Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 562 (2007) 119–129
www.elsevier.com/locate/ejphar

Atorvastatin modulates the profile of proteins released by human atherosclerotic plaques

M. Carmen Durán [a,d,1], Jose L. Martín-Ventura [b,1], Shabaz Mohammed [d], María G. Barderas [a], Luis M. Blanco-Colio [b], Sebastián Mas [a,b], Verónica Moral [a], Luis Ortega [f], Jose Tuñón [c], Ole N. Jensen [d], Fernando Vivanco [a,e], Jesús Egido [b,*]

[a] Department of Immunology, Fundación Jiménez Díaz, Autónoma University, Madrid, Spain
[b] Vascular Research Laboratory, Fundación Jiménez Díaz, Autónoma University, Madrid, Spain
[c] Department of Cardiology, Fundación Jiménez Díaz, Autónoma University, Madrid, Spain
[d] Protein Research Group, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark
[e] Proteomic Unit, Complutense University, Madrid, Spain
[f] Department of Pathology, Hospital Clinico, Madrid, Spain

Received 4 October 2006; received in revised form 18 January 2007; accepted 24 January 2007
Available online 8 February 2007

Abstract

The mechanisms by which hydroxymethylglutaryl CoenzymeA reductase inhibitors (statins) reduce atherosclerotic cardiovascular morbidity and mortality remain poorly understood. Statins have been shown to modulate the levels of different inflammatory proteins both in carotid atherosclerotic plaques and in the blood of patients with atherosclerosis. In this work, we hypothesize that statins could also modulate the levels of the proteins secreted by cultured atherosclerotic plaques. Thus, the secretomes obtained from complicated atherosclerotic plaques incubated in the presence/absence of atorvastatin (10 μmol/l, 24 h) were analysed and compared by two-dimensional electrophoresis, considering the fibrous adjacent areas as controls. In total, 54 proteins (83 protein isoforms) were identified by Mass Spectrometry (MS): 24 proteins were increased and 20 proteins decreased in atheroma plaque supernatants compared to controls. Some of these proteins, like Cathepsin D, could play a significant role in plaque instability, becoming a potential target for therapeutical treatment. Interestingly, 66% of the proteins differentially released by atherosclerotic plaques reverted to control values after administration of atorvastatin, among them, Cathepsin D. Moreover, plaques obtained from patients who received atorvastatin treatment prior to carotid endarterectomy showed decreased Cathepsin D expression relative to plaques from non-treated patients. In conclusion, this proteomic approach has shown that statins are able to modulate the secretome of atherosclerotic plaques, and new therapeutical targets for statins have been characterised.

Keywords: Atherosclerosis; Proteomics; Statins

1. Introduction

Atherosclerosis is a chronic disease which constitutes a leading cause of death in developed countries. Atheroma plaque formation results from the interaction between several cellular and molecular species including connective tissue matrix (collagens) produced by vascular smooth muscle cells (VSMCs), lipids (inside foam cells or free in the tissues), and inflammatory cells (macrophages and T-lymphocytes). Many of these interactions are mediated by a diversity of proteins released during the plaque development. Both, VSMCs and inflammatory cells produce a wide range of proteins, including inflammatory cytokines, procoagulant proteins and proteases such as metalloproteinases or cathepsins, responsible for the degradation of the matrix components, but also others allowing the connection between the endothelium and blood cells. These

* Corresponding author. Vascular Research Lab., Fundación Jiménez Díaz, Avenida Reyes Católicos 2, 28040, Madrid, Spain. Tel.: +34 915504800.
E-mail address: jegido@fjd.es (J. Egido).
[1] Both authors contributed equally to this work.

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.01.077

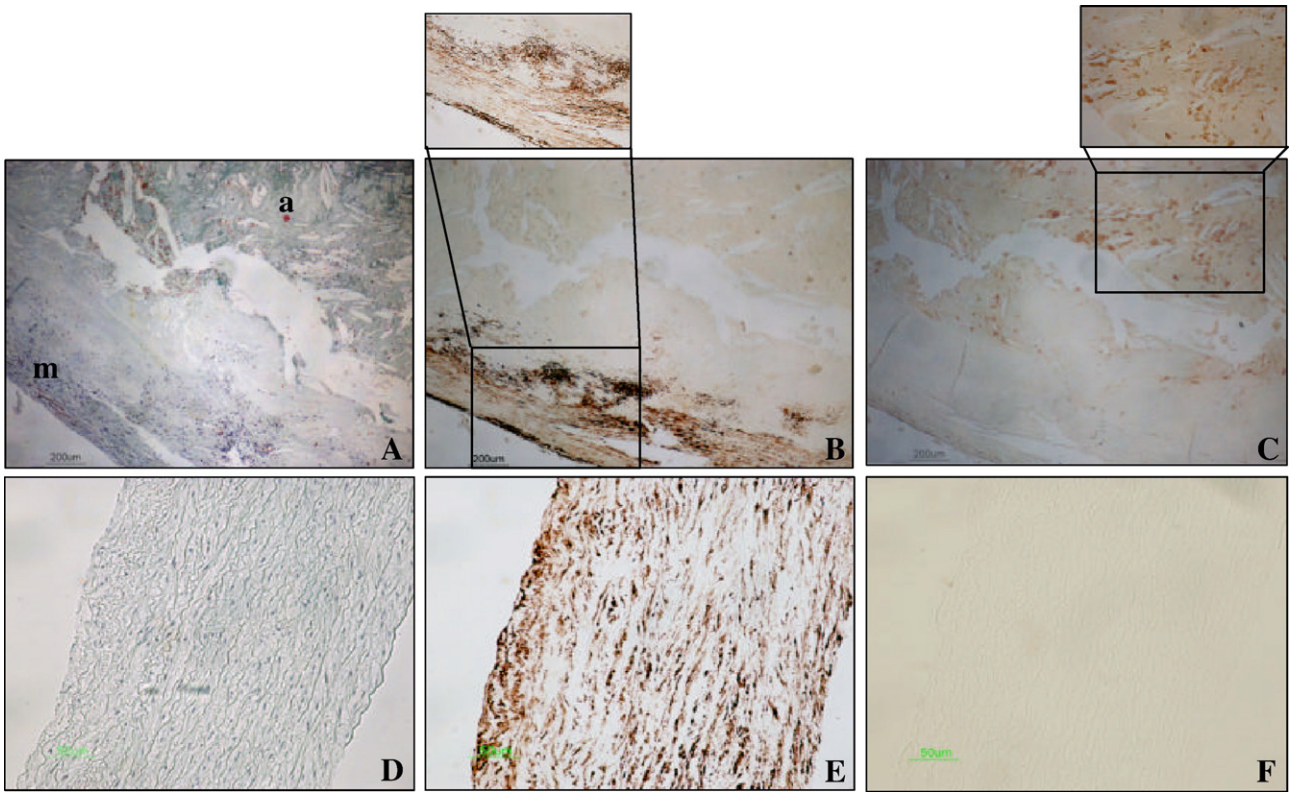

Fig. 1. Histological analysis of human atherosclerotic plaques. Masson's trichrome clearly illustrates the differences between the complicated atherosclerotic plaque (atheroma inflammatory gruel with cholesterol crystals covered by a thin fibrous cap) (A) and the fibrous section (with similar morphology to mammary or radial healthy arteries)(D). Upper panel: The complicated atherosclerotic plaque area contains macrophages (C) together with VSMCs (B). Lower panel: The fibrous adjacent area does not contain macrophages (F), being mainly composed of VSMCs (E). Inset: magnification ×200. a = atheroma, m = media.

blood cells (mainly platelets) contact with the atheromatous gruel after plaque erosion or rupture, triggering thrombosis (Davies, 2001). Therefore, the characterization of the secreted proteins during the atherosclerotic process could add interesting information to a better understanding of the complexity of this pathology.

Emerging proteomic tools, like two-dimensional electrophoresis and MS have become a powerful strategy to characterise new unforeseen proteins, since thousands of them can be separated, detected and identified in a single experiment. By application of these tools, two-dimensional electrophoresis and MS, an alternative strategy to characterise the atherosclerotic process was performed in our laboratory, analysing the supernatants of cultured human atherosclerotic plaques, and comparing the protein secretion profile of atherosclerotic samples with that obtained from healthy arterial segments (Duran et al., 2003). In the present study, the fibrous regions adjacent to the plaques have been considered as controls in order to overcome the potential differences when compared to other arterial territories (such as mammary or radial arteries) and to focus on the identification of those proteins that could be most directly involved in plaque formation and rupture.

Despite the complexity of atherosclerotic plaques, it has been shown that their structure and cellular/protein composition can be therapeutically modified by the use of drugs, such as 3-hydroxy-3-methylglutaryl CoenzymeA reductase inhibitors or statins (Cipollone et al., 2003; Martin-Ventura et al., 2005). Furthermore, these drugs are able to modify the levels of circulating proteins in subjects at different cardiovascular risks (Blanco-Colio et al., 2004). In addition, statins could have some direct effects independently of their lipid lowering effects (Blanco-Colio et al., 2003) although these effects have not been fully elucidated. In this respect, the application of a global proteomic approach to determine the effect of statins on the proteins released by complicated atherosclerotic plaques could help to understand novel mechanisms by which statins promote some of their beneficial effects.

2. Materials and methods

2.1. Tissue sampling

A first group of twenty-one patients undergoing carotid endarterectomy at our institution were included in this study in order to perform the two-dimensional electrophoresis-based proteomic analysis. From each carotid endarterectomy, two different sections, the stenosing complicated zone (origin of the internal carotid artery) and the adjacent fibrous zone (common and external carotid endartery), were dissected as described (Duran et al., 2003). Furthermore, to assess the effect of statins in atherosclerotic plaque secretion profiles, the stenosing complicated region of atherosclerotic plaques were divided into similar pieces (100 mg approximately) and incubated in the presence or absence of atorvastatin (10 μmol/l). All these

samples were incubated separately for 24 h in serum-free RPMI medium at 37 °C. Conditioned media were collected and centrifuged, and protein concentration was determined by Bradford's assay. Supernatants were aliquoted and stored at −80 °C until further analysis by two-dimensional electrophoresis. In some cases, atherosclerotic plaques incubated with/without atorvastatin were fixed in paraformaldehyde and embedded in paraffin. By immunohistochemistry, we have confirmed that the cellular composition of both complicated atherosclerotic pieces was similar (not shown).

In addition, some of the identified proteins were analysed by immunohistochemistry in 20 additional atherosclerotic plaques belonging to a second group composed by 20 patients (9 randomised to usual care and 11 randomised to atorvastatin 80 mg/day during 4–6 weeks before the scheduled carotid endarterectomy), which were available from a previous study (Martin-Ventura et al., 2005). The purpose of this study was to analyse if atorvastatin could modify not only the *ex vivo* release of some of the identified markers, but also their intracellular expression in human atherosclerotic plaques.

These studies were approved by the Hospital's Ethics Committee, according to the institutional and the Good Clinical Practice guidelines, obtaining the informed consents before enrolment in all cases.

2.2. Two-dimensional gel electrophoresis

Supernatants from complicated atherosclerotic plaques incubated in the presence/absence of atorvastatin and control segments were analysed by two-dimensional electrophoresis as previously described (Duran et al., 2003), comparing the resultant proteomic patterns from both mediums. Prior to the first dimension, protein samples were precipitated with trichloroacetic acid (TCA)/acetone. This additional step allowed us to remove contaminating species such as lipids and salts, which would otherwise interfere with the two-dimensional electrophoresis result, and also provided a more concentrated protein sample. From each sample, 600 μg of total protein were precipitated with 10% TCA, 0.07% 2-mercaptoethanol in acetone (1 h, −20 °C). Proteins were centrifuged, and the pellet was washed with 1 ml of cold acetone containing 0.07% 2-mercaptoethanol (−20 °C, 20 min. approximately). The residual acetone was removed by air-drying. The pellet was re-suspended in 300 μl rehydration buffer (8 M Urea, 0.5% 3-[3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 1% Tris-(2-carboxyethyl)phosphine (TCEP), 0.2% Pharmalyte pH 3–10) and two-dimensional gels were processed. For the isoelectrofocusing, strips of 4–7 pH were chosen, employing 12.5% acrylamide in the second dimension. Silver staining was done as described (Blum et al., 1987) and gels were scanned by using a desktop scanner (DuoScan HiD, Agfa). Evaluation and processing of the gel images was done by PD-Quest 7.1.1 (Bio-Rad, Hercules, CA, USA) software, which includes a statistic package. By PD-Quest, gel images were normalized to compensate the background variation due to differences in protein loading, staining or sample preparation.

2.3. In-gel digestion

The spots differentially secreted, according to PD-Quest analysis, were cut from gels with a sterile scalpel and subjected to in-gel trypsin digestion according to Shevchenko et al. (1996) with some modifications. Briefly, after washing, gel pieces were swollen in 50 mM NH_4HCO_3 containing 12.5 ng/μl of sequencing grade modified trypsin, (Promega, Madison, WI, USA) in an ice bath. After 45 min, the supernatant was removed and discarded followed by the addition of 20 μl of 50 mM NH_4HCO_3. The digestion was allowed to proceed at 37 °C overnight. The tryptic peptides were collected and purified for MS analysis.

2.4. Micro-column purification of digested samples

The in-gel digests were purified as described (Gobom et al., 1999). A column consisting of 100–300 nl of POROS R2 material (PerSeptive Biosystems, Framingham, MA) was packed in a constricted GELoader tip (Eppendorf, Hamburg, Germany). A 1.25 ml syringe was used to force liquid through the column by applying gentle air pressure. The column was equilibrated with 20 μl of 5% formic acid followed by the addition of the analyte in an acidified solution. The column was washed with 20 μl of 5% formic acid and the bound peptides subsequently eluted directly onto the Matrix Assisted Laser Desorption/ionisation (MALDI) target with 0.5 μl 2.5-Dihidroxybenzoic acid (DHB) solution (20 μg/μl in acetonitrile (ACN): 0.1% trifluoroacetic acid 70:30, v/v). Reagents and MALDI matrix were provided by Aldrich Chemicals, Milwaukee, WI, USA.

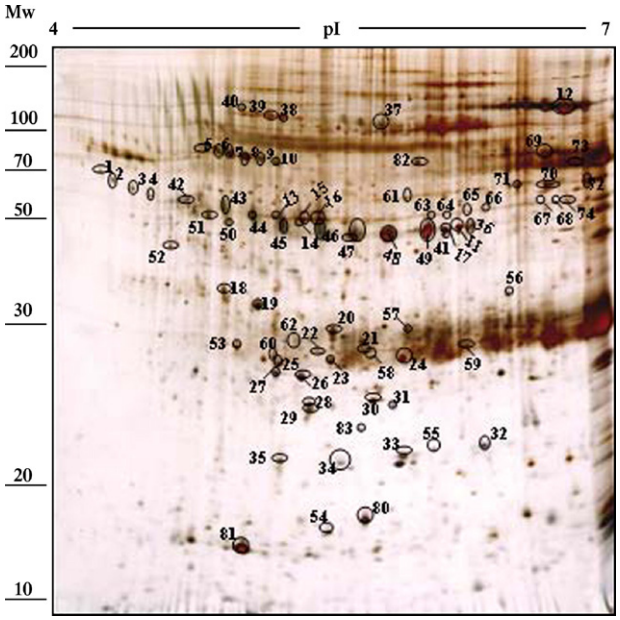

Fig. 2. 2-D map of proteins identified in complicated atherosclerotic samples. A representative 2-D gel from a human atherosclerotic plaque secretion profile is presented. The 83 protein isoforms (54 gen products) identified by MS have been marked with circles, and a number has been assigned to each protein on the gel.

2.5. Protein identification and characterisation by Mass Spectrometry

Peptide mixtures were analysed by MALDI-MS, using a Voyager-DE STR BioSpectometry Workstation instrument (Applied Biosystems, Foster City, CA), in positive reflector mode. Typical instrument settings were: acceleration voltage 20 kV, Grid 72%, delay time 300 ns, 100 shots/spectrum and mass range 800–3500 Da. Spectra were internally calibrated using peaks from trypsin autolysis products, resulting in a mass accuracy of <50 ppm. The spectra were analysed using Data Explorer software (Applied Biosystems, Foster City, CA).

Automated nanoflow liquid chromatography/tandem mass spectrometric analysis was done with a Quadrupole-Time of Flight (Q-TOF) Micromass spectrometer (Waters, Manchester, UK), employing automated data dependent acquisition. A nanoflow-HPLC system (Ultimate; Switchos2; Famos; LC Packings, The Netherlands) was used, employing a 200 nl/min flow rate. A homemade 2 cm fused silica precolumn (75 μm i.d.; 375 μm O.D.; Zorbax® SB-C18 3 μm (Agilent, Wilmington, DE)) was used for chromatographic separation, proceeding as described (Licklider et al., 2002). Sequential elution of peptides was accomplished using a linear gradient from Solvent A (0% ACN in 1% formic acid/0.6% acetic acid/0.005% heptafluorobutyric acid (HFBA)) to 40% of solvent B (90% ACN in 1% formic acid/0.6% acetic acid/0.005% HFBA) in 30 min over the precolumn in-line with a homemade 8 cm resolving column (75 μm ID; 375 μm o.d.;

Table 1
List of increased proteins in atheroma plaque-conditioned media versus the fibrous region and modulation by atorvastatin

PD-Quest Data (Mean±S.E.M)										
No.	Access Number	Identified protein	pI (Theoretical isoelectric point)	Mw (Da) (theoretical)	Sequence coverage (%)	Peptides matched	Score Mascot	Control	Atheroma	Atorvastatin
2	P02750	Leucine rich alpha-2-glycoprotein	5.66	34,553	28	7	93	1981.0±13.0	2719.6±19.8	6076.7±53.6
3	P02750	Leucine rich alpha-2-glycoprotein	5.66	34,553	20	6	84	2498.2±1.6	2358.5±53.1	3360.4±35.9
9	P01009	Alpha-1-Antitrypsin	5.43	44,322	35	12	135	7100.9±29.9	9316.8±25.0	8289.5±28.2
10	P48595	Serpin: protease complex	5.29	37,681	35	12	152	3058.3±29.0	5249.0±83.4	5354.4±29.7
11	P40121	Capping protein, gelsolin like	5.88	38,779	18	8	74	4255.2±12.3	6787.4±54.2	4573.0±12.8
12	P02787	Transferrin	6.81	79,280	24	16	182	3359.3±24.4	8142.2±55.3	
17	P40121	Capping protein, gelsolin like	5.88	38,779	18	10	96	1425.6±51.3	2998.3±55.3	3403.9±56.6
20	P07339	Cathepsin D	5.31	26,457	20	14	110	5673.4±35.2	7978.0±53.4	2287.0±15.6
25	P02647	Apolipoprotein A-l	5.45	28,944	27	7	87	4350.3±22.9	4771.3±43.7	4613.1±52.1
26	P02647	Apolipoprotein A-l	5.45	28,944	37	9	93	14,024.6±13.4	20,253.1±47.0	19,479.6±29.9
27	P02647	Apolipoprotein A-l	5.45	28,944	38	8	84	5909.9±33.4	7635.0±58.1	13,571.1±12.7
28	P02753	Retinol binding protein	5.27	21,287	41	10	114	10,032.9±67.1	16,078.0±0.35	16,165.2±92.2
30	P32119	Thioredoxin peroxidase B	5.44	21,909	27	8	121	23,193.1±1.1	32,348.4±35.7	31,261.0±21.6
32	P00738	Haptoglobin alpha chain	6.25	42,126	16	10	64	4539.2±12.2	18,340.7±13.4	42,869.5±94.2
34	P00738	Haptoglobin	6.25	42,126	27	8	92	1066.7±0.0	91,807.4±0.0	53,486.9±98.7
35	P00738	Haptoglobin	6.25	42,126	25	5	90	14,397.3±24.3	28,938.6±53.0	23,577.4±33.0
36	P02675	Fibrinogen	7.08	36,331	15	7	111	3976.4±38.4	20,887.3±24.7	34,562.8±42.9
39	P04217	Alpha-1B glycoprotein	5.65	52,479	18	7	73	680.5±4.3	1112.1±0.8	1394.3±70.8
40	P02787	Transferrin	6.81	79,280	34	24	198	881.2±11.2	3513.2±16.6	1869.1±60.6
43	P00737	Haptoglobin	6.14	38,722	33	15	167	250.7±56.6	1469.7±62.0	234.9±65.9
44	P00737	Haptoglobin	6.14	38,722	30	13	154	1484.7±14.0	4539.7±62.0	1406.8±50.5
45	P00737	Haptoglobin	6.14	38,722	30	9	98	8363.6±53.4	11,178.7±65.5	27,401.9±16.6
46	P00737	Haptoglobin	6.24	45,860	29	12	105	19,314.8±15.7	29,733.1±0.8	28,402.0±16.7
47	P02675	Fibrinogen	7.08	36,331	56	16	111	26,029.0±0.3	34,085.3±15.1	27,688.6±22.9
48	P02675	Fibrinogen	7.08	36,331	47	17	117	27,022.0±0.6	40,219.0±0.8	41,432.1±51.7
50	P25311	Chain B, Zinc-alpha-2 glycoprotein	5.70	31,854	26	10	76	2511.5±10.1	3072.1±10.4	1246.9±68.2
55	P22392	Nucleoside-diphosphate kinase1, isoform b	5.83	17,309	37	6	76	5505.6±18.6	8328.0±16.1	2617.4±65.2
74	P48735	NADP-dependent isocitrate dehydrogenase	6.34	46,944	14	7	67	287.1±0.0	2497.2±0.0	
77	P04895	Guanine nucleotide binding protein	5.92	44,694	18	6	73	5505.6±18.6	8328.0±0.6	2617.4±65.2
78	P02774	Vitamin D binding protein	5.17	52,794	22	8	128	3058.3±29.0	3149.0±0.3	5354.4±65.2
80	P02766	Transthyretin	5.33	12,835	80	6	68	27,175.5±17.9	37,865.7±60.5	26,823.2±55.1
82	P30101	Protein disulfide isomerase	5.23	57,115	17	8	94	4008.1±10.5	5246.9±69.2	6906.8±60.6
83	P02792	Ferritin light subunit	5.65	16,441	43	4	74	2313.5±42.7	9755.7±58.6	17,787.2±25.7

Proteins are organised by the number assigned on the gels, presenting the corresponding SwissProt database Access Number, Protein name, theoretical isoelectric point (pI) and molecular weight (Mw), sequence coverage or percentage of coincidence between the experimental and the theoretical protein sequence, number of peptides matching with the theoretical sequence, and Mascot database search score or probability of identity (Scores >65 are considered significant). Finally, protein expression levels (mean±S.E.M) provided by PD-Quest software for Control, Atheroma plaques without and after atorvastatin, are presented.

Agilent Zorbax® SB-C18 3.5 μm). The mass spectrometer was operated in the positive ion mode with a resolution of 4000–6000 full-width half-maximum using a source temperature of 80 °C and a counter current nitrogen flow rate of 60 l/h. Data dependent analysis was employed (three most abundant ions in each cycle): 1 s MS (m/z 350–1500) and max 3 s MS/MS (m/z 50–2000, continuum mode), 45 s dynamic exclusion. External mass calibration using NaI resulted in mass errors <50 ppm. Raw data were processed using MassLynx 3.5 ProteinLynx (smooth 3/2 Savitzky Golay and center 4 channels/80% centroid) and the resulting MS/MS data set exported in the Micromass pkl format.

Protein identification was performed by searching a non-redundant protein sequence database (NCBI) using the Mascot program (http://www.matrixscience.com). The following parameters were employed: monoisotopic mass accuracy <50 ppm, 1 missed cleavage, allowed modifications carbamidomethylation of cysteines (complete) and methionine oxidation and pyroglutamic acid (partial).

2.6. Clustering of protein secretion profiles in human carotid atherosclerotic plaques after drug treatment

A hierarchical cluster was done to represent the differential protein secretion levels detected in atheroma plaque supernatants in the presence/absence of atorvastatin. For this purpose, the PD-Quest data were normalized against the fibrous areas, considered as controls, and pre-processed with the Gene Expression Pattern Analysis Suite (GEPAS) v.1.1 Software (Herrero et al., 2003). Clustering itself was done by applying the Self-Organizing hierarchical Neural Network (SOTA) (Herrero et al., 2001) with the following parameters: Euclidian distances for cluster conditions (upper tree) and Euclidean (normal) distance between genes.

2.7. Western blotting

Equal amounts of conditioned medium proteins (15 μg) were loaded onto 12% polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes as described (Martin-

Table 2
List of proteins with lower levels in carotid atherosclerotic plaque-conditioned media than in fibrous regions and modulation by atorvastatin

PD-Quest Data (Mean±S.E.M)										
No.	Access Number	Identified protein	pI (theoretical isoelectric point)	Mw (Da) (theoretical)	Sequence coverage (%)	Peptides matched	Score Mascot	Control	Atheroma	Atorvastatin
1	P02750	Leucine rich alpha-2 glycoprotein	5.66	34,553	17	5	79	1463.3±16.5	1381.8±69.8	3528.7±51.6
4	P02750	Leucine rich alpha-2 glycoprotein	5.66	34,553	14	5	77	1434.6±43.3	1107.3±27.3	2460.3±2.6
5	P01009	Alpha-1-Antitrypsin	5.43	44,322	27	12	163	8217.3±26.8	2962.2±18.7	19,391.1±0.0
6	P01009	Alpha-1-Antitrypsin	5.35	44,307	19	11	111	10,550.1±10.4	9292.2±14.3	16,711.6±34.5
7	P01009	Alpha-1-Antitrypsin	5.43	44,322	27	12	140	21,648.4±34.6	11,262.9±37.9	15,370.1±19.2
8	P01009	Alpha-1-Antitrypsin	5.43	44,322	33	8	104	17,497.4±27.6	12,618.6±57.6	15,901.4±36.1
13	Q96B34	Beta-Actin	5.56	41,321	23	9	99	14,063.5±43.6	11,102.2±18.3	8640.4±21.3
18	P28830	Complex-forming glycoprotein HC	5.84	20,592	37	7	78	507.2±1.8	3766.1±13.4	6133.2±18.5
19	P28830	Complex-forming glycoprotein HC	5.84	20,592	37	5	70	16,021.6±35.8	12,429.4±3.9	12,487.9±36.3
21	P02743	Serum amyloid P component	6.12	23,358	26	6	92	10,375.0±0.4	2189.9±30.9	4131.6±4.8
22	P02743	Serum amyloid P component	6.10	25,485	21	6	88	9011.8±44.2	6585.8±44.6	6232.2±12.6
23	P04792	HSP27	7.83	22,427	29	5	85	9868.4±39.4	5851.0±0.2	3058.7±27.9
24	P04792	HSP27	7.83	22,427	30	5	90	15,625.7±11.5	13,857.5±37.7	
29	P02753	Retinol binding protein	5.27	21,287	41	9	110	8263.5±22.0	3711.4±22.0	3470.0±64.5
31	P32119	Thioredoxin peroxidase B	5.44	21,909	28	6	84	9242.9±39.9	7167.1±10.1	8250.7±39.4
33	P00738	Haptoglobin-2-alpha	6.25	42,126	16	5	64	19,518.7±0.0	96,573.5±0.0	58,769.8±0.0
37	P02790	Hemopexin	6.43	49,948	23	12	149	14,031.9±21.8	3604.2±13.7	8320.4±17.1
38	P04217	Alpha-1B-glycoprotein	5.65	52,479	30	10	94	1209.4±30.1	1111.3±25.5	3018.0±9.0
41	P06396	Similar to gelsolin	4.85	31,052	21	5	69	2713.5±10.2	737.4±38.3	2133.5±0.0
49	P02675	Fibrinogen	7.08	36,331	48	16	108	33,606.8±25.2	19,366.5±25.3	28,021.6±29.7
51	P25311	Chain B, Zinc-alpha-2 glycoprotein	5.70	31,854	30	6	69	25,611.8±28.4	20,721.4±39.5	6678.0±3.5
53	P01834	IgG-kappa chain	8.45	36,535	29	6	71	7114.8±19.3	3008.0±0.1	5967.4±10.6
54	P04792	HSP27	7.83	22,427	37	7	94	16,485.6±40.9	6116.3±13.0	6179.5±47.7
58	P04792	HSP27	7.83	22,427	24	5	66	2872.1±4.0	500.0±0.8	18,664.3±0.0
59	P04792	HSP27	7.83	22,427	37	7	98	22,794.4±12.6	14,121.8±25.7	3036.5±30.8
60	P04792	HSP27	7.83	22,427	25	5	76	3336.6±42.8	2530.3±22.1	3098.1±1.9
61	–	Crystal structure of PedP	–	–	–	11	116	5326.7±48.4	2195.1±16.7	2395.1±1.8
63	P08107	HSP70	5.48	70,052	37	10	105	2096.5±10.8	1623.2±19.4	766.4±33.8
71	P06733	Enolase 1	7.01	47,481	22	11	83	4466.8±38.4	3181.2±16.5	9742.6±54.7
72	P06733	Enolase 1	7.01	47,481	29	12	90	5519.7±3.1	4788.1±10.5	1919.6±4.2
81	P09382	Beta-galactoside soluble lectin	5.34	14,917	59	7	104		52,200.4±23.8	51,167.8±15.6

(See Table 1 for table contents description).

Ventura et al., 2004). Blots were incubated with anti-Cathepsin D antibody (ab-6313, abcam), dilution 1:100. Ponceau S was performed to confirm equal amounts.

2.8. Immunohistochemistry

Atherosclerotic plaques from the *in vivo* study were fixed with paraformaldehyde and embedded in paraffin. Masson's trichrome was performed on 4 μm sections as previously described (Duran et al., 2003). Immunohistochemistry was performed using a monoclonal anti-human smooth muscle actin (clone 1A4, DAKO) and anti-human macrophages (CD68, DAKO) and a goat anti-Cathepsin D (ab-6313, abcam) antibodies at 1:100 dilution, as previously described (Martin-Ventura et al., 2005).

Results were evaluated by a computer-assisted morphometric analysis with the Olympus semiautomatic image analysis system Micro Image software (version 1.0 for Windows). This analysis was done by a pathologist who was blinded to the patient's group which the atherosclerotic plaques belong to, as previously described (Martin-Ventura et al., 2005). Results are expressed as percentage of positive staining per mm^2.

2.9. Statistical analysis

PD-Quest and immunohistochemistry results are expressed as mean ± S.E.M. The Wilcoxon paired test was used to analyse differences in *ex vivo* protein secretion between complicated atherosclerotic plaques (treated or untreated) and fibrous regions. The non-parametric Mann–Whitney test was used to analyse the effect of treatment with atorvastatin *in vivo*. Differences were considered significant at $P<0.05$.

3. Results

3.1. Identification of proteins differentially released by atherosclerotic plaques in culture

The supernatants obtained by incubation of human complicated atherosclerotic plaques and the fibrous adjacent areas were compared by two-dimensional electrophoresis with the aim to identify novel released proteins potentially related with atheroma plaque formation and rupture. Histological analysis clearly shows that complicated plaques contained a variable, but important proportion of inflammatory cells. The paired control adjacent area was composed of fibrous thickening mainly containing VSMCs (Fig. 1).

The analysis of the supernatants by two-dimensional electrophoresis reported an average of 620 spots detected per gel, from which we focused on 260 spots for this analysis, choosing those located in the central area of the gel, and excluding those in the extremes, normally more difficult to identify by MS. According to PD-Quest analysis, a total of 217 protein spots were present at higher levels in the conditioned media of atherosclerotic plaques than in the fibrous region, while 43 proteins spots were decreased or remained unchanged. From a total of 54 proteins (83 isoforms) identified by MS, 24 proteins showed higher levels in atheroma plaque secretome, while another 20 proteins presented lower levels compared to the fibrous control-conditioned media. The rest of the proteins remained unchanged. A representative gel including all the identified protein isoforms is shown (Fig. 2). The corresponding protein names and characteristics are included in Tables 1 and 2. The identification of diverse isoforms for several identified proteins suggested the presence of post-translational modifications, affecting to their isoelectric point (pI) and/or molecular weight (Mw). Examples of these proteins include Leucine rich α-2-glycoprotein (2 isoforms); Transferrin (2 isoforms); Apolipoprotein A-I (3 isoforms); Fibrinogen (3 isoforms); α-1-Antitrypsin (4 isoforms), Complex-forming glycoprotein HC (2 isoforms), Serum Amyloid P component (2 isoforms), HSP27 (3 isoforms) or Enolase 1 (2 isoforms).

3.2. Modulation of the protein profile of atheroma plaque secretome by atorvastatin

In previous reports we and others have shown that statins could modulate the levels of different inflammatory proteins both in carotid atherosclerotic plaques (Cipollone et al., 2003;

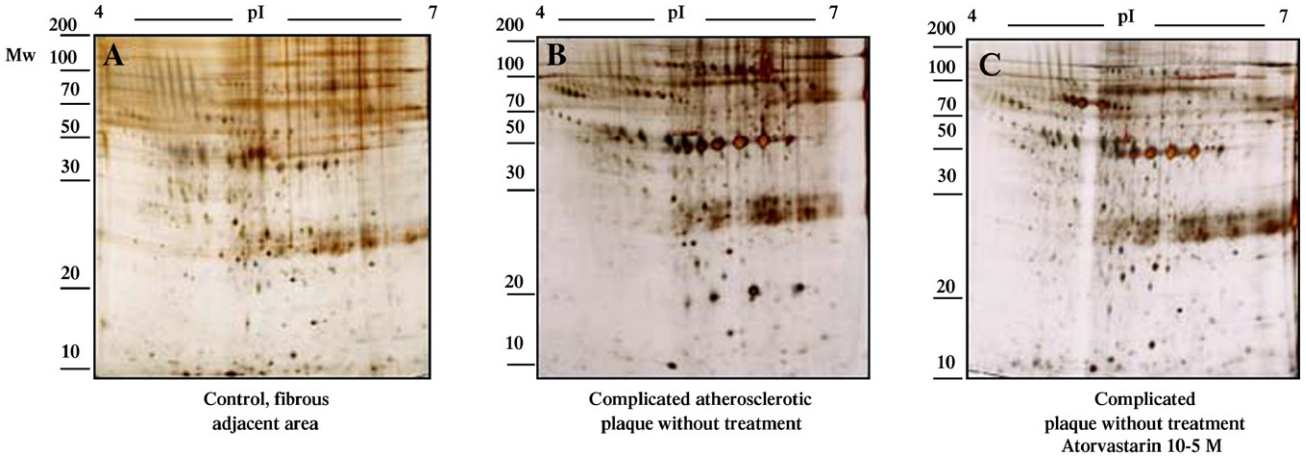

Fig. 3. Effect of atorvastatin in human atheroma plaques followed by 2DE. Representative 2-D gels corresponding to the protein secretion profile detected for fibrous regions (A), complicated atherosclerotic plaques segments (B) and the same complicated areas after incubation with atorvastatin (C).

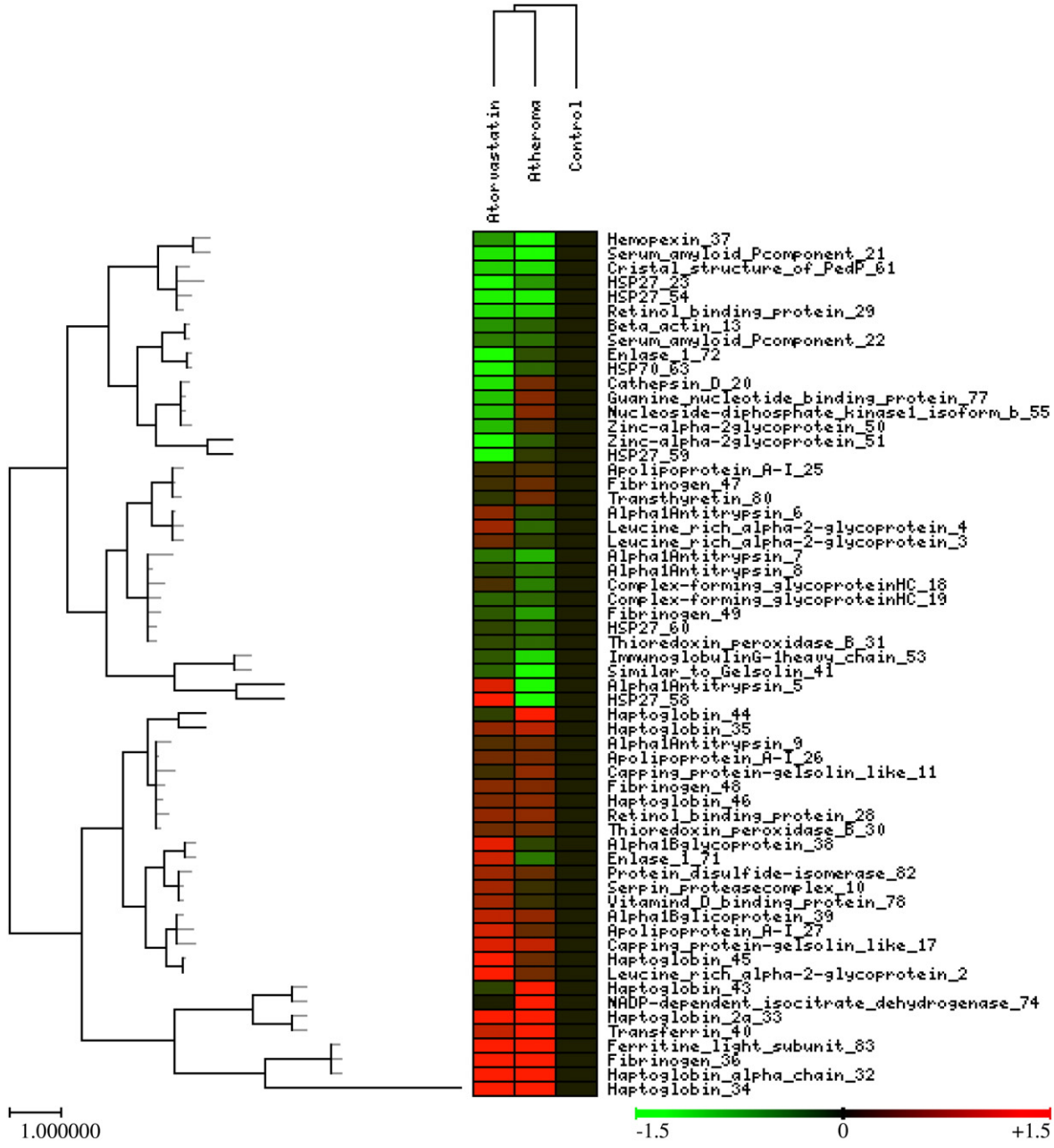

Fig. 4. Modulation by atorvastatin of atherosclerotic plaque secretion levels. This hierarchical cluster reflects the differential protein secretion profile detected by proteomic analysis for complicated atherosclerotic plaques cultured in the presence/absence of atorvastatin (10 μmol/l, 24 h). Following the colour scale, proteins isoforms with secretion levels lower than controls are into the green range and the ones with higher secretion are coloured in red. Dark green corresponded to controls.

Martin-Ventura et al., 2005) and in the blood of patients with carotid atherosclerosis (Martin-Ventura et al., 2005; Blanco-Colio et al., 2004). We hypothesized that the addition of atorvastatin to complicated atherosclerotic plaques could modulate some of the novel identified proteins released by the plaques in culture. To test this hypothesis, two-dimensional electrophoresis gels were performed from the conditioned media obtained after incubation of complicated atherosclerotic plaques with or without atorvastatin (10 μmol/l), considering the fibrous segments as controls (Fig. 3). The addition of atorvastatin to the complicated atherosclerotic segments promoted different effects in protein levels of tissue-conditioned media, depending on the particular protein. Interestingly, the majority of the proteins detected by PD-Quest as differentially released by atherosclerotic plaques versus fibrous areas reverted to control values after

Fig. 5. Modulation of Cathepsin D levels in atheroma plaque-conditioned media by atorvastatin. Western blot showing increased protein secretion for Cathepsin D in atheroma plaque samples (AT) versus fibrous regions (C). Levels are reverted after administration of atorvastatin (AT + ATV).

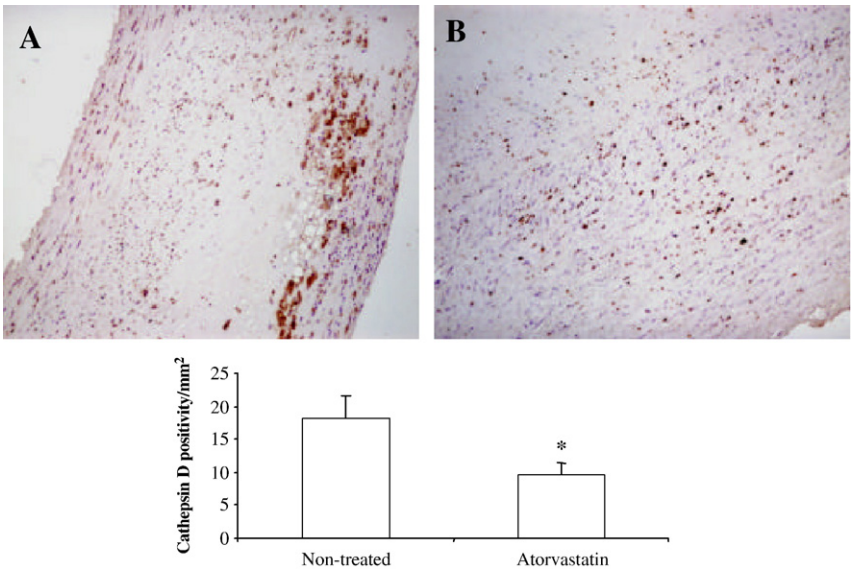

Fig. 6. Cathepsin D levels in human carotid atherosclerotic plaques. Modulation by atorvastatin. Cathepsin D immunostaining was diminished in human carotid atherosclerotic plaques of patients treated with atorvastatin (B) in relation to non-treated patients (A). * $P<0.05$ vs non-treated.

atorvastatin administration (66%). The modulation by atorvastatin of the identified proteins (Tables 1 and 2) has been represented in a hierarchical cluster, where differences between all experimental conditions can be observed (Fig. 4).

3.3. Validation by western blotting of proteomic results

In order to validate some of the proteins differentially released by atherosclerotic plaques and to evaluate the modulation by atorvastatin treatment, immunoblottings were done (not shown). In this respect, we focused on Cathepsin D, a protein that could play a significant role in atheroma plaque instability. This lysosomal protease participates in the degradation of the extracellular matrix and could induce hydrolytic modifications of low density lipoprotein (LDL) and foam cell formation (Hakala et al., 2003). Interestingly, this protein was secreted at higher levels by complicated atherosclerotic plaques compared to controls, but reverted to similar levels as control segments after

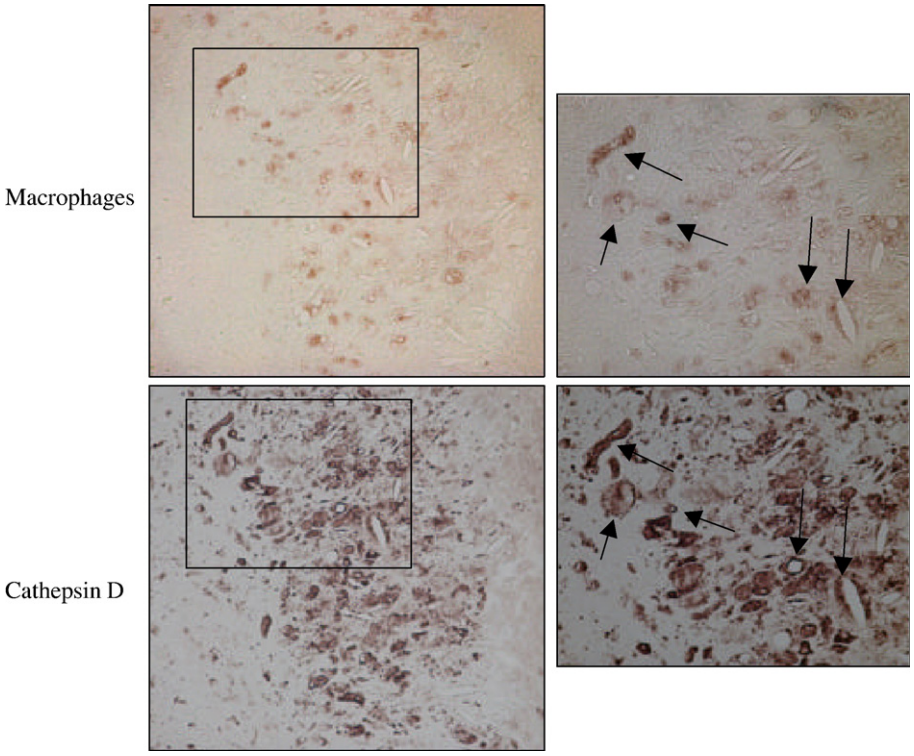

Fig. 7. Colocalization of Cathepsin D and macrophages in human carotid atherosclerotic plaques. Cathepsin D was highly expressed in macrophage-rich areas of atherosclerotic plaques, but also in other cells of the atherosclerotic plaques.

administration of atorvastatin (Fig. 5), in agreement with the proteomic analysis (Fig. 4).

3.4. Modulation of Cathepsin D in human atherosclerotic plaques by atorvastatin

Finally, we evaluated the effect of atorvastatin in Cathepsin D expression levels by immunohistochemistry. Thus, plaques obtained from patients who received atorvastatin treatment prior to carotid endarterectomy showed decreased Cathepsin D expression relative to plaques from non-treated patients (10 ± 1 vs 18 ± 3 positive staining/mm^2, $P < 0.05$, Fig. 6). Negative controls with the corresponding IgG were done, and no staining was detected. In accordance with previous studies (Hakala et al., 2003), we observed that Cathepsin D was mainly expressed in macrophage-rich areas of advanced carotid atherosclerotic plaques (Fig. 7). On the whole, these results suggest that atorvastatin can regulate the levels of Cathepsin D *in-vivo*.

4. Discussion

The atherosclerotic plaque is a complex structure where the cells conforming the vascular wall (endothelial cells and VSMCs) and inflammatory cells, are involved (Anderson, 1999). Different strategies have been carried out to analyse the mechanisms involved in atheroma plaque formation and rupture. In this regard, we have focused on the study of proteins released by the vascular wall. In a preliminary study, we showed that cultured carotid atherosclerotic plaques are able to release proteins into the medium, and a differential protein profile between normal artery segments and atherosclerotic plaques was detected by two-dimensional electrophoresis (Duran et al., 2003). In the present work, we have compared the protein secretion profiles of carotid atherosclerotic plaques versus the non-damaged adjacent fibrous region, with the aim to overcome the differences when comparing different arterial territories (such as mammary or radial arteries) and focusing on the identification of these proteins which would be more directly involved in plaque formation and rupture. Thus, the inclusion in this study of a higher number of samples and several improvements in two-dimensional electrophoresis protocols have allowed us to characterise a major number of proteins differentially released by carotid atherosclerotic plaques versus the fibrous section. Among the 54 proteins unambiguously identified by MS, 24 proteins showed increased secretion levels in atheroma plaques, while 20 were secreted in lower levels compared to the fibrous area. The remaining proteins did not present significant differences between both arterial sections. Some of the proteins were identified in several spots, corresponding to different isoforms, suggesting that post-translational modifications could be present on them. Post-translational modifications are normally associated with protein activity. The fact that several isoforms from the same protein presented a changing behaviour, with increased or decreased secretion in atherosclerotic plaques versus the control segments, could reflect the differential activation/inactivation stage of those proteins during the atherosclerotic phenomenon. Nevertheless, further studies should be done to consider any of the identified isoforms.

Interestingly, many of the proteins with increased levels in the atheroma plaque secretome have been previously related to the pathophysiology of atherothrombosis, among them: thioredoxin peroxidase, an antioxidant protein which has been recently suggested to have extracellular anti-inflammatory functions (Billiet et al., 2005); structural proteins like macrophage capping protein, involved in the actin assembly and reorganization during atherosclerosis (Dabiri et al., 1992); typical iron transporters like ferritin or transferrin, which may promote an increase in iron levels and the subsequent production of free oxygen radicals (Auer et al., 2002); protein disulfide isomerase, a protein whose over-expression could suppress the activation of NF-κB (Higuchi et al., 2004), a key transcription factor involved in atherosclerosis (Collins and Cybulsky, 2001); and finally, proteins related to cholesterol metabolism, like Apolipoprotein A-IV or Zinc-α-2-glycoprotein. The increase of Apopolipoprotein A-IV in atheroma could be related with a protective role, promoting the efflux of cholesterol from the damaged tissue. In the same way, Zinc-α-2-glycoprotein may favour the regression of the plaque structure by stimulating lipid degradation in adipocytes and reducing the lipidic deposits. On the other hand, we detected decreased levels for Apolipoprotein A-I, a component of anti-atherogenic high density lipoprotein (HDL), in agreement with reports that associate reduced levels of Apolipoprotein A-I with increased cardiac events (Walldius and Jungner, 2004). In summary, the application of a global proteomic approach to study a complex process such as atherosclerosis suggest that the final balance between the different pro- or anti-atherosclerotic functions of all these proteins may be involved in the progression or not of atherothrombosis.

One of the identified proteins that could play a more significant role in the atherosclerotic phenomenon is Cathepsin D, a lysosomal aspartic protease which degrades intracellular and endocytosed proteins. We previously demonstrated that this protein was increased in monocytes and plasma of patients with acute coronary syndromes (Vivanco et al., 2005). Interestingly, higher levels of Cathepsin D were also detected in the conditioned media of advanced atherosclerotic plaques. This increasing could be associated to the presence of macrophages and oxidized LDL in the atheroma gruel. An increased presence of Cathepsin D into the macrophage-derived foam cells located in the necrotic cores of atherosclerotic plaques has been described (Kaesberg et al., 1993), which has been related with the transformation of macrophages into apoptotic foam cells (Li and Yuan, 2004). Moreover, macrophages seem to release Cathepsin D to the extracellular medium, where it could participate in hydrolytic modifications of LDL and foam cell formation in the human arterial intima (Hakala et al., 2003). All these data, together with the presented herein, suggest that Cathepsin D could be significantly involved in plaque vulnerability and rupture. In this regard, Cathepsin D has been shown to participate together with other proteases in the degradation of the extracellular matrix of atherosclerotic plaques, a determinant of plaque stability. In addition,

Cathepsin D appeared to be involved in the apoptosis of macrophages/foam cells (Li et al., 2001), which have been detected at the site of plaque rupture in sudden coronary death (Kolodgie et al., 2000).

Despite its complexity, atherosclerotic plaques are not immutable and can be therapeutically modified. The administration of drugs such as statins has promoted a diminution in the mortality and morbidity levels in atherosclerotic patients but also in other cardiovascular diseases. We and other groups have previously shown that statins are capable of modulating the levels of different inflammatory proteins in both carotid atherosclerotic plaques (Cipollone et al., 2003; Martin-Ventura et al., 2005) and in the blood of patients with carotid atherosclerosis (Martin-Ventura et al., 2005). In this sense, we hypothesized that atorvastatin could also modulate the levels of the proteins differentially released by cultured atherosclerotic plaques. To test this hypothesis, we followed the same proteomic approach, analysing and comparing the protein secretion levels in complicated atherosclerotic plaques incubated in the presence or absence of atorvastatin. Interestingly, a high number of the proteins detected by PD-Quest analysis as differentially released by atherosclerotic plaques reverted to normal values after addition of atorvastatin (66%). In this regard, the differential secretion profile in the presence/absence of atorvastatin was represented in a hierarchical cluster. Although it doesn't reflect the high percentage observed for the total of proteins analysed, it is significant how administration of atorvastatin to atherosclerotic plaques is closer to the secretion profile detected in controls than the one seen for the atherosclerotic plaques without treatment.

In the case of Cathepsin D, the addition of atorvastatin to atherosclerotic plaques promoted a reversion to control levels for this protein when compared with the untreated paired complicated atherosclerotic plaque. Furthermore, plaques obtained from patients who received atorvastatin treatment prior to carotid endarterectomy showed decreased Cathepsin D expression relative to plaques from non-treated patients. Such diminution of Cathepsin D *in vivo* after atorvastatin administration could be understood in two different ways. It has been seen that lipid levels are decreased in patients treated with atorvastatin (Cipollone et al., 2003; Martin-Ventura et al., 2005), and this could promote a diminution in Cathepsin D, which seems to be up-regulated by oxidized LDL (Kaesberg et al., 1993). On the other hand, statins have been shown to stabilize atherosclerotic plaques by diminishing its inflammatory component. Since Cathepsin D is preferentially expressed by macrophages, the diminished levels observed intracellularly could be related to the significant decrease of macrophages observed in atherosclerotic plaques of patients treated with statins (Cipollone et al., 2003; Martin-Ventura et al., 2005). However, the potential mechanism(s) by which atorvastatin could modulate Cathepsin D release from cultured atherosclerotic plaques remain still unknown. On the whole, our data suggest that the reduction of Cathepsin D levels by statins in atherosclerotic plaques could represent another potential mechanism by which these drugs decrease the apoptotic component and increase the collagen content in human carotid plaques (as previously shown by the diminution of other proteases (Cipollone et al., 2003)) finally contributing to plaque stabilization.

5. Conclusion

Novel proteins released by atherosclerotic plaques that could be potentially involved in atheroma plaque instability have been characterised. Furthermore, we have also analysed for the first time the effect of drug administration in atheroma plaque protein secretion, suggesting that this proteomic approach could be used as a tool to better understand the mechanisms by which statins exert their beneficial effects in cardiovascular diseases.

Acknowledgements

We would like to thank Prof. J. Timms for his critical comments and mainly for his support. Special thanks to Dr Javier Serrano and Dra. Melina Vega for providing endarterectomy samples.

Source of support: Pfizer-Spain, SAF 2004/06109, CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Red RECAVA (RD06/0014/0035), Fundacion Mutua Madrileña, INDAS, Marie Curie Fellowship, European Commission (HPMT-GH-00-00153-00), University of Southern Denmark, Odense (Denmark) and Complutense and Autonoma University, Madrid (Spain).

References

Anderson, T.J., 1999. Assessment and treatment of endothelial dysfunction in humans. J. Am. Coll. Cardiol. 34, 631–638.

Auer, J.W., Berent, R., Weber, T., Eber, B., 2002. Iron metabolism and development of atherosclerosis. Circulation 106 (e7; author reply e7).

Billiet, L., Furman, C., Larigauderie, G., Copin, C., Brand, K., Fruchart, J.C., Rouis, M., 2005. Extracellular human thioredoxin-1 inhibits lipopolysaccharide-induced interleukin-1beta expression in human monocyte-derived macrophages. J. Biol. Chem. 280, 40310–40318.

Blanco-Colio, L.M., Tunon, J., Martin-Ventura, J.L., Egido, J., 2003. Anti-inflammatory and immunomodulatory effects of statins. Kidney Int. 63, 12–23.

Blanco-Colio, L.M., Martin-Ventura, J.L., Sol, J.M., Diaz, C., Hernandez, G., Egido, J., 2004. Decreased circulating Fas ligand in patients with familial combined hyperlipidemia or carotid atherosclerosis: normalization by atorvastatin. J. Am. Coll. Cardiol. 43, 1188–1194.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrilamide gels. Electrophoresis 8, 93–99.

Cipollone, F., Fazia, M., Iezzi, A., Zucchelli, M., Pini, B., De Cesare, D., Ucchino, S., Spigonardo, F., Bajocchi, G., Bei, R., Muraro, R., Artese, L., Piattelli, A., Chiarelli, F., Cuccurullo, F., Mezzetti, A., 2003. Suppression of the functionally coupled cyclooxygenase-2/prostaglandin E synthase as a basis of simvastatin-dependent plaque stabilization in humans. Circulation 107, 1479–1485.

Collins, T., Cybulsky, M.I., 2001. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J. Clin. Invest. 107, 255–264.

Dabiri, G.A., Young, C.L., Rosenbloom, J., Southwick, F.S., 1992. Molecular cloning of human macrophage capping protein cDNA. A unique member of the gelsolin/villin family expressed primarily in macrophages. J. Biol. Chem. 267, 16545–16552.

Davies, M.J., 2001. Going from immutable to mutable atherosclerotic plaques. Am. J. Cardiol. 88, 2F–9F.

Duran, M.C., Mas, S., Martin-Ventura, J.L., Meilhac, O., Michel, J.B., Gallego-Delgado, J., Lazaro, A., Tunon, J., Egido, J., Vivanco, F., 2003. Proteomic analysis of human vessels: application to atherosclerotic plaques. Proteomics 3, 973–978.

Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R., Roepstorff, P., 1999. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. J. Mass Spectrom. 34, 105–116.

Hakala, J.K., Oksjoki, R., Laine, P., Du, H., Grabowski, G.A., Kovanen, P.T., Pentikainen, M.O., 2003. Lysosomal enzymes are released from cultured human macrophages, hydrolyze LDL in vitro, and are present extracellularly in human atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. 23, 1430–1436.

Herrero, J., Valencia, A., Dopazo, J., 2001. A hierarchical unsupervised growing neural network for clustering gene expression patterns. Bioinformatics 17, 126–136.

Herrero, J., Al-Shahrour, F., Diaz-Uriarte, R., Mateos, A., Vaquerizas, J.M., Santoyo, J., Dopazo, J., 2003. GEPAS: a web-based resource for microarray gene expression data analysis. Nucleic Acids Res. 31, 3461–3467.

Higuchi, T., Watanabe, Y., Waga, I., 2004. Protein disulfide isomerase suppresses the transcriptional activity of NF-kappaB. Biochem. Biophys. Res. Commun. 318, 46–52.

Kaesberg, B., Harrach, B., Dieplinger, H., Robenek, H., 1993. In situ immunolocalization of lipoproteins in human arteriosclerotic tissue. Arterioscler. Thromb. 13, 133–146.

Kolodgie, F.D., Narula, J., Burke, A.P., Haider, N., Farb, A., Hui-Liang, Y., Smialek, J., Virmani, R., 2000. Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death. Am. J. Pathol. 157, 1259–1268.

Li, W., Yuan, X.M., 2004. Increased expression and translocation of lysosomal cathepsins contribute to macrophage apoptosis in atherogenesis. Ann. N. Y. Acad. Sci. 1030, 427–433.

Li, W., Dalen, H., Eaton, J.W., Yuan, X.M., 2001. Apoptotic death of inflammatory cells in human atheroma. Arterioscler. Thromb. Vasc. Biol. 21, 1124–1130.

Licklider, L.J., Thoreen, C.C., Peng, J., Gygi, S.P., 2002. Automation of nanoscale microcapillary liquid chromatography-tandem mass spectrometry with a vented column. Anal. Chem. 74, 3076–3083.

Martin-Ventura, J.L., Duran, M.C., Blanco-Colio, L.M., Meilhac, O., Leclercq, A., Michel, J.B., Jensen, O.N., Hernandez-Merida, S., Tunon, J., Vivanco, F., Egido, J., 2004. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. Circulation 110, 2216–2219.

Martin-Ventura, J.L., Blanco-Colio, L.M., Gomez-Hernandez, A., Munoz-Garcia, B., Vega, M., Serrano, J., Ortega, L., Hernandez, G., Tunon, J., Egido, J., 2005. Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. Stroke 36, 1796–1800.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.

Vivanco, F., Martin-Ventura, J.L., Duran, M.C., Barderas, M.G., Blanco-Colio, L., Darde, V.M., Mas, S., Meilhac, O., Michel, J.B., Tunon, J., Egido, J., 2005. Quest for novel cardiovascular biomarkers by proteomic analysis. J. Proteome Res. 4, 1181–1191.

Walldius, G., Jungner, I., 2004. Apolipoprotein B and apolipoprotein A-I: risk indicators of coronary heart disease and targets for lipid-modifying therapy. J. Intern. Med. 255, 188–205.