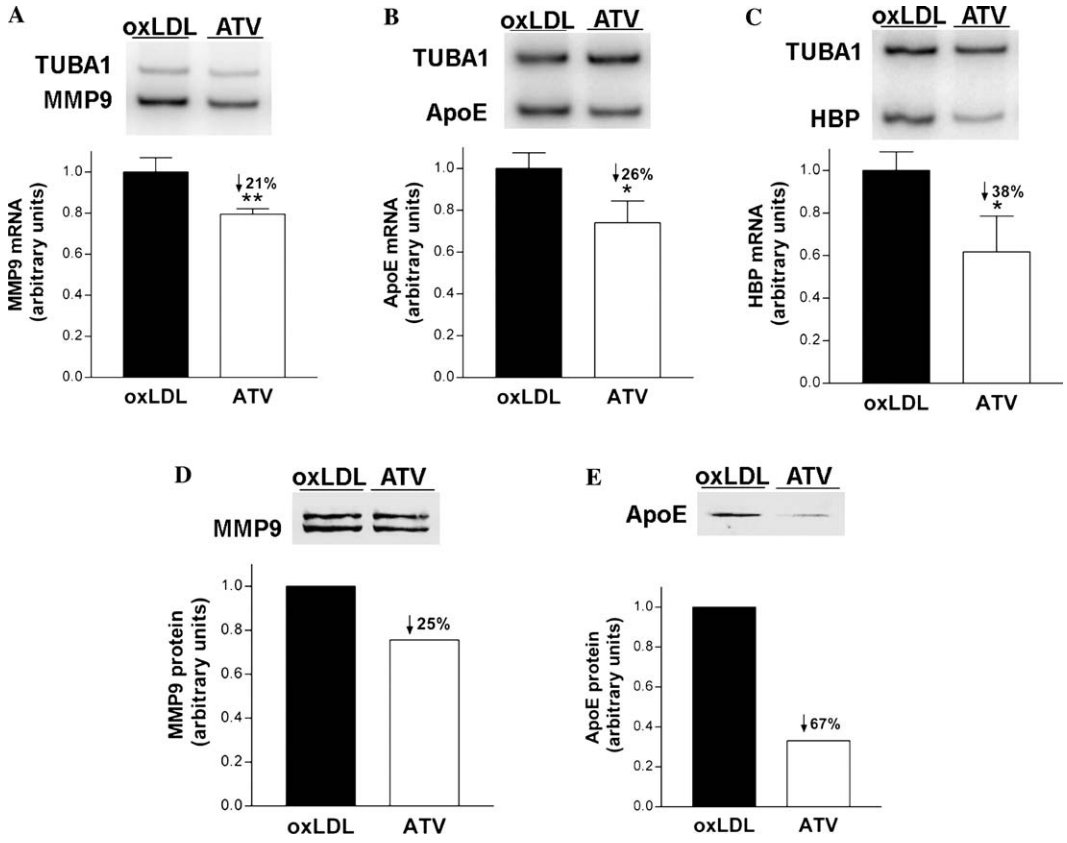

Fig. 3. Validation of MMP9, ApoE, and HBP expression by RT-PCR and Western blot. Analysis of MMP9 (A), ApoE (B), and HBP (C) mRNA levels and MMP9 (D) and ApoE (E) protein expression in PMA-treated THP-1 cells incubated with oxLDL (100 μg/ml) + ATV (5 μM) versus oxLDL-treated cells. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of the mRNA levels normalized using TUBA-1 as reference, are shown. Data represent means ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the corresponding control situation. Total protein extracts (20–50 μg) were resolved in 10–15% SDS–polyacrylamide gel. The blots were analyzed with the corresponding specific antibodies that detect a ~92 and a ~34 kDa proteins for MMP9 and ApoE, respectively. Representative autoradiograms and quantifications of two independent experiments are shown.

macrophages, and in primary human and mouse monocytes [37]. Our data show an increase in FABP4 expression after treatment of THP-1 macrophages with oxLDL, in agreement with Fu et al. [38]. Although the mechanism is not fully understood, the effect could be attributed to PPARγ activation by some components of oxLDL, as it has been reported that PPARγ agonists induce FABP4 in monocytes [39] and human macrophages [40]. Studies in transgenic mice have shown that FABP4 deficiency protects apo E-deficient mice from atherosclerosis development [41]. Specifically, macrophages lacking FABP4 show a reduced expression of several pro-inflammatory cytokines, and accumulate less cholesteryl esters when they are exposed to modified lipoproteins; moreover, bone marrow transplantation experiments showed that apo $E^{-/-}$ mice reconstituted with $FABP4^{-/-}$ macrophages had significant reductions in atherosclerotic lesions compared to apo $E^{-/-}$ mice reconstituted with $FABP4^{+/+}$ macrophages [42]. Therefore, it is believed that macrophage FABP4 expression promotes foam cell formation. Our results show that atorvastatin significantly prevents the increase in FABP4 expression caused by oxLDL, both at the mRNA and protein levels. It has been proposed that FABP4 shuttles fatty acids to intracellular enzymes such as ACAT, resulting in an increase in cholesteryl ester formation [37]. On the other hand, it is well known that statins reduce macrophage cholesteryl ester synthesis, an effect that has been traditionally attributed to an indirect inhibition of ACAT, by limitation of cholesterol availability [43]. It is possible that statins also reduce cholesteryl ester accumulation in macrophages through the inhibition of FABP4 expression, as this would reduce the amount of free fatty acids available for the esterification reaction. This effect may constitute a novel direct mechanism for the antiatherosclerotic actions of statins, independent of a reduction in plasma lipid levels.

The expression of three additional genes involved in the genesis and disruption of atherosclerotic plaques, namely MMP9, HBP, and apo E, was also reduced by atorvastatin, in comparison with macrophages treated with oxLDL alone. HBP, as its chicken orthologue vigilin, is expressed as a cytosolic protein (150-kDa) [44,45] which is processed to a 110-kDa form that can be

attached to the plasma membrane, where it binds HDL [46]. However, it does not seem to act as a classic cell surface receptor, and its function has not yet been established. Some evidences suggest that HBP may play a role in cellular sterol metabolism and in atherosclerosis. For example, in fibroblasts from subjects with familial HDL deficiency, HBP expression is higher than in cells from control subjects, reflecting an impairment in cholesterol transport [47]. Moreover, Chiu et al. [48] demonstrated that HBP was highly expressed in endothelial and macrophage-derived foam cells from human atherosclerotic lesions. As HBP contains KH domains that confer the ability to bind to RNA [49], it has been proposed that HBP could modulate the synthesis of proteins involved in cellular cholesterol mobilization processes [48]. Therefore, the reduction of HBP expression elicited by atorvastatin could restore these altered pathways of cholesterol trafficking, a mechanism that may contribute to the beneficial effects of statins in the atherosclerotic process.

Interestingly, macrophage foam cells of human atherosclerotic lesions expressing HBP, express also apo E [48]. According to our results, both genes are underexpressed by atorvastatin. Only a few studies focused on the effect of statins on macrophage apo E mRNA levels, and the results were contradictory. Thus, Cignarella et al. [50] showed that lovastatin increased apo E mRNA in human monocyte-derived macrophages whereas Castilho et al. [51] showed the opposite in THP-1 cells. Our results are in agreement with the latter, and the discrepancies with the results of Cignarella et al. could be attributed to the use of different cellular models. Macrophage-secreted apo E is regarded as an antiatherogenic protein, because it stimulates the efflux of free cholesterol from these cells [52]. However, the relative importance of this apoE-dependent cholesterol efflux pathway, compared to the efflux mediated by apo AI is currently unknown [53]. On the other hand, the inhibition of apo E production by statins may simply reflect a beneficial effect of this class of drugs, that is, the reduction of macrophage cholesterol levels.

Finally, MMPs are a family of proteolytic enzymes that can be produced by all cell types present in human atherosclerotic plaques: smooth muscle cells, endothelial cells, and macrophages. The ability of macrophage-secreted MMPs to degrade collagen, the major component of the extracellular matrix in fibrous caps, may predispose vulnerable atherosclerotic plaques to rupture. Therefore, the observed reduction in MMP-9 expression produced by atorvastatin may contribute to the plaque-stabilizing properties of this drug. From several studies [14,54–56] it has been concluded that statins reduce MMP-9 secretion and activity. However, the effects of statins on MMP gene expression have been scarcely investigated. Bellosta et al. [54] showed an increase in MMP-9 mRNA levels by statins, accompanied by a reduction in MMP-9 protein detected in the media. However, that study was performed in human macrophages not exposed to oxLDL. It has been described that statins inhibit NF-κB activation [57], and that MMP genes are regulated by this transcription factor [58]. Therefore, it is conceivable that the reduction in MMP-9 mRNA expression by atorvastin reported here is caused by inhibition of NF-κB signalling pathways.

In summary, in this study we used cDNA arrays to screen for genes differentially expressed in atorvastatin-treated macrophages under atherogenic conditions. The main finding is that atorvastatin inhibits the oxLDL-dependent induction of CD68 and FABP4, and reduces HBP RNA expression. These genes may be involved in foam cell formation and atherogenesis, although their exact role in these processes is not fully understood. Therefore, the inhibitory effect of atorvastatin on the expression of these genes may constitute novel mechanisms to understand the protective effect of statins against development of atherosclerosis.

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