research articles Journal of proteome research

Proteomic Study of Plasma from Moderate Hypercholesterolemic Patients

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Received April 10, 2006

Proteomics is a technology to detect and identify several proteins and their isoforms in a single sample. We used proteomics to analyze modifications in the protein map of plasma after simvastatin treatment of moderate hypercholesterolemic patients. Plasma from hypercholesterolemic patients ($n = 9$) was compared before and after 12 weeks of simvastatin treatment (40 mg/day). Patients with similar cardiovascular risk factors were used as controls (CR group). By using two-dimensional electrophoresis and mass spectrometry, we identified the different protein isoforms. The plasma expression of three fibrinogen gamma chain isoforms (FGG) was enhanced, whereas the expression of two isoforms of the fibrinogen beta chain (FGB) was reduced in the hypercholesterolemic patients compared with the CR group. The expression of apolipoprotein A-IV and three haptoglobin isoforms was higher in hypercholesterolemic patients. Simvastatin treatment modified the plasma expression of FGG chain isoform 1, FGB chain isoforms 1 and 2, vitamin D binding protein isoform 3, apo A-IV, and haptoglobin isoform 2. The modification of FGG chain isoform 1 and FGB chain isoforms 1 and 2 was positively correlated with total plasma cholesterol level. Proteomic analysis of plasma may help to know more in depth the molecular mechanism modified by simvastatin treatment.

Keywords: hypercholesterolemia • plasma proteins • proteomics • simvastatin

Introduction

Hypercholesterolemic is the major risk factor for the development of atherosclerotic disease.[1] Reduction of circulating cholesterol levels is associated with a significant in cardiovascular mortality and morbidity. HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway through which cells synthesize cholesterol from acetate moieties.[3] Inhibition of HMG-CoA reductase by statins and the subsequent reduction in cholesterol synthesis has been associated with prevention of coronary events.[4] In this regard, the Scandinavian simvastatin survival study (4S) was the first study with an HMG-CoA reductase inhibitor demonstrating mortality reduction in hypercholesterolemic patients[3] statins treatment has been now associated with a consistent reduction in coronary events of ~30−40%.[5,6]

Epidemiological studies have shown that long-term morbidity and mortality in coronary heart disease is directly related to circulating levels of atherogenic lipoproteins, in particular low-density lipoprotein (LDL) cholesterol.[7] In addition, long-term prospective randomized trials of statins in coronary heart disease patients have also shown a direct relation between the LDL concentration achieved during treatment and the risk of new ischaemic cardiovascular events.[8] On the other hand, it is assumed that an increase in high-density lipoproteins (HDL) will lead to reduction in coronary heart disease incidence.[9] In this regard, in the Framingham study, an association between reduced HDL and increased cardiovascular risk was established.[10]

Independently of their cholesterol-lowering effects, statins, including simvastatin, seem to act through several other molecular mechanisms by modifying the expression of different proteins.[11−13] Such cholesterol-independent effects have been termed as "pleiotropic" effects. Between such pleiotropic effects have been included improvement of endothelial functionality, antioxidant effects, anti-inflammatory properties, and stabilization of the atherosclerotic plaque.[14] However, most of these studies have been performed in vitro or using animal models with statin concentrations that exceed those achieved in plasma in pharmacokinetics studies.[15] Moreover, until now, it has been difficult to monitor plasma protein changes associated with the attributed pleiotropic effects of statins in humans.

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10.1021/pr060159w CCC: $33.50

Published on Web 08/02/2006

Table 1. Clinical Characteristics of Patients and CR Group and Lipid Values in Plasma

	CR group ($n = 9$)	hypercolesterolemic group ($n = 9$)
age (years)	60.11 ± 1.42	68.3 ± 4.8
male/female	4/5	4/5
controlled hypertension	5 (56%)	4 (44%)
tobacco smoking	2 (22%)	2 (22%)
calcium antagonist	2 (22%)	2 (22%)
diuretics	4 (44%)	3 (33%)
ACEI	3 (33%)	3 (33%)
total Cholesterol (mg/dL)	180.0 ± 28.28	PR: 246.0 ± 29.55[b] AS: 196.0 ± 29.66[c]
LDL-Cholesterol (mg/dL)	90.0 ± 22.62	PR: 134.2 ± 33.16[b] AS: 86.85 ± 39.13[c]
HDL-Cholesterol (mg/dL)	41.0 ± 22.62	PR: 39.4 ± 10.26 AS: 45.1 ± 16.12
triglycerides (mg/dL)	135.0 ± 28.28	PR: 184.0 ± 99.50 AS: 152.8 ± 82.28

[a] PR: prior to simvastatin; AS: after simvastatin; results are represented as mean ± SD. [b] $p < 0.05$ with respect to CR group. [c] $p < 0.05$ with respect to prior to simvastatin treatment.

Proteomics is a powerful tool to describe changes in protein expression developing a map of proteins, and more importantly, proteomics allow us to identify protein isoforms in plasma. However, to our knowledgment, there are no proteomic studies in plasma from hypercholesterolemic patients. Thus, the aims of the present study were: (a) to identify by proteomics in moderate hypercholesterolemic patients, in which the pleiotropic effects of simvastatin could be more evident, changes in the expression of plasma proteins; (b) to determine whether the expression of such plasma proteins could be modified after 12 weeks of simvastatin treatment; and (c) to analyze if the changes observed in the plasma proteome map after simvastatin treatment could be attributed to its lipid lowering effects.

Materials and Methods

Patients. The patient population consisted of 9 moderate hypercholesterolemic patients (4 male and 5 female) (total cholesterol level > 200 mg/dl) from the Internal Medicine Department of Fundación Jiménez Díaz. Patients were treated with simvastatin (40 mg/day) for 12 weeks. A peripheral blood sample was obtained at inclusion and 12 weeks after simvastatin treatment. The clinical features of the included patients are shown in Table 1. Five of the hypercholesterolemic patients had controlled hypertension (SBP: 132 ± 4 mmHg; DBP: 73 ± 4 mmHg). The drugs that the patients were taking remained unchanged during the study.

A group of 9 patients with a similar incidence of controlled hypertension (SBP: 130 ± 6 mmHg; DBP: 75 ± 3 mmHg) but with normal cholesterol plasma levels (<200 mg/dL) were enrolled as control group (CR group). The drugs that the CR group patients were taking were unchanged during the study. Exclusion criteria for both groups of patients included malignant disease, infections, hematological disorders, anticoagulant therapy, and exposure to any lipid-lowering therapy within six months prior to inclusion. The selected patients were recommended to follow a low-cholesterol and low-fat diet during the statin treatment period. All patients and control subjects signed a fully informed consent, and the study was approved by the Institutional Ethics Committee (13/02).

Two-Dimensional Electrophoresis (2DE). Blood samples were collected in EDTA, and plasma was obtained by centrifugation. For 2DE, 500 μg of proteins contained in the plasma were diluted in 350 μL of 8 mol/L urea, 2% CHAPS w/v, 40 mmol/L dithiothreitol, 0.2% Bio-Lyte ampholyte (Bio-Rad), and 0.01% w/v bromophenol blue. The proteins contained in the plasma were quantified by BCA technique (Pierce, Rockford Illinois). The samples were loaded on immobilized linear gradient IPG strips (pH 4−7), and isoelectric focusing was performed using a Protean IEF Cell System (Bio-Rad). The gels were actively rehydrated at 50V for 60 h and submitted to rapid and linear voltage ramping steps[16]. In the second dimension, the proteins from the strips were resolved on 10% SDS−PAGE gels using a Protean II XL system (Bio-Rad). Then, the gels were fixed and silver stained using a Silver Stain Plus Kit (Bio-Rad) as reported [16].

Image Acquisition Analysis. The stained gels were scanned in a UMAX POWERLOOK III Scanner operated by the software, Magic Scan V 4.5. Intensity calibration was carried out using an intensity stepwedge prior to gel image capture. Image analysis was carried out using the PD Quest 6.2.1 and Quantity One 4.2.3 (Bio-Rad). Image spots were initially detected, matched, and then manually edited. Each spot intensity volume was processed by background subtraction, and the total spot volume was normalized by the corresponding spot volume of albumin.

Mass Spectrometry. As reported,[16] the spots of interest were manually excised from the gels using biopsy punches. To identify the spots of interest by mass spectrometry, spots from two different gels were obtained. The spots were washed twice with water, shrunk with 100% acetonitrile, and dried in a SpeedVac. The samples were then reduced with 10 mmol/L dithiothreitol in 25 mmol/L ammonium bicarbonate and subsequently alkylated with 55 mmol/L iodoacetamide in 25 mmol/L ammonium bicarbonate. They were digested with 12.5 ng/μL sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mmol/L ammonium bicarbonate (pH 8.5) overnight at 37°C. After digestion, the supernatant was collected, and 1 μL was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μL of 3 mg/mL of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in a Voyager-DE™ STR instrument (PerSeptives Biosystems), a model fitted with a 337-nm nitrogen laser and operated in reflector mode, with an accelerating voltage of 20 000 V. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin. The analysis by MALDI-TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed with a signal-to-noise greater than 20 can be collected and represented as a list of monoisotopic molecular weights. Proteins ambiguously identified by peptide mass fingerprints were subject to MS/MS sequencing analysis using the MALDI-tandem time-of-flight mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). So, from the MS spectra, suitable precursors were selected for MS/MS analyses with CID on (atmospheric gas was used) 1 Kv ion reflector mode and precursor mass Windows ± 10 Da. The plate model and default calibration were optimized for the MS/MS spectra procesing. For protein identification, we used Mascot database 1.9 (http://www.matrixscience.com) through the global protein Server v3.5 from Applied Biosystems. Search parameters were carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; peptide mass tolerance 50−100

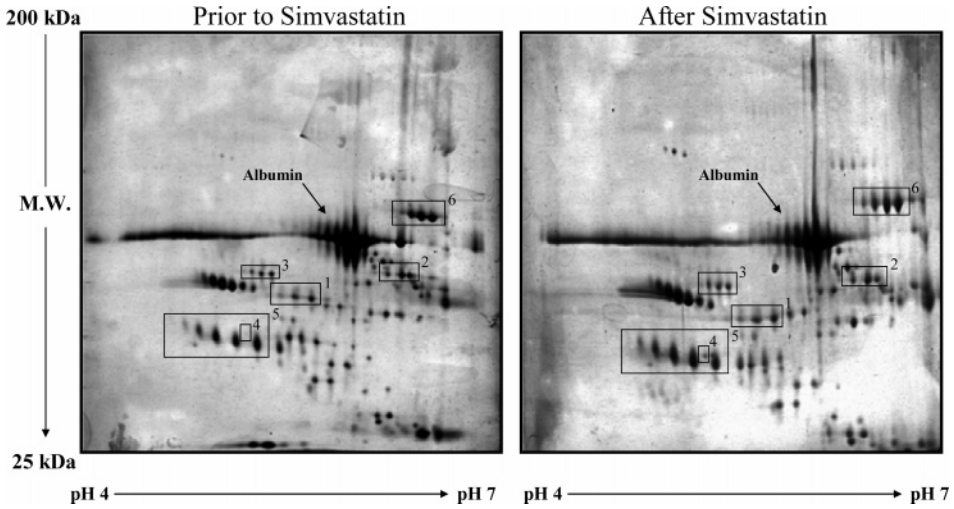

Figure 1. Representative 2D gels of plasma from a moderate hypercholesterolemic patient before and three months after simvastatin treatment. The first dimension electrophoresis was performed using a pH range between 4 and 7. Six areas were analyzed (1−6) in each subject. In area 7, albumin was identified.

ppm; 1 missed trypsin cleavage site and MS/MS fragments tolerance 0.3 Da. In all protein identifications, the probability scores were greater then the score fixed by Mascot as significant with a p value < 0.05.

Statistical Methods. Results are expressed as means ± standard deviation (SD). In the hypercholesterolemic patients, statistical differences before and after simvastatin treatment were calculated by Wilcoxon's test. To determine statistical differences between control and hypercholesterolemic patients, Mann−Whitney;s test was used. Pearson's correlation analysis was used to determine associations between the level of protein expression and the lipid parameters. A p value < 0.05 was considered statistically significant.

Results

Clinical Features and Lipid Characteristics of the Patients. Table 1 shows the clinical features of the CR and hypercholesterolemic patients groups and the lipid laboratory values before and after 12 weeks of simvastatin treatment. The hypercholesterolemic patients showed a moderate although significantly higher level of total cholesterol and LDL-cholesterol compared with the CR group (Table 1). Simvastatin treatment significantly reduced both total cholesterol and LDL-cholesterol levels (Table 1). Although plasma triglyceride levels tended to be higher in hypercholesterolemic patients than in the CR group, they did not reach statistically significant differences (Table 1). There were no differences in the plasma level of HDL-cholesterol between the groups (Table 1).

Protein Identification. Figure 1 shows a representative 2D gel of plasma from a patient before and after simvastatin treatment. In a previous study, we have observed that the major number of proteins present in the human plasma was detected in a pH range between 4 and 7.[16] We observed 153.7 ± 7.6 spots in the proteomic map of plasma from hypercholesterolemic patients before and 173.6 ± 9.1 spots after simvastatin treatment. In the plasma from CR patients, 185.8 ± 11.6 spots (p not significant between the different groups) were observed. The most remarkable spots were densitometrically measured normalized by the corresponding spot volume of albumin and identified by comparison with those shown in the Swiss-2D database plasma map (http://www.expasy.ch/ch2d/). Seven different areas containing the best-defined spots were initially analyzed (Figure 1). The identity of some of these spots was also confirmed by mass spectrometry (Table 2).

In 2D electrophoresis, three different fibrinogen gamma chain (FGG) isoforms were detected in area 1 (Figures 1 and 2). The presence of FGG isoform 1 was greater in the plasma from hypercholesterolemic patients in the CR group. There were no statistically significant differences in the expression of FGG isoforms 2 and 3 between the CR group and the hypercholesterolemic patients (Table 3). After simvastatin treatment, the plasma level of FGG isoform 1 was significantly decreased with respect to that detected prior to simvastatin treatment (Table 3). The level of expression of FGG isoforms 2 and 3 was not modified by simvastatin treatment (Table 3).

Three different isoforms of fibrinogen beta chain were detected in area 2 (FGB, Figures 1 and 2). FGB chain isoforms 1 and 3 were significantly reduced in the plasma from hypercholesterolemic patients when compared with the CR group (Table 3). Simvastatin treatment did not modify the level of expression of FGB isoform 3 with respect to that observed before simvastatin administration but enhanced the expression of FGB isoform 1 when compared with that observed in the CR group (Table 3). The expression of the FGB isoform 2 was significantly increased in the simvastatin-treated hypercholesterolemic patients when compared with the CR group (Table 3).

In area 3, we observed three spots that were identified by mass spectrometry as vitamin D-binding protein (DBP) (Figures 1 and 3). There was no modification in any of the DBP isoforms in the plasma of hypercholesterolemic patients when compared with that from the CR group (Table 3). However, the plasma level of the DBP isoform numbered as 3 was significantly enhanced after simvastatin treatment (Table 3).

One apolipoprotein A-IV isoform was detected in the plasma proteome (apo A-IV, Figures 1 and 4). Apo A-IV expression was significantly higher in plasma from hypercholesterolemic patients than in that from the CR group (Table 3). The expression of apo A-IV was enhanced in the hypercholesterolemic patients after simvastatin treatment when compared with them before simvastatin treatment and with the CR group (Table 3).

Table 2. Methods Followed to Identify the Different Proteins

protein	score	peptides submitted/ peptides matched	sequence peptides matched	confirmation method	sequence coverage
fibrinogen gamma chain (FGG)					
isoform 1	95%	1/1	YLQEIYNSNNQK	MS/MS[c], plasma map[b]	2%
isoform 2	95%	2/2	YLQEIYNSNNQK/VELEDWNGR	MS/MS[c], plasma map[b]	7.6%
isoform 3	95%	30/8	DNCCILDER/YEASILTHDSSIR/ YLQEIYNSNNQK/VELEDWNGR/ TSTADYAMFK/VGPEADKYR/ IIPFNR/LTIGEGQQHHLGGAK	MS/MS[a], plasma map[b]	18%
fibrinogen beta chain (FGB)					
isoform 1	−	−	−	plasma map[b]	−
isoform 2	−	−	−	plasma map[b]	−
isoform 3	−	−	−	plasma map[b]	−
vit D binding protein (DBP)					
isoform 1	95%	1/1	EVVSLTEACCAEGADPDCYDTR	MS/MS[c]	4%
isoform 2	95%	2/2	EVVSLTEACCAEGADPDCYDTR/ SLGECCDVEDSTTCFNAK	MS/MS[c]	9.4%
isoform 3	95%	67/12	EVVSLTEACCAEGADPDCYDTR/ EVVSLTEACCAEGADPDCYDTR/ EVVSLTEACCAEGADPDCYDTR/ EVVSLTEACCAEGADPDCYDTR/ SCESNSPFPVHPGTAECCTK/ VCSQYAAYGEK/NSKFEDCCQEK/ VCSQYAAYGEK/NSKFEDCCQEK/ SLGECCDVEDSTTCFNAK/ SLGECCDVEDSTTCFNAK/ SDFASNCCSINSPPLYCDSEIDA	MS[a]	13−22%
apolipoprotein A IV (APO A-IV)	95%	47/20	LGEVNTYAGDLQK/LVPFATELHER/ LLPHANEVSQK/LEPYADQLR/ TQVNTQAEQLR/TQVNTQAEQLRR/ QLTPYAQR/ENADSLQASLRPHADELK/ IDQNVEELKGR/LTPYADEFK/ LTPYADEFKVK/IDQTVEELRR/ SLAPYAQDTQEK/ISASAEELR/ QRLAPLAEDVR/LAPLAEDVR/ SLAELGGHLDQQVEEFR/RVEPYGENFNK/ ALVQQMEQLR/ALVQQMEQLR	MS[a], plasma map[b]	48%
albumin	−	−	−	plasma map[b]	−
haptoglobin (HPT)					
isoform 1	−	−	−	plasma map[b]	−
isoform 2	95%	1/1	YVMLPVADQDCIR	MS/MS[c], plasma map[b]	4%
isoform 3	−	−	−	plasma map[b]	−
isoform 4	−	−	−	plasma map[b]	−
isoform 5	−	−	−	plasma map[b]	−
serotransferrin (SER)					
isoform 1				plasma map[b]	−
isoform 2	−	−	−	plasma map[b]	−
isoform 3	−	−	−	plasma map[b]	−
isoform 4	−	−	−	plasma map[b]	−

[a] MS: mass spectrometry. [b] Plasma map: Swiss-Prot 2D database. [c] MS/MS: tandem mass spectrometry.

In area 5, five haptoglobin (HPT) isoforms were identified by using the Swiss-2D database plasma map. In addition, MS/MS was used to identify HPT isoform 2 (Figures 1 and 4). Plasma from hypercholesterolemic patients showed an increased expression of the haptoglobin isoforms 1, 2, and 3 with respect to the CR group (Table 3). The HPT isoform 2 was significantly increased in plasma after simvastatin treatment compared with that found prior to simvastatin (Table 3).

Four serotransferrin protein isoforms (SER) were detected in area 6 (Figures 1 and 5). There were no differences in the level of expression of any of the SER isoforms between the different groups (Table 3).

Relationship between Cholesterol Lowering and the Changes in Protein Expression. We further analyzed whether the changes observed in the level of plasma expression of FGG isoform 1, FGB isoforms 1 and 2, DBP isoform 3, apo A-IV, and HPT isoform 2 after simvastatin treatment of hypercholesterolemic patients were associated with the cholesterol-lowering effect of simvastatin. Therefore, we analyzed if total plasma cholesterol and plasma LDL-cholesterol levels, which were modified by simvastatin treatment, were correlated with the expression of the above-mentioned protein isoforms. Pearson's analysis demonstrated a positive correlation between total cholesterol levels and FGG chain isoform 1 and between LDL

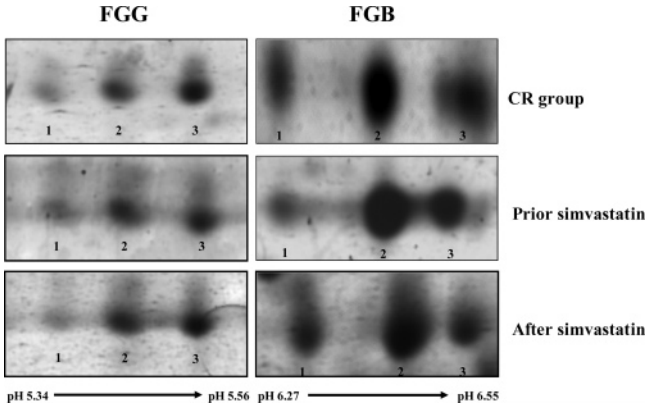

Figure 2. Representative pattern of fibrinogen gamma (FGG) and beta (FGB) chains isoforms in plasma from hypercholesterolemic patients before and after simvastatin treatment. Images were obtained from the bidimensional electrophoresis gels using a pH range of 4−7. Numbers 1−3 were only used to identify each FGG and FGB isoform.

cholesterol and FGG chain isoform 1 (Table 4). There was also a negative correlation between total plasma cholesterol and the level of expression of FGB isoforms 1 and 2 (Table 4). There was no correlation between LDL cholesterol and the plasma expression of these FGB chain isoforms (Table 4). There was no correlation between the plasma expression of DBP isoform 3, apo A-IV, and HPT isoform 2 with neither total cholesterol nor LDL cholesterol (Table 4).

Discussion

Using proteomics, we here analyzed the changes in different proteins expressed in the plasma of moderate hypercholesterolemic patients after simvastatin treatment. To our knowledge, this is the first time that proteomics has been used to study hypercholesterolemia and to analyze the effect of the treatment with a statin such as simvastatin.

The first identified proteins in the plasma proteome were FGG and FGB chains. High fibrinogen plasma levels have been associated with an increased risk of cardiovascular disease because fibrinogen has been linked to thrombogenesis, inflammation, and, hence, vascular disease.[17,18] In hypercholesterolemic patients, FGG chain isoform 1 was increased with respect to the CR group, and it was reduced after simvastatin treatment. An increased plasma expression of FGG chain isoform 1 has been also observed in the plasma from patients during an acute coronary syndrome.[16]

We also observed that there were differences in the plasma expression of FGB chain isoforms 1 and 3 in hypercholesterolemic patients with respect to the CR group, but whereas the plasma expression of FGB chain isoform 1 was reduced by simvastatin treatment, the expression of FGB chain isoform 3 was not modified.

In vitro studies have suggested that the FGB chain limits the rate of production of mature fibrinogen.[19] In this regard, a strong association between FGB chain polymorphisms and fibrinogen plasma concentration has been reported.[20] In contrast, most of the studies did not find any correlation between FGB chain levels and the risk of cardiovascular disease.[21]

No changes, reductions, and even increases in fibrinogen plasma levels have been observed after statin treatment.[22,23] Therefore, further studies are needed to assess whether the observed changes in both FGG and FGB chain isoforms before and after simvastatin treatment may have any relevance on fibrinogen activity in hypercholesterolemic patients.

We identified three DBP isoforms in plasma. Other authors have also observed three DBP isoforms in serum and demonstrated binding differences between them when incubated with 25-hydroxycholecalciferol.[24] DBP isoform 3 was increased in plasma after simvastatin treatment. This may seem paradoxical because a function attributed to DBP has been neutrophil chemotaxis[25] and experimental works have shown anti-inflammatory properties for statins.[9,26] However, others studies have also demonstrated that DBP is a multi-functional protein involved in functions such as reduction of actin-induced platelet activation.[27] In this regard, although there are conflicting findings on the effects of statins on thrombosis, different reports have suggested a reduction of prothrombotic-markers by simvastatin.[26,28]

Apo A-IV plays an important role in reverse cholesterol transport. Apo A-IV binding to peripheral cells promotes cholesterol afflux and enhances the formation of small HDL particles.[29] Studies in fat-fed mice have demonstrated that apo A-IV is also a vascular protective protein because in transgenic mice, human apo A-IV expression is protective of murine vasculature.[30] We detected one apo A-IV isoform in the plasma that was significantly increased after simvastatin treatment, suggesting a protective effect of simvastatin on the vascular wall. Several studies have demonstrated the protective effect of simvastatin on vascular functionality,[31,32] and an association has even been demonstrated between low plasma apo A-IV concentrations and coronary artery disease in humans.[33] Paradoxically, the plasma from hypercholesterolemic patients showed a higher expression of apo A-IV than that from the CR group. Because apo A-IV exerts antiatherogenic properties, the enhanced apo A-IV plasma levels observed in hypercholesterolemic patients may be related to an endogenous protective vascular response to hypercholesterolemia that could be promoted by simvastatin.

We further analyzed the effects induced by simvastatin treatment on two proteins involved in iron homeostasis: haptoglobin and serotransferrin. Transition metals, such as iron and copper, are very effective in catalyzing free-radical oxidation of lipids and proteins which are well-known to be involved in atherogenesis [34−36].

Haptoglobin is an acute-phase reactant protein that binds free hemoglobin, exerting antioxidant properties.[37] Three of the five haptoglobin isoforms found in plasma were increased in hypercholesterolemic patients, which may occur as a defensive response against the increased oxidative stress associated with hypercholesterolemia. Because haptoglobin isoform 2 was significantly increased after simvastatin treatment, it is possible that simvastatin further enhanced the antioxidant response in the hypercholesterolemic patients. In vitro studies have accordingly demonstrated an antioxidant effect of simvastatin.[38] Unfortunately, as occurred for the other protein isoforms identified in the present study, at present there are not reports about the functional role of each haptoglobin isoform. It is also important to remember that the different proportion of haptoglobin isoforms may reflect a different haptoglobin phenotype. In this regard, a strong association has been demonstrated between haptoglobin phenotype and the risk of developing premature coronary and peripheral vascular diseases.[39,40]

Serotransferrin is a soluble protein that carries iron in the blood. Epidemiological data have suggested that excess iron may contribute to the development of cardiovascular diseases.[41]

Table 3. Densitometric Analysis of the Plasma Expression of Fibrinogen Gamma Chain (FGG), Fibrinogen Beta Chain (FGB), and Vitamin D-Binding Protein (DBP) Isoforms in Plasma[a]

protein	map area	experimental mass (kDa/pI)	CR group (A.U.)	prior to simvastatin (A.U.)	after simvastatin (A.U.)
FGG isoform 1	1	49.51/5.34	56.5 ± 35.01	104.1 ± 47.0[b]	68.0 ± 24.3[c]
FGG isoform 2	1	49.38/5.44	136.2 ± 89.7	177.2 ± 67.7	169.4 ± 61.09
FGG isoform 3	1	49.38/5.56	161.4 ± 134.1	139.0 ± 47.4	155.6 ± 49.21
FGB isoform 1	2	55.49/6.27	87.8 ± 24.32	54.8 ± 24.60[b]	74.5 ± 22.91[c]
FGB isoform 2	2	55.05/6.41	162.1 ± 48.36	181.4 ± 57.42	223.0 ± 63.92[b]
FGB isoform 3	2	54.05/6.55	124.9 ± 45.82	83.5 ± 40.44[b]	74.0 ± 20.08[b]
DBP isoform 1	3	54.52/5.30	63.3 ± 41.57	75.6 ± 58.38	71.1 ± 44.41
DBP isoform 2	3	54.51/5.34	131.6 ± 83.72	132.4 ± 67.03	179.2 ± 123.03
DBP isoform 3	3	54.52/5.40	175.0 ± 102.95	160.9 ± 74.10	205.7 ± 106.91[c]
APO A IV	4	43.36/5.22	38.2 ± 25.73	167.7 ± 134.63[b]	280.6 ± 207.61[b,c]
HPT isoform 1	5	44.52/4.81	54.3 ± 27.72	137.0 ± 104.65[b]	142.1 ± 142.55[b]
HPT isoform 2	5	38.94/4.90	165.1 ± 60.81	250.7 ± 76.08[b]	328.7 ± 144.25[b,c]
HPT isoform 3	5	42.22/4.97	336.8 ± 94.46	478.2 ± 132.94[b]	592.5 ± 183.84[b]
HPT isoform 4	5	41.49/5.08	506.3 ± 177.62	555.3 ± 206.76	609.0 ± 133.50
HPT isoform 5	5	41.41/5.21	521.7 ± 294.72	671.0 ± 382.40	562.3 ± 132.37
SER isoform 1	6	80.55/6.28	96.2 ± 71.90	70.63 ± 36.20	71.2 ± 31.11
SER isoform 2	6	80.55/6.34	193.5 ± 76.08	167.1 ± 78.35	146.5 ± 64.77
SER isoform 3	6	80.55/6.40	335.2 ± 179.32	304.2 ± 143.12	322.1 ± 144.25
SER isoform 4	6	79.27/6.45	274.9 ± 191.29	392.7 ± 146.51	386.7 ± 162.35

[a] Results are represented as mean ± SD; A.U.: arbitrary units. [b] $p < 0.05$ with respect to CR group. [c] $p < 0.05$ with respect to prior to simvastatin treatment.

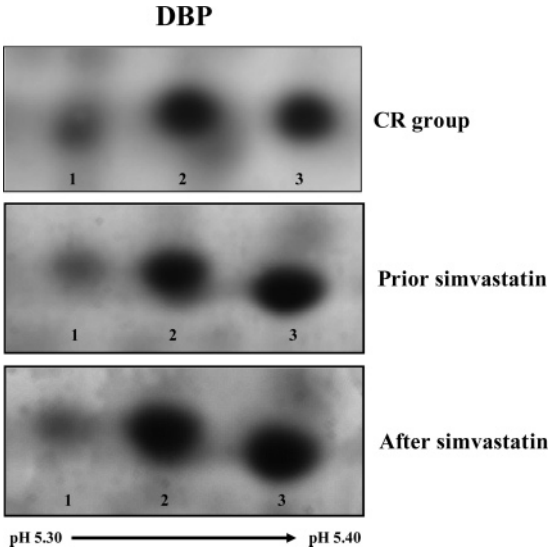

Figure 3. Representative pattern of vitamin D binding protein (DBP) isoforms in plasma from hypercholesterolemic patients before and after simvastatin treatment. Images were obtained from the bidimensional electrophoresis gels using a pH range of 4−7. Numbers 1−3 were only used to identify each DBP isoform.

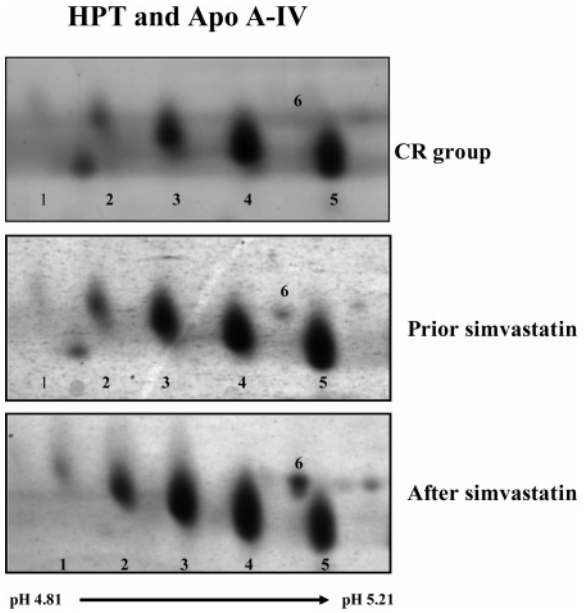

Figure 4. Representative pattern of haptoglobin (HPT) isoforms and apolipoprotein A-IV (apo A-IV) in plasma from hypercholesterolemic patients before and after simvastatin treatment. Images were obtained from the bidimensional electrophoresis gels using a pH range of 4−7. Numbers 1−5 were only used to identify each HPT isoform. Number 6 was only used to identify the apo A-IV.

None of the four serotransferrin isoforms detected in plasma were modified between the different studied groups.

The beneficial effects of statins are assumed to result from their ability to reduce cholesterol synthesis. However, experimental data have suggested that statins may modify other molecular pathways independently of their cholesterol-lowering effect. The plasma level of FGG chain isoform 1 was positively correlated with total cholesterol and LDL-cholesterol levels, which may suggest that the modification of this protein isoform was related to the lipid-lowering effect of simvastatin. The changes in FGB chain isoforms 1 and 2 were also associated with total plasma cholesterol levels. However, the modifications observed after simvastatin treatment in apo A-IV, DBP isoform 3, and haptoglobin isoform 2 may be related to a nonlipid effect of this drug because there was not a correlation between their plasma level and the total cholesterol level.

Several considerations about the present study should be done. First, we have performed the study in hypercholesterolemic patients where following a low-cholesterol and low-fat diet was recommended although no control was kept. However, with the present experimental design, we cannot discard that this type of diet may favor the observed plasma protein

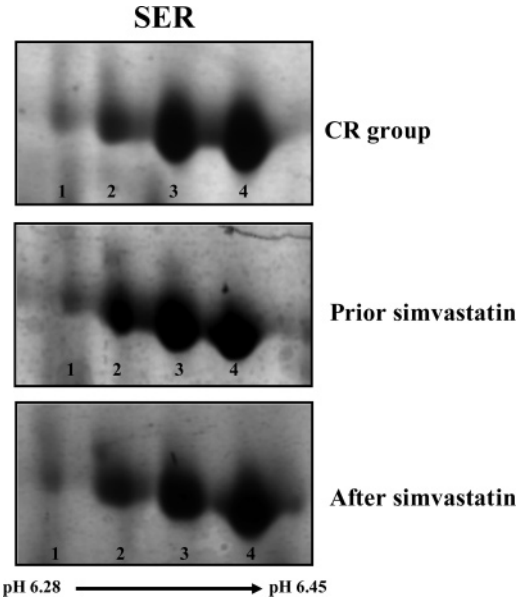

Figure 5. Representative pattern of serotransferrin (SER) isoforms in plasma from hypercholesterolemic patients before and after simvastatin treatment. Images were obtained from the bidimensional electrophoresis gels using a pH range of 4−7. Numbers 1−4 were only used to identify each SER isoform.

Table 4. Pearson's Correlation Analysis for the Protein Isoforms Modified in Plasma after Simvastatin Treatment

	total cholesterol		LDL cholesterol	
protein[a]	Pearson's coefficient	statistical significance	Pearson's coefficient	statistical significance
FGG isoform 1	0.740	0.002	0.669	0.006
FGB isoform 1	−0.685	0.007	−0.255	0.379
FGB isoform 2	−0.602	0.018	−0.417	0.122
DBP isoform 3	0.102	0.707	−0.029	0.915
APO A-IV	0.191	0.478	−0.103	0.706
HPT isoform 2	−0.266	0.358	−0.452	0.105

[a] FGG: fibrinogen gamma chain; FGB: fibrinogen beta chain; DBP: vitamin D-binding protein; APO A-IV: apolipoprotein A-IV; HPT: haptoglobin.

modifications induced by simvastatin treatment. However, Thuraisingham et al. have demonstrated that a low-cholesterol diet does not confer additional advantage on the lipid profile once simvastatin therapy has been intrated.[42] Moreover, other authors have showed that the effect of simvastatin on different mechanisms was not observed with a lipid-lowering diet.[43] Second, the modifications observed in the plasma proteome could be related to a different gene expression and/or post-translational modifications. Indeed FGG, FGB, and haptoglobin are all glycoproteins that could be modified by sulfation, which can change protein p*I* and even protein functionality. In this regard, statins inhibit the mevalonate pathway, which not only decreases the hepatic circulation of cholesterol but decreases isoprenoids as well. Isoprenoids are critical in the post-translational modification of many proteins.[44] Third, with the present experimental design, it is difficult to assess the clinical impact of the here-reported findings. The pleiotropic effects of statins including simvastatin are under continued investigation to fully establish their role in the prevention of cardiovascular events by statins. In this regard, the clinical importance of the noncholesterol-lowering effects of simvastatin in patients has been notoriously difficult to determine. Same trials have suggested that there was no apparent association between the prevention of coronary event rate and the level of LDL cholesterol reduction reached with statins.[45,46] Accordingly, we have observed that simvastatin treatment modified the expression of plasma protein isoforms associated with different cardiovascular protective mechanisms i.e., vascular, thrombosis, and oxidative stress protection, which was unrelated to the lipid effect of simvastatin. Then, these findings may help to explain mechanisms by which simvastatin reduces the evidence of acute vascular events and improves the clinical outcome of patients with hyperchholesterolemic. Therefore, our findings using proteomics may enhance the knowledgment of the mechanisms of action of simvastatin in the hypercholesterolemic patient, mainly to identify its pleiotropic mechanisms, and may even open new area for exploration of novel targets for drug development.

In summary, our study used, for the first time, proteomics to demonstrate that simvastatin treatment of moderate hypercholesterolemic patients modified the plasma expression of protein isoforms of biomarkers associated with thrombosis, oxidative stress, and vascular protection. The present study is only one example to show that proteomics may be a useful technique to know more in depth the molecular modifications induced by drug treatment of cardiovascular patients. Further studies with a larger number of patients are then needed to establish the clinical relevance of the plasma proteomic modifications.

Acknowledgment. This work has been supported by a Medical School Grant from Merck Sharp and Dohme and Fondo de Investigaciones de la Seguridad Social (Redes temáticas de Cooperación Red Heracles G03/045). S.A.-O. is a fellow of Red Heracles. We thank Begoña Larrea for the secretarial assistance. Mass spectrometry was performed in a facilities center (Complutense University, Madrid, Spain).

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PR060159W