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Effects of two lipid lowering therapies on immune responses in hyperlipidemic subjects

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ABSTRACT

Aims: To compare the effects of two of the most effective lipid-lowering therapies with similar LDL-cholesterol reduction capacity on the innate and adaptive immune responses through the evaluation of autoantibodies anti-oxidized LDL (anti-oxLDL Abs) and electronegative LDL [LDL(−)] levels.
Main methods: We performed a prospective, randomized, open label study, with parallel arms and blinded endpoints. One hundred and twelve subjects completed the study protocol and received rosuvastatin 40 mg or ezetimibe/simvastatin 10/40 mg for 12 weeks. Lipids, apolipoproteins, LDL(−), and anti-oxLDL Abs (IgG) were assayed at baseline and end of study.
Key findings: Main clinical and laboratory characteristics were comparable at baseline. Lipid modifications were similar in both treatment arms, however, a significant raise in anti-oxLDL Abs levels was observed in subjects treated with rosuvastatin ($p = 0.026$ vs. baseline), but not in those receiving simvastatin/ezetimibe. ($p = 0.233$ vs. baseline), thus suggesting modulation of adaptive immunity by a potent statin. Titers of LDL(−) were not modified by the treatments.
Significance: Considering atherosclerosis as an immune disease, this study adds new information, showing that under similar LDL-cholesterol reduction, the choice of lipid-lowering therapy can differently modulate adaptive immune responses.

Introduction

Despite the established association between cardiovascular disease and LDL cholesterol levels, atherosclerosis is considered a chronic inflammatory disease of blood vessels involving activation of immune responses. Oxidation leads to the release of bioactive lipids and causes physicochemical changes in the remaining LDL particles, generating not a single molecular species but a spectrum of modified LDL particles. Adaptive immune response is also triggered, as a consequence of LDL antigenic epitopes (Hansson and Hermansson, 2011).

The relevance of innate and adaptive immune responses in the context of atherosclerosis has been acknowledged, but evaluation of oxidation and immune parameters are less reported following lipid lowering therapies that promote similar and very low levels of LDL-c (Robinson, 2013).

Regarding innate immunity, an LDL sub-fraction, named electronegative LDL [LDL(−)], that is considered minimally oxidized and more negatively charged than the native LDL particle has been proposed as a new biomarker (Avogaro et al., 1988). It represents less than 10% of total LDL in healthy subjects, and more than 10% in patients at high cardiovascular risk (Sanchez-Quesada et al., 2002).

Immunoglobulin G autoantibodies against oxLDL (anti-oxLDL Abs), have been reported in human plasma and atheromas of subjects with coronary atherosclerosis and are considered markers of adaptive immunity. Titers of these antibodies seem related to the severity of atherosclerosis and they can be changed following medical therapies (Steinerova et al., 2001; Gounopoulos et al., 2007).

Thus, our study aimed to compare the effects of two of the most effective lipid lowering strategies on the immune responses.

Methods

Design and study population

We performed a prospective, randomized, open label study, with parallel arms and blinded endpoints. Patients were recruited from the outpatient unit of dyslipidemias of our university. The trial protocol

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was conducted in accordance to the ethical standards of the institution on human experimentation and approval was obtained from the local ethics committee. All participants have signed the written informed consent prior their inclusion in the study. Eligible patients were men and women, 30 to 75 years of age, in primary or secondary prevention of coronary heart disease, who had an indication for lipid-lowering therapy in accordance to the National Cholesterol Education Program/Adult Treatment Panel (NCEP/ATP III, 2002) guidelines. One-hundred and twelve subjects completed the study protocol. Patients with liver, renal or gastrointestinal disease, malignancies, uncontrolled metabolic disorder, that might affect the tolerability or safety of the treatments were excluded. Exclusion criteria during the study were low adherence (less than 80%) to the lipid-lowering regimen. The major characteristics of the study population are listed in Table 1. Risk factors and metabolic syndrome were defined by the NCEP/ATP III guidelines. The 24-hour dietary recall was obtained at the beginning and end of the study (Bingham et al., 1994). Before treatment, all patients received nutritional counseling based on the Therapeutic Lifestyle Changes of the NCEP/ATP III.

Study drugs

Rosuvastatin (Crestor®, IPR Pharmaceuticals, Puerto Rico), Simvastatin/Ezetimibe (Zetsim®, Schering-Plough Products, Las Piedras, Puerto Rico) were gifts from AstraZeneca and Merck Co, respectively.

Biochemistry, serum lipids and apolipoproteins

Twelve-hour fasting samples were obtained for all patients at baseline and 12 weeks after treatment and were assayed in a central laboratory of our university using automated techniques (Advia 2400, Siemens Healthcare Diagnostics, Tokyo, Japan). LDL-cholesterol was estimated using the Friedewald formula (Friedewald et al., 1972). Glycated hemoglobin was assayed by high-performance liquid chromatography (Tosho G2, Tosho Inc., Tokyo, Japan), apolipoproteins A1 and B, and highly-sensitive C-reactive protein were determined by nephelometry (Array 360 CE/AL, Beckmann Coulter, Inc. Brea, CA).

Determination of anti-oxLDL Abs

To determine the antibodies of IgG type against oxidized LDL (anti-oxLDL Abs), we used a previously described method (Fernvik et al., 2004). A 96-well ELISA plate was coated with 50 μl of the copper-induced oxidized LDL [7.5 μg/ml per well] in 0.1 mol/l carbonate/bicarbonate buffer (pH 9.6) and left overnight at 4 °C. After washing with PBS, the plate was blocked with 3% gelatin at room temperature for 24 h. Patients' serum samples (50 μl) were diluted 1:400 before addition to the wells. After 2-h incubation, the plate was washed with PBS containing 0.05% Tween, and peroxidase-conjugated goat anti-human IgG (dilution 1:1000 — Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. After washing, tetra-methyl-benzidine (250 μl 3,3′5,5′ 6.5% in DMSO), plus H_2O_2 in citrate phosphate buffer, (0.1 mol/l, pH 5.5) were added as substrate. The reaction was stopped by the addition of 2 mol/l H_2SO_4 and measured at 450 nm in optical density (OD).

To improve the quantification of anti-oxLDL Abs by the ELISA method, due to intra-plate variation, we used a control with a manufactured IgG (purified human IgG – 10 mg/ml – Pierce Protein Research Products, Thermo Scientific, Rockford, IL) and a buffer blank (PBS). All the samples were processed in the same period of time, at the end of the clinical protocol. Antibody titers were expressed as the Index of Reactivity (IR) for each sample and calculated as follows: IR = (sample OD − blank OD) / (control IgG − blank OD), in order to minimize the possible detection of false positive values due to cross-reactivity with antigen naive epitopes. Inter-assay reproducibility was assessed and Pearson's correlation coefficient for this experiment was 0.889 ($p < 0.001$). All samples were run in triplicate and an average of the three obtained values was calculated.

Detection of electronegative LDL in plasma

The concentrations of LDL(−) in plasma were determined by ELISA using a human monoclonal antibody (mAb3D1036) anti-LDL(−) produced in our laboratory. The mAb3D1036 recognizes an epitope formed due to slight loss of the apo-B100 secondary structure on minimally modified LDL particles (Damasceno et al., 2006). Briefly, the microplates were coated with 50 μl mAb3D1036 (1 μg/well) in a carbonate–bicarbonate buffer (pH 9.4, 0.1 M) and incubated overnight at 4 °C. Then, the microplate was washed three times with a PBS buffer containing Tween 20 (0.05%) and blocked with 2% non-fat dry milk for 24 h at 37 °C. Plasma diluted in PBS containing 1% non-fat milk and 0.01% Tween 20 was added to the plates and incubated for 1.5 h at 37 °C. The plates were washed and incubated with the anti-LDL(−) monoclonal antibody biotinylated for 2 h at 37 °C. After washing, the microplates were incubated with streptavidin–HRP conjugate (Southern Biotech, Birmingham, USA) for 1 h at 37 °C then, the washed plates were incubated with 3,3′,5,5′ tetramethylbenzidine (TMB, Sigma Chemical Co, St. Louis, MO) for 10 min at 37 °C. The reaction was stopped by adding 2 M sulfuric acid, and the absorbance at 450 nm was measured by spectrophotometry. All samples and standards were run in triplicate. The calibration curve was made with LDL(−) obtained from human plasma as previously described (Sevanian et al., 1999).

Table 1
Baseline characteristics and treatment effects, according to treatment arm.

	Ezetimibe 10 mg/simvastatin 40 mg (E10/S40) (n = 55)		p post vs pre	Rosuvastatin 40 mg (R40) (n = 57)		p post vs pre
	Pre-treatment	Post-treatment		Pre-treatment	Post-treatment	
SBP (mm Hg)	130 (2)	126 (2)	0.092	130 (2)	126 (2)	0.080
DBP (mm Hg)	78 (1)	77 (1)	0.348	78 (1)	77 (1)	0.460
Glucose (mg/dl)*	107 (5)	106 (4)	0.967	101 (2)	100 (2)	0.657
Hb1Ac (%)*	5.7 (0.1)	5.8 (0.1)	0.083	5.5 (0.1)	5.6 (0.1)	0.608
Total cholesterol (mg/dl)	243 (6)	145 (4)	< 0.001	251 (6)	143 (4)	<0.001
HDL-c (mg/dl)	53 (2)	52 (2)	0.555	54 (2)	53 (2)	0.306
LDL-c (mg/dl)*	160 (6)	70(3)	<0.001	161 (5)	66 (3)	<0.001
TG (mg/dl)*	151 (9)	109 (6)	<0.001	175 (11)	117 (6)	<0.001
Apo A (mg/dl)*	143 (3)	147 (4)	0.064	150 (4)	153 (4)	0.169
Apo B (mg/dl)	134 (4)	68 (3)	<0.001	134 (4)	67 (3)	<0.001
Apo B/Apo A ratio	0.95 (0.03)	0.46 (0.02)	<0.001	0.92 (0.03)	0.45 (0.02)	<0.001
hsCRP (mg/l)*	3.1 (0.3)	1.8 (0.3)	<0.001	3.2 (0.4)	1.2 (0.3)	<0.001

Data are expressed as mean (SEM). HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, triglycerides; and Apo, apolipoprotein. There were no differences between groups at baseline and 12 weeks.

* Log-transformed variables.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software version 17.0 was used for all analyses.

For normally distributed data (skewness and kurtosis between −2.0 to 2.0) the paired or the independent samples *t*-test was used. For non-normally distributed variables, data were log transformed. For categorical variables Chi-square test or Fisher's exact test was performed. Data presented in the text and tables are mean values (SEM). Associations were calculated by Pearson's correlation test. Probability value under 0.05 was considered significant.

Results

The two treatments were equally effective on lipid parameters. Twelve-week treatment with lipid-lowering agents (rosuvastatin or ezetimibe/simvastatin) was associated with comparable decrease in total cholesterol (42% vs 38%), LDL-cholesterol (58% vs 53%) and triglycerides (28% vs 32%), with no changes in HDL-cholesterol levels (Table 1). In addition, apolipoprotein B levels decreased similarly (49% vs 47%), without changes on apolipoprotein A1 (1% vs 3%) when compared to baseline.

Highly-sensitive C-reactive protein serum levels were elevated at baseline and decreased similarly between treatment groups (41% vs 44%) for rosuvastatin and simvastatin/ezetimibe, respectively.

Immune responses

Antibodies against oxidized LDL (anti-oxLDL Abs) and electronegative LDL [LDL(−)]

No differences between groups were observed at baseline for the titers of anti-oxLDL Abs. However, a significant raise in anti-oxLDL Abs levels was observed after the treatment with rosuvastatin, but not after the simvastatin/ezetimibe therapy (Fig. 1).

Conversely, the LDL(−) titers were comparable at baseline between the two groups and were not modified after treatments (Fig. 2).

Discussion

The treatment with two intensive pharmacologic strategies to reduce the cholesterol to similar levels showed different patterns of oxidative responses. In the rosuvastatin group the observed increase of anti-oxLDL antibodies after the treatment has been previously reported by our group, as a consequence of a decrease in immune complexes formation probably related to lower consumption of antibodies following the decrease in LDL particles (Santos et al., 2009; Izar et al., 2013). This scenario has been related to clinical stability and lower severity of coronary atherosclerosis, suggesting a decrease in the deposition of immune complexes in the intima of the arteries. Indeed, increased titers of anti-oxLDL Abs has been described in healthy subjects as well as after hypertension control (Garrido-Sanchez et al., 2009; Brandão et al., 2010).

The adaptive immune system has a more specificity to antibody response and seems to have a crucial role for decrease of atherosclerosis through the production of antibodies by the B-cells (Perry et al., 2012, 2013). The dynamic formation of immune complexes is rapidly up regulated via either antigenic-specific stimulation of memory cells or recruitment of virgin B-cells as in the case of a primary response (Libby et al., 2009). Thus, the immune responses produced by subtypes of B cells seem to protect against atherosclerosis and impairment of these responses may increase T lymphocytes recruitment and plaque development (Perry et al., 2013; Libby et al., 2009, 2011).

Electronegative LDL has been associated with the severity and extent of angiographic evidence of coronary artery disease (Niccoli et al., 2012). However, its clinical significance is still not clear, appearing to be related to risk factors and inflammation (Mello et al., 2011). It is considered that only mildly modified LDL particles can be altered by statins (Zhang et al., 2008, 2009). Thus, the measurements of electronegative LDL and antibodies against oxidized LDL can be considered complementary information: the former involving characteristics of the particle and the latter the immune responses related to specific epitopes of apolipoprotein B.

Despite marked differences in some pleiotropic effects, these two lipid-lowering strategies have been reported as equally effective on decrement of CRP levels (Morrone et al., 2012). Statins decrease isoprenoids formation reducing the activation of small proteins related to inflammation transcription genes (Liu et al., 2009). Conversely, the

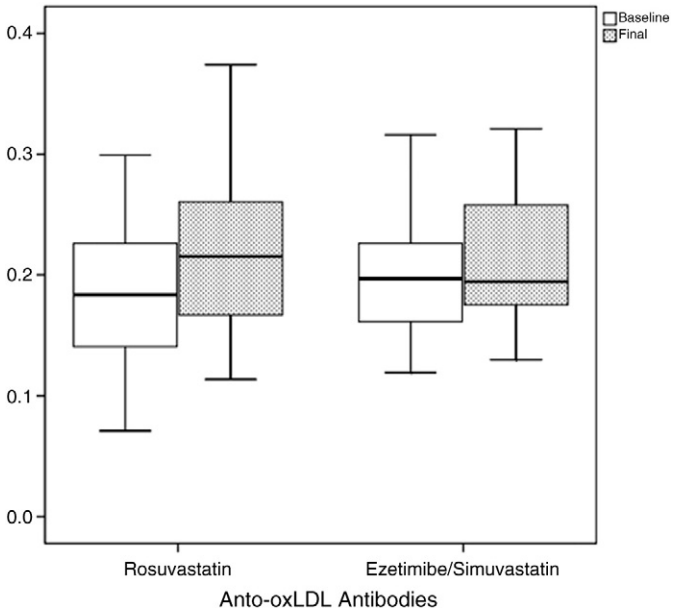

Fig. 1. Box plots showing medians and 25th and 75th percentiles for antibodies against oxidized LDL(anti-oxLDL) at baseline and after 12 weeks in both groups. For rosuvastatin group there was an increase in anti-oxLDL Abs titers ($p = 0.026$); and for ezetimibe/simvastatin group there were no changes in anti-oxLDL Abs titers ($p = 0.233$).

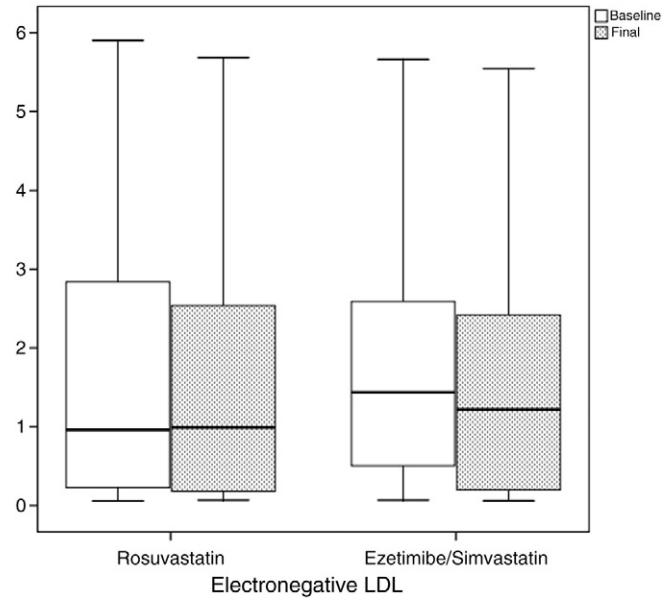

Fig. 2. Box plots showing medians and 25th and 75th percentiles for electronegative LDL [LDL(−)] at baseline and after 12 weeks in both groups. There were no differences between groups at baseline and no changes in anti-oxLDL Abs titers during the study. For rosuvastatin group ($p = 0.248$) and for ezetimibe/simvastatin group ($p = 0.109$) there were no changes in LDL(−) levels.

reduction on CRP levels observed with ezetimibe seems dependent on cholesterol reduction and occurs only when combined with statins. In fact, by blocking intestinal cholesterol absorption, there is an increment on cholesterol synthesis, an effect counterbalanced by the concomitant use of statins (Barbosa et al., 2013).

The absence of HDL-C increase in our study can be expected due to the relatively high HDL-C levels at baseline. In fact, in the large JUPITER trial, also showing normal baseline levels of HDL-C, only modest increase was observed (Ridker et al., 2008). Interestingly, recent analysis of the effects of rosuvastatin in this trial revealed changes in some characteristics of HDL particles, and in particular, the HDL-particle number was better related to cardiovascular outcomes than the chemical determinations of HDL-C or apoA1 (Mora et al., 2013).

The electronegative LDL particle has been characterized as a modified LDL particle enriched with hydroperoxides (Sevanian et al., 1997) and the dense LDL particles detected in plasma seem particularly prone to oxidation (Sevanian et al., 1996). The relationship between plasma oxidized LDL and macrophage-rich atherosclerotic plaques was reported by Nishi et al. (2002). Taken together, these studies suggest that the oxidation of LDL particles starts in the blood and undergoes further oxidation in the intima. More recently, a very interesting study showed that B1 lymphocytes have crucial role in atherosclerosis by producing, natural antibodies (IgM) against oxidized LDL. Thus, these observations suggest that part of the LDL oxidation occurs in the plasma, with the degree of immune responses (innate and adaptive) apparently related to plaque development (Perry et al., 2013; Libby et al., 2009, 2011; Grundtman and Wick, 2011).

Differences in the immune responses can be related to the pattern of LDL particles. On this regard, Berneis et al. (2010) reported that ezetimibe use (alone or combined with simvastatin), in healthy subjects, increased the more atherogenic small and dense LDL particles. This finding can be related to differences in the immune responses, although the studies in this area are still controversial (Winkler et al., 2012; Rizzo et al., 2009).

Limitations and strengths of the study

Our study reported differences in the immune responses following two lipid-lowering regimens with the same efficacy on LDL-C reduction. These differences in the immune response are interesting and possibly relevant, but the mechanisms and subpopulations of lymphocytes involved, phenotype of lipoproteins, and impact in the development of atherosclerosis were not addressed in this paper.

Conclusion

Strategies with two equally effective lipid lowering regimens can differently alter the adaptive immune response to oxidized LDL, despite similar findings for plasma electronegative LDL.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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