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ORIGINAL ARTICLE

Reduction of Heat Shock Protein Antibody Levels by Statin Therapy

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Abstract Atherosclerosis is a disease whose pathogenesis involves inflammatory and immunological mechanisms, including an autoimmune reaction against heat shock proteins (Hsps). The purpose of this study was to analyze whether the antiatherogenic effect of statin therapy was not limited to its lipid lowering effect, but also included anti-inflammatory and immunomodulatory effects, paying special attention to the measurement of circulating concentrations of anti-Hsp70 and anti-Hsp60 antibodies previously related to vascular disease. Two-hundred and seventy-five subjects aged 40–60 years, randomly selected in an epidemiological study on the incidence of vascular risk factors, were studied. Laboratory tests included a complete lipid profile after a 12-h fast and measurements of glucose, C-reactive-protein, anti-Hsp70 and anti-Hsp60 antibodies. Subjects with hypercholesterolemia had significantly higher concentrations of anti-Hsp70 antibodies as compared to subjects with normal cholesterol concentrations. Statin therapy was associated with 11.63 and 15.3% reductions in total and LDL-cholesterol ($P = 0.005$ and 0.017, respectively) as compared to untreated subjects, and with lower concentrations of circulating anti-Hsp70 ($P = 0.016$) antibodies. No differences were found in C-reactive-protein values. Since statin therapy not only reduces lipid profile, but also anti-Hsp70 and anti-Hsp60 antibody concentrations, without changing C-reactive-protein values, it is suggested that such an effect could not be accounted for by the anti-inflammatory properties of statins, but by their direct immunomodulatory properties through their effects on lymphocyte function.

Keywords Atherosclerosis · Inflammation · Immunology · Hyperlypidemia · Cholesterol · HMG-CoA reductase

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Introduction

Cardiovascular disease is the leading cause of death in most western countries [1]. Experimental and epidemiological studies and genetic forms of hypercholesterolemia have shown the association between serum cholesterol, and particularly LDL cholesterol, and atherosclerosis [2]. Hypercholesterolemia causes focal endothelial activation in large and middle-sized arteries. LDL infiltration and retention in the arterial intima starts an inflammatory response in the arterial wall [3]. LDL cholesterol reduction using lipid lowering drugs is associated to significant decreases in cardiovascular morbidity and mortality both in primary and secondary prevention [4], and therefore represents the first pharmacological target for the adult treatment panel III (ATP III) [2]. The advent of statins at the end of the 1980s and the beginning of the 1990s revolutionized treatment of hypercholesterolemia, since they were safe drugs that were administered once daily and

decreased LDL cholesterol levels by 25–35% [5]. Statins also decreased endothelial dysfunction, had direct anti-inflammatory effects, reducing content of inflammatory cells and their production of proinflammatory cytokines in the atherosclerotic plaque [6], and even exerted immunomodulatory effects, interfering with lymphocyte T function [7].

Both adaptive and innate immunity are also involved in the etiopathogenesis of atherogenesis [8]. Specific antigens starting immune response in atherosclerosis include oxidized LDL [9], heat shock proteins (Hsps) [10], and β2-glycoprotein I [11].

Hsps have been involved in the pathogenesis of various diseases, and their role in atherosclerosis is among the most widely studied. Special attention has been paid to Hsp60 and Hsp70 [10]. Various studies have shown Hsp60 to be selectively located in atherosclerotic lesions rather than non-atherosclerotic areas of the arterial wall [12]. In advanced atherosclerotic lesions, Hsp70 are overexpressed in different cell types such as monocytes, macrophages, dendritic cells, and smooth muscle cells. Hsps have also been identified in soluble form in serum together with anti-Hsps antibodies, and various studies have shown a correlation between levels of these antibodies and severity of atherosclerosis. Anti-Hsp70 antibody concentrations are elevated in patients with established hypertension as compared to the normotensive control group [13] in patients with abdominal aortic aneurysm or peripheral vascular disease [14]. High titers of anti-Hsp60 antibodies have been found in patients with carotid atherosclerosis, coronary disease, or stroke [15].

The aim of this study was to explore whether statins not only achieved lower total and LDL cholesterol values in treated subjects as compared to an untreated population, but could also have anti-inflammatory and immunomodulatory effects in addition to their antiatherogenic effect, by measuring the circulating concentrations of anti-Hsp70 and anti-Hsp60 antibodies previously related to vascular disease.

Methods

Study Population and Design

This was an observational, cross-sectional, epidemiological study on the incidence of vascular risk factors. Inclusion criteria: randomly selected voluntary subjects of both sexes aged 40–60 years, employees (either active or not) of the Hospital General Universitario Gregorio Marañón in January 2004, who signed the informed consent. The study was approved by the Research Committee and the Clinical Research Ethics Committee of the center. All participants provided a clinical history and answered an epidemiological survey including age, personal and family medical history, smoking (number of cigarettes per year and smoking duration; if former smokers, number of years elapsed since smoking cessation), alcohol intake (if yes, grams of alcohol daily), treatments, and occurrence or presence of disease of atherosclerotic etiology. Blood pressure (BP) was measured, and blood was taken for the appropriate laboratory measurements. Exclusion criteria included pregnancy or breast-feeding, any systemic infection in the past 3 months, current oncological disease or radiotherapy/chemotherapy, autoimmune disease (rheumatoid arthritis, systemic lupus erythematosus, sarcoidosis), endocrine disorders (except for diabetes), liver disease, renal failure, glomerulonephritis, congenital heart disorder, oncohematological disease, or allergic disorders.

Laboratory Tests

Venous blood was drawn after a 12-h fast and centrifuged, and serum samples were frozen at −70 °C for subsequent testing. In order to assess causal and conditional vascular risk factors [4], total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and glucose were quantified in a Hitachi Modular Analytic SVA autoanalyzer, Roche Diagnostics S.L., Barcelona, Spain. C-reactive protein (CRP) was quantified using a commercial ELISA (CRP-MTPL-EIA, DRG Instruments GMBH, Marburg, Germany) according to the manufacturer's instructions. Final absorbances were measured at 450 nm (Bio-Rad 3550 Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA). Values under 5 μg/mL were considered normal. CRP concentrations in the samples were calculated by interpolation in the standard curve obtained (range 1.62–25 μg/mL). Homocysteine has been quantified by high performance liquid chromatography (HPLC); the established reference values (P_{95}) were 13 μmol/L for men and 11 μmol/L for women.

Definition of Vascular Risk Factors [2, 16]

Obesity

Obesity was defined as a body mass index (BMI kg/m^2) >30, and overweight when the BMI was >25.

Blood Pressure

The blood pressure (BP) of all participants was measured with an automated BP recording device after an individual had been sitting quietly for 5 min. Hypertension has been defined as a systolic blood pressure ≥140 mmHg and/or a diastolic ≥90 mmHg in at least two measurements taken separately.

Blood Lipids

Hypercholesterolemia was defined as a fasting total cholesterol level of ≥200 mg/dL, or LDL-cholesterol ≥130 mg/dL. By hypertriglyceridemia we understand a condition in which a fasting total triglyceride level is ≥150 mg/dL.

Blood Glucose

Diabetes has been defined as a fasting glycemia level ≥120 mg/dL, or post-prandial glucose of 130–160 mg/dL.

Measurement of Circulating Anti-Hsp70 and Anti-Hsp60 Antibodies

Titers of anti-Hsp70 and anti-Hsp60 antibodies in serum (diluted 1:1,000) were measured using two commercial enzymimmunoassays [EKS-750, Anti-Human Hsp70 (IgG/IgM/IgA) ELISA Kit and EKS-650, Anti-Human Hsp60 (total) ELISA Kit, Stressgen Biotechnologies Corporation, Victoria, Canada] in a microplate coated with recombinant human Hsp70 or Hsp60, capturing anti-Hsp70 and anti-Hsp60 antibodies present in serum. The resulting absorbance was measured at 450 nm (Bio-Rad 3550 Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA). Anti-Hsp antibody concentrations in the samples, expressed as μg/mL, were obtained by interpolating in the standard curve the absorbances obtained in the samples.

Statistical Analysis

Results were analyzed using their means, medians, standard deviations, standard error of the mean (SEM), and ranges for quantitative variables, and using absolute frequencies and percentages for qualitative variables with a

Table 1 Characteristics of normocholesterolemic and hypercholesterolemic subjects

	Normocholesterolemic patients ($n = 116$)				Hypercholesterolemic patients ($n = 159$)				P*
Anti-Hsp70 antibodies (μg/mL)	392.53 ± 23.13				451.33 ± 21.50				0.049
Patients under no treatment	404.27 ± 24.34 ($n = 101$)				466.87 ± 22.48 ($n = 140$)				0.041
Anti-Hsp60 antibodies (μg/mL)	57.99 ± 5.84				49.94 ± 3.48				NS
Age	47.66 ± 0.75				49.36 ± 0.48				NS
Sex									
Females	52				82				NS
Males	64				77				
Total cholesterol (mg/dL)	179.59 ± 2.03				233.96 ± 1.91				<0.001
LDL-cholesterol (mg/dL)	100.23 ± 3.11				146.48 ± 1.98				<0.001
HDL/LDL	0,63 ± 0.02				0.047 ± 0.01				<0.001
Blood glucose (mg/mL)	90.00 ± 1.53				89.84 ± 1.03				NS
Triglycerides (mg/mL)	107.11 ± 5.77				115.98 ± 5.47				NS
Homocysteinemia (mmol/L)	9.37 ± 0.71				8.77 ± 0.24				NS
CRP (μg/mL)	4.55 ± 0.75				4.50 ± 0.59				NS
Hypertension									
Yes	21				37				NS
No	95				122				
Smoking[a]									
Degree	0	1	2	3	0	1	2	3	NS
Number	89	17	10	0	109	35	17	1	
Alcohol intake[b]									
Degree	0	1	2	3	0	1	2	3	NS
Number	77	33	6	0	96	55	7	1	

Results given as mean ± SEM

Hsp heat shock proteins, *CRP* C-reactive protein

* Referring to the effect of treatment on parameters analyzed in the Mann–Whitney test for two independent samples or an ANOVA test with Bonferroni correction

[a] Smoking degree: 0: non-smoker; 1: 0–20 cigarettes/day; 2: 20–40 cigarettes/day; 3: >40 cigarettes/day

[b] Alcohol intake degree: 0: none or less than 20 g of alcohol/day; 1: 20–40 g of alcohol/day; 2: 40–80 g of alcohol/day; 3: >80 g of alcohol/day

95% confidence interval. Variable means were compared using non-parametric tests. A Mann–Whitney's *U* test for two independent samples, a one-way ANOVA test with Bonferroni correction for three independent samples, or a Kruskal–Wallis test was used for quantitative variables. Qualitative variables were analyzed using a Spearman's correlation test. The statistical significance level selected was $P < 0.05$. SPSS 12.0 software for Windows was used for statistical analysis.

Results

A total of 275 subjects were included in the study, 134 females (age 48.95 ± 0.57 years) and 141 males (48.35 ± 0.62). Of these, 159 participants (57.8%) [82 females (51.57%) and 77 males (48.42%)] had total cholesterol levels higher than 200 mg/dL or LDL cholesterol levels higher than 130 mg/dL.

Hypercholesterolemic individuals had significantly higher titers of circulating anti-Hsp70 antibodies (451.32 ± 21.5 μg/mL) as compared to subjects with normal cholesterol levels (392.53 ± 23.13 μg/mL) ($P < 0.05$, Mann–Whitney's test), with no differences between both groups in the incidence of other population (age, sex, and social level) or vascular risk factors (glucose, hypertension, triglyceridemia, homocysteinemia and CRP, smoking, alcohol intake and its extent). Both effects, hypercholesterolemia and titers of anti-Hsp70i antibodies, therefore appear to be directly related and not influenced by other potential factors acting on such antibodies (Table 1).

Thirty-four subjects (11 females and 23 males) were being treated with simvastatin 10 mg/day at the time of the study. This treatment was associated to a significant 11.63% lower total cholesterol levels ($P = 0.013$, Mann–Whitney's test) and a 11.07% LDL cholesterol levels as compared to subjects with no statin therapy ($P = 0.011$, Mann–Whitney's test). Patients treated with simvastatin also showed higher glucose, triglyceride, BP, and CRP values (Table 2), probably due to the presence of additional vascular risk factors [4]. In this latter case, when population was segmented based on the degree of vascular risk according to the Task Force [16] or considering the cases of established atherosclerosis, statin therapy caused no

Table 2 Differences between subjects with or without statin therapy at the time of study

	Patients with treatment ($n = 34$)				Patients without treatment ($n = 241$)				P*
Total cholesterol (mg/dL)	197.15 ± 6.1				212.98 ± 2.26				0.013
LDL cholesterol (mg/dL)	113.79 ± 5.60				129.07 ± 2.14				0.011
Anti-Hsp70 antibodies (μg/mL)	330.84 ± 34.43				440.57 ± 17.30				0.016
Anti-Hsp60 antibodies (μg/mL)	43.26 ± 5.23				54.84 ± 3.58				NS
Sex									
Females	11				123				0.042
Males	23				118				
Blood glucose (mg/mL)	98.82 ± 3.74				88.65 ± 0.89				0.002
Triglycerides (mg/mL)	133.44 ± 10.842				109.26 ± 4.27				0.005
Homocysteinemia (mmol/L)	9.44 ± 0.74				8.93 ± 0.36				NS
CRP (μg/mL)	7.42 ± 1.68				4.12 ± 0.47				0.04
Hypertension									
Yes	12				46				0.30
No	22				195				
Smoking[a]									
Degree	0	1	2	3	0	1	2	3	NS
Number	21	11	2	0	177	38	25	1	
Alcohol intake[b]									
Degree	0	1	2	3	0	1	2	3	NS
Number	21	11	2	0	152	77	11	1	

Results given as mean ± SEM

Hsp heat shock proteins, *CRP* C-reactive protein

* Referring to the effect of treatment on parameters analyzed in the Mann–Whitney test for two independent samples or an ANOVA test with Bonferroni correction

[a] Smoking degree: 0: non-smoker; 1: 0–20 cigarettes/day; 2: 20–40 cigarettes/day; 3: >40 cigarettes/day

[b] Alcohol intake degree: 0: none or less than 20 g of alcohol/day: 1: 20–40 g of alcohol/day; 2: 40–80 g of alcohol/day; 3: >80 g of alcohol/day

Table 3 Differences in CRP between subjects with or without statin therapy by vascular risk (Task Force [16])

Group	Patients with treatment ($n = 34$)	Patients without treatment ($n = 241$)	P*
No VRF			0.918
N	5	141	
CRP (μg/mL)	2.83 ± 1.93	2.59 ± 0.44	
Moderate VRFs (10%)			0.722
N	12	80	
CRP (μg/mL)	5.34 ± 2.56	5.35 ± 0.93	
Established atherosclerosis			0.845
N	17	20	
CRP (μg/mL)	10.11 ± 2.64	10.26 ± 2.60	

Results given as mean ± SEM

CRP C-reactive protein, *VRF* vascular risk factors

* Referring to the effect of treatment on parameters analyzed in the Mann–Whitney test for two independent samples

significant changes in CRP concentrations (Table 3). However a significant trend to a lowering in the concentration of anti-Hsp70i antibodies in individuals with VRF or established atherosclerosis has been proven ($P = 0.024$, Kruskal–Wallis test). Patients with atherosclerosis show the lowest levels of circulating anti-Hsp70i antibodies [($P = 0.008$, Mann–Whitney test). Hsp70i is the inducible form of the HSP70 family and is also known as Hsp72, based on its molecular weight [17]. Therapy with statins reduces anti-Hsp70i antibodies concentrations significantly in patients with VRF and atherosclerosis in comparison to an identical group of patients without such therapy ($P = 0.043$, one-way ANOVA) (Table 4).

Treatment with simvastatin was very significantly associated with lower concentrations s of circulating anti-Hsp70 antibodies ($P = 0.016$, Mann–Whitney's test). In order to rule out the potential effect of other population and vascular risk factors on the decrease in concentrations of circulating anti-Hsp70 antibodies seen in the statin-treated group, the potential association between such factors and anti-Hsp antibodies was tested in the study population. Circulating concentrations of anti-Hsp70i antibodies in the overall population were not associated with factors such as age, sex, blood glucose, triglycerides, BP, homocysteinemia, or CRP (Table 5).

Discussion

Monocytes–macrophages and T lymphocytes are involved in atherosclerosis, a chronic inflammatory disease. Hsps are immunomodulatory molecules that act as potent autoantigens. Hsps recognition by T lymphocytes would trigger an autoimmune response potentially involved in the inflammatory etiopathogenesis of atherosclerosis [18]. The finding of a significant increase in anti-Hsp70 antibody concentrations in hypercholesterolemic patients as compared to normocholesterolemic subjects in age- and sex-matched populations, in the absence of a hypothetical etiopathogenetic role of other associated vascular risk factors, agrees with reports by other authors [19]. This could be explained by an enhanced immunoactivation status associated to atherosclerosis in this group. High cholesterol levels not only activate or damage endothelium per se but may modulate the function of T and B cells. Cholesterol has been shown to increase the antigen presentation function of monocytes, and oxidized LDL may activate dendritic cells and promote in vitro the proliferation of T lymphocytes mediated by dendritic cells [20].

Both the recommendations by the National Cholesterol Education Program [2] and those derived from the Second Joint Task Force of European societies [16] emphasize the need for controlling individual risk of coronary disease. Dyslipidemia is clearly a major risk factor for coronary disease, though it is often modulated by the presence of other additional risk factors. Both recommendations state that reduction of LDL cholesterol levels represents a recognized target, essential for reducing coronary risk. Drugs affecting lipoprotein metabolism include HMG-CoA reductase inhibitors (statins), fibrates, bile acid sequestrants (resins), and nicotinic acid and its derivatives. They have all been shown to decrease coronary disease progression in

Table 4 Differences in Anti-Hsp70 antibodies concentration (μg/mL) between subjects with or without statin therapy according to the degree of vascular risk (Task Force [16])

Group	Global	Patients with treatment	Patients without treatment	P*	P**
No VRF	458.72 ± 21.52 ($n = 146$)	510.65 ± 127.89 ($n = 5$)	456.87 ± 21.90 ($n = 141$)	0.024	0.043
Moderate VRFs (10–20%)	414.36 ± 27.99 ($n = 92$)	372.85 ± 44.77 ($n = 12$)	420.66 ± 31.54 ($n = 80$)		
Established atherosclerosis	349.93 ± 41.27 ($n = 37$)	265.95 ± 43.85 ($n = 17$)	421.31 ± 31.35 ($n = 20$)		

Results are given as means ± SEM

VRF vascular risk factors

* P refers to anti-HSP70i Abs level and their relevance in vascular disease (Kruskas–Wallis test)

** P refers to the effects of the treatment on Anti-Hsp70i Abs concentration in the different groups analyzed by the Anova-one-way test

Table 5 List of populational and biochemical parameters and anti-Hsp70 antibody levels in a randomly selected general population Anti-Hsp70 antibodies (μg/mL)

					*P**
Sex					
Females ($n = 134$)	435.55 ± 22.07				NS
Males ($n = 141$)	418.88 ± 22.83				
Age					
40–44	375.65 ± 26.4				NS
45–49	465.62 ± 31.6				
50–54	432.04 ± 40.02				
55–60	448.56 ± 37.28				
Blood glucose (mg/mL)					
Normoglycemic	428.32 ± 16.83				NS
Hyperglycemic	411.86 ± 44.55				
Triglycerides (mg/mL)					
Normotriglyceridemia	432.46 ± 17.38				NS
Hypertriglyceridemia	394.94 ± 38.79				
Hypertension					
No	436.47 ± 18.93				NS
Yes	391.55 ± 25.16				
Homocysteinemia (mmol/L)					
Normal	419.56 ± 16.92				NS
Hyperhomocysteinemia	455.32 ± 42.09				
Smoking[a]					
Degree	0	1	2	3	NS
Number	198	48	27	1	
Anti-Hsp70 Abs (μg/mL)	435.96 ± 18.51	446.92 ± 35.41	518.41 ± 53.79	10,900	
Alcohol intake[b]					
Degree	0	1	2	3	NS
Number	173	88	13	1	
Anti-Hsp70 Abs (μg/mL)	420.82 ± 8.78	434.09 ± 31.76	458.65 ± 69.44	460.86	
CRP (μg/mL)					
Females ($n = 134$)	4.56 ± 0.72				NS[c]
Males ($n = 141$)	4.49 ± 0.60				
Anti-Hsp60 antibodies (μg/mL)					
Females ($n = 134$)	52.57 ± 4.47				NS[c]
Males ($n = 141$)	54.22 ± 4.61				

Results given as mean ± SEM

Hsp heat shock proteins, *CRP* C-reactive protein

* Referring to the effect of treatment on parameters analyzed in the Mann–Whitney test for two independent samples or an ANOVA test with Bonferroni correction

[a] Smoking degree: 0: non-smoker; 1: 0–20 cigarettes/day; 2: 20–40 cigarettes/day; 3: >40 cigarettes/day

[b] Alcohol intake degree: 0: none or less than 20 g of alcohol/day: 1: 20–40 g of alcohol/day; 2: 40–80 g of alcohol/day; 3: >80 g of alcohol/day

[c] Spearman's correlation coefficient

angiographic studies. However, both the most convincing angiographic evidence and the best evaluation criteria in clinical trials have been obtained using statins [21]. These drugs are also the safest to date and the easiest to use. Thus, statins currently are the first-choice lipid lowering drugs [22].

The antiatherogenic effect of statins is not limited to their effects on the reduction of circulating total and LDL cholesterol levels. This study has shown that patients treated with simvastatin not only had significantly lower total and LDL cholesterol levels, but also showed significant lower anti-Hsp70 antibody concentrations and

relevant but not statistically significant lower anti-Hsp60 concentrations which agrees with reports from similar studies [23]. Since anti-Hsp antibodies are positively related to the risk of vascular disease and involved in the etiopathogenesis of atherosclerosis, their reduction may decrease the chronic inflammation characteristic of atherosclerosis and contribute to stabilization of the atherosclerotic plaque. The mechanism by which statin therapy is associated to a decrease in circulating anti-Hsp antibodies could be the pleiotropic effects of these drugs, including immunomodulation, that are independent from the decrease in cholesterol levels, as also shown in this study. Statins have also been shown to be able to inhibit binding of the lymphocyte function-associated antigen-1 (LFA-1) to the intercellular adhesion molecule-1 (ICAM-1), thereby preventing leukocyte adhesion and extravasation at inflammation sites, as well as antigen presentation, and lymphocyte T costimulation [24]. This would explain the significant lower circulating anti-Hsp70 antibodies detected in the group of treated subjects.

The immunosuppressant effect of statins is not only independent of their lipid lowering effect, but also of their documented anti-inflammatory properties, resulting from a decreased activation of the nuclear factor kappa B (NF-kB) that induces expression of multiple proinflammatory molecules [25]. Since statin therapy reduced not only the lipid profile, but also circulating concentrations of anti-Hsp70 and anti-Hsp60 antibodies without changing—even in patients with established atherosclerotic disease—CRP concentrations, a systemic inflammation marker attributed by some authors to an active role not only in the onset of atherosclerosis, but also as a biomarker of its course and prognosis [26], it is suggested that such an effect cannot be completely explained by its anti-inflammatory effects, but by its direct immunomodulatory properties.

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