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High LDL levels are associated with increased lipoprotein-associated phospholipase A_2 activity on nitric oxide synthesis and reactive oxygen species formation in human endothelial cells

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ABSTRACT

Objective: To evaluate *in vitro* the effects of serum and LDL fractions isolated from hypercholesterolemic patients on nitric oxide (NO) synthesis and reactive oxygen species (ROS) production by human umbilical vein endothelial cells (HUVECs).

Design and methods: Serum and LDL isolated from subjects with high ($n=18$) and normal ($n=21$) LDL-cholesterol levels were analyzed on NO synthesis and ROS production *in vitro* models of HUVECs. LDL was furthers characterized in their chemical composition and activities of lipoprotein-associated phospholipase A_2 (Lp-PLA_2), cholesteryl ester transfer protein (CETP) and paraoxonase.

Results: NO bioavailability was significantly lower and ROS production higher in HUVECs incubated with serum samples from patients with high LDL-cholesterol levels in comparison to control subjects. Moreover, hypercholesterolemic patients presented higher CETP and Lp-PLA_2 activities than control subjects. LDL fractions isolated from patients and controls were not different in their chemical composition, Lp-PLA_2 activity, and their capacity to reduce NO synthesis and increase ROS production.

Conclusion: Alterations of serum from hypercholesterolemic patients could be due to the increment in LDL concentration, main Lp-PLA_2 carrier, and not to LDL composition or intrinsic Lp-PLA_2 activity.

Introduction

Atherosclerosis is a clinical condition frequently associated to high plasma levels of low-density lipoprotein-cholesterol (LDL-C), which is considered one of the major risk factors for endothelial dysfunction and subsequent cardiovascular disease [1,2]. This fact is supported by the high incidence of premature atherosclerosis observed in patients with familial hypercholesterolemia who show extremely high LDL-C levels [3–5]. Moreover, it has been reported that hypercholesterolemic patients do not efficiently respond to vasodilator agents, suggesting the presence of endothelial dysfunction [6–9]. Therefore, the effect exerted by high LDL-C levels on endothelial functions is clearly a key event in endothelial dysfunction and the early stages of atherosclerosis [10–12].

The mechanisms by which oxidized LDL (oxLDL) particles could induce endothelial dysfunction would include: a) promotion of cell adhesion molecule expression, which would facilitate monocyte infiltration into the intima [13–15], b) induction of vascular endothelial cell, smooth muscle cell and macrophage apoptosis and necrosis, c) reduction of nitric oxide (NO) synthesis, and d) increase of reactive oxygen species (ROS) formation. ROS could cause deleterious effects on DNA, membrane lipids and proteins and decrease NO bioavailability [13–16] and also ROS inhibit both NO and endothelium-derived hyperpolarizing factor (EDHF) release [17]. However, although high levels of ROS have deleterious effects on endothelial cell (apoptosis and death) [18] low levels of ROS play an important role in regulating ischemic process promoting proliferation and migration of endothelial cells essentials for angiogenesis process [19].

Furthermore, studies carried out *in vivo* have demonstrated that hypercholesterolemic and oxLDL induce endothelial dysfunction, as shown by high levels of soluble intracellular adhesion molecule-1 (sICAM-1), soluble E-selectin [20] oxidative stress markers [21] and high activity of lipoprotein-associated phospholipase A_2 (Lp-PLA_2) [22] and cholesteryl ester transfer protein (CEPT) [23].

Lp-PLA_2 is produced by macrophages, T lymphocytes, and mast cells, it circulates bound to low-density lipoprotein (LDL) or high-density lipoprotein (HDL). Lp-PLA_2 increases the circulating levels of

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oxidized free fatty acids and lysophosphatidylcholine and can then have adverse effects on endothelial function due an increase of ROS [24,25]. Several studies have shown that elevated Lp-PLA_2 levels are associated with an increased risk of endothelial dysfunction and cardiovascular risk [22,24–26].

Cholesteryl ester transfer protein (CETP) is a plasma glycoprotein that interchanging triglycerides and cholesteryl esters between lipoproteins. An increase in CETP activity leads to the formation of LDL triglyceride-enriched more small and dense and susceptible to undergo oxidation [27,28]. High CETP activity has been related to atherosclerosis in several clinical studies and plays an important role in atherogenesis by modifying the arterial intima cholesterol content [27–30].

However, up to our knowledge, no studies have evaluated the effects of whole serum or LDL fraction from hypercholesterolemic patients on NO synthesis or ROS formation *in vitro* models of endothelial cells and its association with CETP and Lp-PLA_2 activity. Therefore, the aim of the present study was to explore if serum and/or LDL fraction isolated from patients with high LDL-C levels (>160 mg/dL) can affect NO synthesis and/or ROS formation in HUVECs and its correlation to traditional and novel atherogenic risk factors such as lipid profile, LDL chemical composition, CETP and Lp-PLA_2 activity and paraoxonase (PON) levels.

Methods

Subjects

In a cross-sectional study, we evaluated a cohort of 39 subjects who were consecutively recruited during a period of about 6 months from Institute of Cardiology and Cardiovascular Surgery of the Favaloro Foundation, Buenos Aires, Argentina. Subjects were included in the present study when satisfying the following criteria: 1) age between 20 and 75 years old, 2) triglyceride levels<400 mg/dL, 3) lack of diabetes evidenced by at least 2 determinations of fasting glucose levels; 4) normal thyroid function evaluated by plasma levels of thyroid-stimulating hormone and clinical examination of the thyroid gland; 4) normal renal function evaluated by plasma levels of urea, creatinine and proteinuria; and 5) normal hepatic function evaluated by different biochemical hepatic parameters and absence of hepatomegalia confirmed by clinical examination. Special care was taken to avoid including subjects with additional causes of dyslipidemia such as excessive tobacco (>10 cigarettes/day) or ethanol consumption (>30 g/day), therapy with drugs that could affect lipoprotein or carbohydrate metabolism and familial history of diabetes mellitus. Individuals presenting infectious processes or under acute stressing situations were not included. Subjects were classified into two groups according to the ATPIII definition [31]: hypercholesterolemic patients (LDL-C levels>160 mg/dL) ($n=18$) and control subjects (LDL-C levels<160 mg/dL) ($n=21$). Informed consent was signed from all participants and the protocol was approved by the Ethical Committees from School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina and Faculty of Pharmacy, University of Concepción, Concepción, Chile.

Study protocol and samples

After a 12-hour overnight fast, venous blood was drawn from the antecubital vein. Samples were centrifuged at 1500 × *g* for 15 min at 4 °C and serum was stored at 4 °C to be used within 24 h for glucose and lipid profile evaluation as well as for lipoprotein isolation by ultracentrifugation. Isolated LDL fractions were immediately employed for lipid and protein characterization and they were further sterilized and stored at 4 °C until they were used in functional assays. Serum aliquots were also stored at −70 °C for the determination following of cholesteryl ester transfer protein (CETP), paraoxonase (PON) 1, arylesterase (ARE), and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) activities; sICAM-1 and sE-selectin levels, and serum capacity to modulate NO synthesis and ROS production by HUVECs.

Analytical procedures

Glucose, triglycerides and total cholesterol were quantified by standardized methods (Roche Diagnostics, Mannheim, Germany) in Hitachi 917 autoanalyzer. Within-run precision (CV) for triglycerides and total cholesterol was 1.3% and 1.1%, respectively. Between-day precision (CV) was 2.4% and 1.5%, respectively. Laboratory bias was 1.1% and −1.7%, respectively. Low-density lipoprotein-cholesterol (LDL-C) level was determined as the difference between total cholesterol and the cholesterol contained in the supernatant obtained after selective precipitation of LDL with 10 g/L polyvinylsulphate in polyetilenglycol (M.W. 600; 2.5% w/v; pH = 6.7) [32]. Within-run and between-day precision (CV) was 4.7% and 5.0%, respectively. HDL was isolated from the supernatant obtained following precipitation of apolipoprotein (apo) B-containing lipoproteins with 0.44 mmol/L phosphotungstic acid in the presence of magnesium ions [33]. Within-run and between-day precision (CV) for HDL-C were 3.2% and 3.8%, respectively and laboratory bias was −2.0%. Very low-density lipoprotein-cholesterol (VLDL-C) was calculated as the difference between total cholesterol and the cholesterol contained in LDL and HDL measurements. Quality control was performed by RIQA Program (Ireland). In the isolated LDL fractions, triglycerides and cholesterol were measured by the methods described above by the Bartlett method [34], and proteins by the Lowry method [35]. Total CV for phospholipid determination was 3.1%. Within-run and between-day precision (CV) for protein measurement were 3.0% and 4.2%, respectively. Lipoprotein mass was estimated as the summatory of all components (triglycerides, cholesterol, phospholipids and total proteins) and then the percentage was calculated. The following ratios were calculated: total cholesterol/HDL-C, LDL-C/HDL-C, and triglycerides/HDL-C.

Lipoprotein isolation

LDL (d: 1.019–1.063 g/mL) were isolated from total plasma by sequential preparative ultracentrifugation [36] in a XL-90 Beckman ultracentrifuge, with a type 90 Ti rotor, at 105 000 × *g*, for 18 h, at 4 °C. LDL purity was tested by as agarose gel electrophoresis and albumin content was tested by inmunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Albumin content was less than 2% of total protein.

CETP activity

CETP activity was determined in serum from control and hypercholesterolemic patients following a general procedure [37] with few modifications. Briefly, CETP activity was assessed by the ability of serum to promote the transfer of tritiated cholesteryl esters from biosynthetically labeled HDL_3 (^{3}H-CE-HDL_3) (NEN Life Science Products, Boston, USA) towards serum apo B-containing lipoproteins. Samples were incubated with ^{3}H-CE-HDL_3 (50 μmol/L cholesterol) and 1.5 mmol/L iodoacetate for 3 h, at 37 °C. After incubations, lipoproteins were separated by a selective precipitation with phosphotungstic acid (0.44 mmol/L) [33]. Radioactivity was measured in the precipitates and in the supernatant in a Liquid Scintillation Analyzer (Packard 210TR; Packard Instruments, Meridian, CT). Results were expressed as percentage of ^{3}H-cholesteryl esters transferred from HDL_3 to apo B-containing lipoproteins per mL per hour. Measurements were carried out in duplicate within the same assay. Within-run precision (CV) was 4.9%.

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Paraoxonase 1 (PON)/arylesterase (ARE) activity

The enzyme PON was evaluated employing two different substrates: paraoxon (Sigma Chemical Co.; PON1 activity) and phenylacetate (Sigma Chemical Co.; ARE activity). Both activities were measured in serum samples following the method of Furlong et al. [38].

PON1 activity was assessed by adding serum samples (20 μL) to 2 mL Tris/HCl buffer (100 mmol/L, pH = 8.0) containing 2 mmol/L $CaCl_2$, 2.6 mmol/L paraoxon (O-diethyl-O-p-nitrophenylphosphate) and 1.0 mol/L NaCl. The rate p-nitrophenol generation was determined at 405 nm at 25 °C, in a Hitachi U-1100 spectrophotometer. Increases in the optical densities (OD) were recorded at 45-s intervals during 5 min, after 30 s of initial pre-incubation. Enzymatic activity was calculated from the molar extinction coefficient (17,000 mol/l/cm) and results were expressed as nmol/mLmin. Measurements were carried out within the same assay. Within-run precision (CV) was 5.5%.

ARE activity was measured by adding serum samples (20 μL of 1/20 dilution in distilled water) to 2 mL Tris/Acetate buffer (50 mmol/L, pH = 7.8) containing 20 mmol/L $CaCl_2$ and 4.4 mmol/L phenylacetate. The rate of phenol generation was determined at 270 nm at 25 °C, in a Hitachi U-1100 spectrophotometer. Increases in the OD were recorded at 30 s intervals during 5 min, after 30 s of initial pre-incubation. Blanks were included to correct the spontaneous hydrolysis of phenylacetate. Enzymatic activity was calculated from the molar extinction coefficient (1310 mol/L/cm) and results were expressed as μmol/mL min. Measurements were carried out within the same assay. Within-run precision (CV) was 4.8%.

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2)

Lp-PLA_2 activity was measured following the radiometric assay described by Blank et al. [39] with few modifications. Briefly, each incubation mixture contained 50 μL of 1/50 diluted serum or LDL fraction and 10 μmol/L 1-hexadecyl-2-[^{3}H]acetyl-glicero-3-phosphocholine (New England Nucleotides, Specific Activity = 25 μCi/μmol) in a total volume of 0.5 mL of phosphate-buffered saline (pH = 7.4) and non-tritiated (Cayman Chemical). Once the substrates were mixed, solvents were evaporated under N_2 flow and sample was redissolved in phosphate-buffered saline, with a sonication step of 5 min. The enzymatic reactions were carried out for 5 min at 37 °C and stopped the addition of 1.5 mL of chloroform in an ice bath. Then, 0.5 mL of a saturated sodium bicarbonate solution was added. After centrifugation, the aqueous phase was washed twice with 1.5 mL of chloroform and the radioactivity of the aqueous phase sample and blanks was measured by liquid scintillation using a Liquid Scintillation Analyzer (Packard 210TR; Packard Instruments, Meridian, CT). Radioactivity of the substrate-buffer was also measured. Results were expressed as μmol/mLh. Measurements were carried out within the same assay. Within-run precision (CV) for Lp-PLA_2 activity was 5.1%.

Human umbilical vein endothelial cell culture (HUVECs)

HUVECs were isolated from freshly acquired human umbilical veins by digestion with collagenase (0.25 mg/mL), as described [40,41]. Informed consent was provided according to the Declaration of Helsinki [42]. HUVECs were cultured (37 °C, 5% CO_2) in 199 medium containing 5 mmol/L D-glucose, 10% (v/v) new born calf serum, 10% (v/v) foetal calf serum, 3.2 mmol/L L-glutamine, 100 μmol/L L-arginine, and 100 U/mL penicillin–streptomycin. Cells were grown to confluence (75–100%) and washed with phosphate buffer saline (PBS), to be were cultured for 12 h with 199 medium containing 1% (v/v) new born calf serum. Then, cells were incubated in serum-free 199 medium for 16 h with serum or LDL fractions (50 μg/mL) from hypercholesterolemic patients or control subjects.

NO synthesis

HUVECs were cultured in 96-well plates (10.000 cells/well), until subconfluency. Then, they cells were incubated for 16 h with total serum or LDL fractions (50 μg/mL) and 199 medium containing 4,5-diaminofluorescein diacetate (DAF-2DA, 1 μM, 30 min), a membrane permeable dye that reacts with NO to produce a fluorescent stable compound inside the cells. Fluorescence was measured in histamine stimulated HUVECs in a fluorescence spectrometer (Sinergy 2, Viotec, l_{ex}:495, l_{em}:510). Results were expressed as units of DAF-2DA fluorescence per cell protein content.

ROS formation

ROS formation was assessed using the probe 2,7-dichlorofluorescein diacetate (DCF) (Calbiochem) probe. HUVECs were exposed to whole serum or LDL fractions (50 μg/mL) for 16 h. Then, cells were washed with PBS and loaded with 100 μM DCF for 30 min at 37 °C in 199 medium. ROS formation was measured by evaluating DCF emission at 510 nm (excitation 495 nm) in a fluorescence spectrometer (Sinergy 2, Viotec). Results were expressed as units of DCF fluorescence per cell protein content. When required, inhibitors of ROS formation (vitamin C) were added 1 h before HUVEC exposure to serum or LDL fractions.

Statistical analyses

Statistical analyses were performed with Graph Pad Prism (GraphPad, San Diego, CA) and result were expressed as mean ± SD. Comparisons between groups were carried out by one-way ANOVA Test. Differences were considered significant at $P<0.05$ in the bilateral situation. Correlations between all variables were carried out by Pearson test.

Results

Clinical and biochemical characteristics of hypercholesterolemic patients and control subjects

As shown in Table 1, anthropometric and metabolic parameters are similar in hypercholesterolemic patients and control subjects, except for total cholesterol, triglyceride and LDL-C which were significantly higher in hypercholesterolemic patients. In accord with these differences, ratios indicating atherogenic risk (total cholesterol/HDL-C and LDL-C/HDL-C), as well as triglycerides/HDL-C, an indicator

Table 1
Clinical and biochemical characteristics from control subjects and hypercholesterolemic patients.

	Control subjects ($n=21$)	Hypercholesterolemic patients ($n=18$)	P
Age (years)	56 ± 13	57 ± 8	NS
Women/men	12/9	12/6	NS
BMI (kg/m^2)	27 ± 6	26 ± 4	NS
Waist circumference (cm)	95 ± 19	88 ± 13	NS
Glucose (mg/dL)	80 ± 9	82 ± 9	NS
TC (mg/dL)	200 ± 23	290 ± 44	0.0001
TG (mg/dL)	97 ± 52	150 ± 52	0.001
VLDL-C (mg/dL)	20 ± 7	23 ± 7	NS
LDL-C (mg/dL)	127 ± 24	217 ± 41	0.0001
HDL-C (mg/dL)	52 ± 13	49 ± 13	NS
TC/HDL-C	4.0 ± 1.1	6.1 ± 1.4	0.0001
LDL-C/HDL-C	2.6 ± 0.2	4.5 ± 0.3	0.0001
TG/HDL-C	2.0 ± 1.7	3.3 ± 1.5	0.001

Data are expressed as mean ± SD. TC, total cholesterol; TG, triglycerides; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NS, not significant.

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of insulin resistance and of the proportion of small and dense LDL particles, were significantly increased in hypercholesterolemic patients than in controls.

On the other hand endothelial dysfunctions markers, such as soluble cell adhesion molecules, sVCAM-1 (57 ± 44 vs. 44 ± 34 ng/mL, patients and controls, respectively) and sE-selectin (61 ± 24 vs. 62 ± 21 ng/mL, patients and controls, respectively) were also evaluated, showing no statistical difference between both groups.

Table 2 shows CETP and Lp-PLA_2 activities, which were significantly higher in hypercholesterolemic patients than in control subjects. In contrast, no significant differences were detected in PON1 and ARE activities between both groups (Table 2).

Effect of human serum or isolated LDL fraction on NO production by HUVECs

The effect of human serum on NO and ROS production was tested in cultured HUVECs during 16 h-incubations. Fig. 1A shows that when HUVECs were previously stimulated with histamine and then exposed to sera from hypercholesterolemic patients or from control subjects NO synthesis diminished significantly, being this decrease more significant for the hypercholesterolemic group (10% and 40%, respectively). As a control, L-NAME, known to inhibit eNOS, significantly reduced the histamine-induced NO production in HUVEC ($P<0.001$).

To evaluate the effect of isolated LDL fraction on NO, HUVECs were treated for 16 h with LDL obtained from hypercholesterolemic patients or control subjects (Fig. 1B). Both LDL fractions significantly decreased NO synthesis reaching a 50% of inhibition when compared to cells incubated only with histamine. This inhibition mimicked the effect L-NAME on NO production (Fig. 1B).

Effect of human serum or isolated LDL fraction on ROS production by HUVECs

As shown in Fig. 2A, ROS production was significantly higher in HUVECs exposed to human sera, being more important when incubated with serum from hypercholesterolemic patients than from control subjects (5- and 2.5-fold, respectively) (Fig. 2A). Vitamin C, a well-known free radical scavenger, efficiently blocked the effect of both hyper and normocholesterolemic sera on ROS synthesis ($P<0.05$ for both of them) (Fig. 2A).

On the other hand, ROS production was significantly increased by LDL fractions isolated from both hypercholesterolemic patients and control subjects in comparison to basal ROS synthesis ($P<0.01$ for both of them) (Fig. 2B). In both conditions, vitamin C inhibited ROS synthesis by HUVECs ($P<0.01$ and $P<0.05$ in control and hypercholesterolemics LDL fractions, respectively).

Correlation analyses were performed between NO and ROS determinations and the different clinical and biochemical parameters previously shown. Interestingly, LDL-C levels were inversely associated with NO synthesis ($r_s = -0.39$, $P<0.01$) and directly related with ROS production ($r_s = 0.35$, $P<0.02$) (Fig. 3A and B, respectively). Furthermore, CETP and Lp-PLA_2 enzymatic activities were inversely

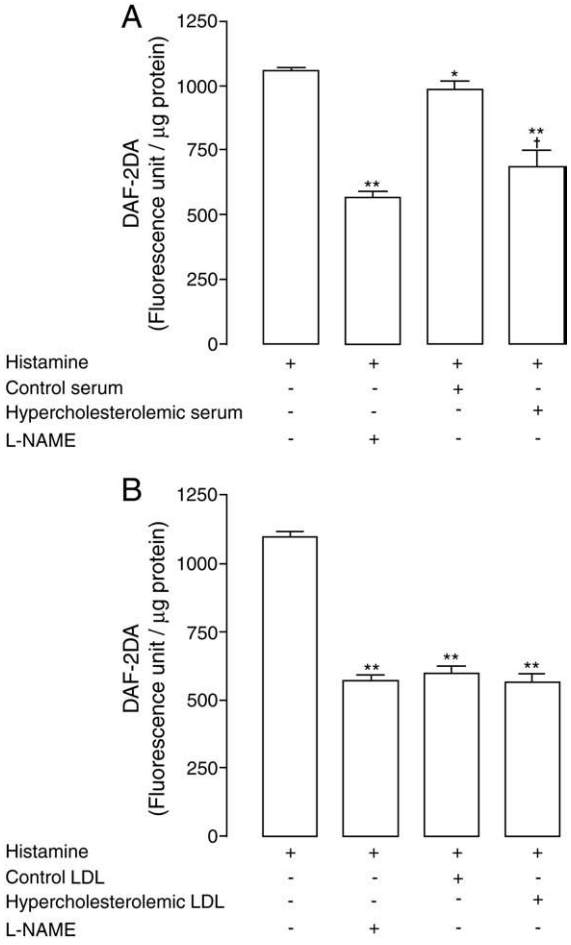

Fig. 1. Effect of human serum and isolated LDL on NO synthesis by HUVECs. Serum-starved HUVECs were incubated with human serum (A) or isolated LDL (B) from hypercholesteromic patients or control subjects during 16 h. NO was determinated using 4,5-diaminofluorescein diacetate (DAF-2DA, 1 µM, 30 min) as described in Methods in human umbilical vein endothelial cells. *$P<0.05$ and **$P<0.01$ vs. HUVECs only treated with histamine; †$P<0.05$ serum from hypercholesterolemic patients vs. serum from control subjects.

associated to NO synthesis ($r_s = -0.48$, $P<0.005$ and $r_s = -0.26$, $P<0.05$, respectively) and directly associated to LDL-C levels ($r_s = 0.35$, $P<0.01$ and $r_s = 0.47$, $P<0.005$, respectively).

LDL composition

LDL fractions isolated from hypercholesterolemic and control subjects were characterized in terms of their chemical composition (cholesterol, triglycerides, proteins and phospholipids). These analyses revealed no differences between both groups (Fig. 4). Moreover, no statistical differences were detected in Lp-PLA_2 activities measured in both LDL fractions hypercholesterolemic and control (9.6 ± 5.4 vs. 8.7 ± 4.5 µmol/mg LDL protein, patients and controls, respectively).

Table 2
Lipoprotein-associated transfer proteins and enzymes from control subjects and hypercholesterolemic patients.

	Control subjects ($n=21$)	Hypercholesterolemic patients ($n=18$)	P
CETP (% trans./mLh)	234 ± 53	293 ± 75	0.01
Lp-PLA_2 (µmol/mLh)	11.2 ± 2.7	15.0 ± 3.4	0.0005
PON1 (nmol/mLmin)	382 ± 249	444 ± 316	NS
ARE (µmol/mLmin)	151 ± 38	157 ± 48	NS

Data are expressed as mean ± SD. CETP, cholesteryl ester transfer protein; Lp-PLA_2, lipoprotein-associated phospholipase A_2; PON, paraoxonase; ARE, arylesterase; NS, not significant.

Discussion

Atherosclerosis is a multifactorial disease that is triggered by numerous cardiovascular risk factors including smoking, hypertension and hypercholesterolemia, all associated with impaired endothelial function. Evaluation of endothelial dysfunction would be a useful tool to detect subclinical atherosclerosis. Thus, the use of human endothelial cells culture systems is pivotal as an experimental model for clinical analysis [12,43].

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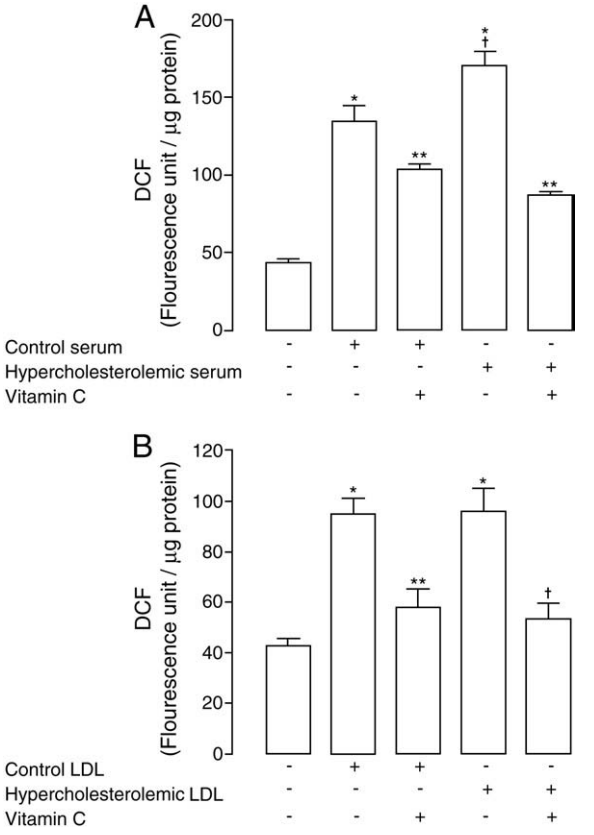

Fig. 2. Effect of human serum and isolated LDL on ROS production by HUVECs. Serum-starved HUVECs were incubated with human serum (A) or isolated LDL (B) from hypercholesteromic patients or control subjects during 16 h. Reactive oxygen synthesis was determinated using 2,7-dichlorofluorescein diacetate (DCF, 100 µM). In panel A, *P<0.05 vs. non-treated HUVECs; †P<0.05 vs. serum from control subjects; **P<0.001 vs. HUVECs cultured with serum and without vitamin C. In panel B, *P<0.001 vs. non-treated HUVECs; **P<0.01 and †P<0.05 vs. HUVECs cultured with serum and without vitamin C.

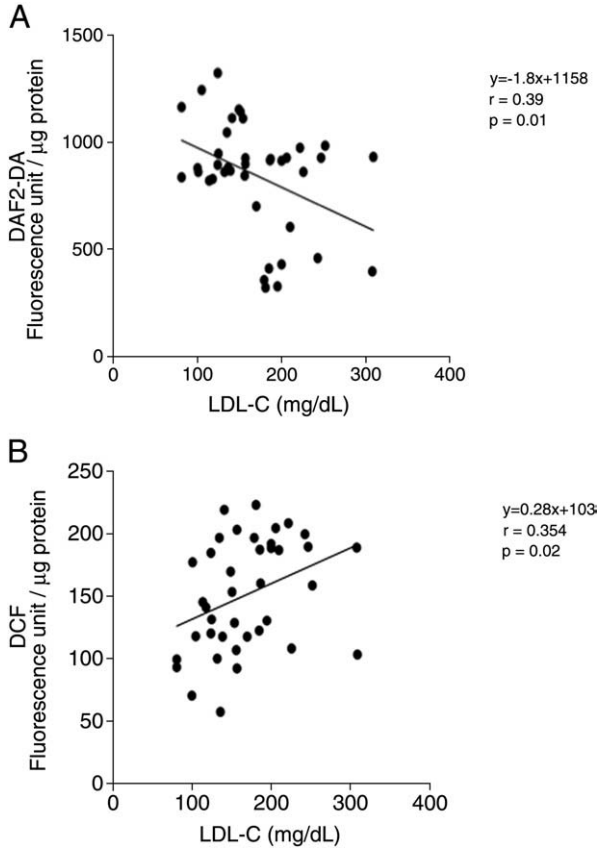

Fig. 3. Association of LDL-cholesterol with NO synthesis (A) and ROS formation (B) by HUVECs. LDL-C, low-density lipoprotein-cholesterol; NO, nitric oxide; ROS, reactive oxygen species; HUVECs, human umbilical vein endothelial cells.

Using *in vitro* culture of HUVECs, we have shown that: i) serum samples from hypercholesterolemic patients were more efficient in reducing NO synthesis and increasing ROS formation than normolipemic sera, ii) isolated LDL fractions from both hypercholesterolemic patients and control subjects showed similar behaviour with respect to NO synthesis and no statistically significant differences were detected in LDL composition and Lp-PLA_2 activities, iii) both NO synthesis and ROS formation were significantly associated to total cholesterol and LDL-C levels, iv) NO synthesis was inversely associated to serum Lp-PLA_2 and CETP activity, and v) LDL-C levels were positively associated to serum Lp-PLA_2 and CETP activity.

Our findings are consistent with other studies, suggesting that alterations in NO pathway, specially associated to high ROS formation, play a central role in endothelial dysfunction induced by hypercholesterolemia [14,44]. In fact, several *in vivo* and *in vitro* studies evidenced that high LDL-C levels induce endothelial dysfunction [45–47], though the exact mechanisms underlying this impairment have not been fully elucidated.

When the effect of whole serum was tested on HUVECs, we detect a decrease in NO synthesis and the increase in ROS formation. This effect could be attributed to several factors inherent to hypercholesterolemic sera, which could confer these samples some properties for endothelial dysfunction in patients with high LDL-C levels, such as LDL concentration, LDL chemical composition, LDL modification and subpopulation (oxidized LDL, and small dense LDL particles), lipoprotein-associated transfer proteins and enzymes, such as CETP and Lp-PLA_2, among others. Regarding Lp-PLA_2, which is mainly transported by LDL particles, this enzyme has been proven to act exclusively on oxidized phospholipids, thus releasing oxidized fatty acids and lysophophatidylcholine [22,25,26]. The fact that previous LDL oxidation by ROS is required for Lp-PLA_2 activation and the knowledge that the generated lysophophatidylcholine inhibits NO synthesis via endothelial NO synthase [28–30,48] could constitute an explanation for the diminished NO synthesis and the increased ROS formation observed in this study when HUVECs were incubated with hypercholesterolemic serum. Regarding CETP, this lipoprotein-associated protein interchanges triglycerides and cholesteryl esters between lipoproteins and is partially responsible for the formation of

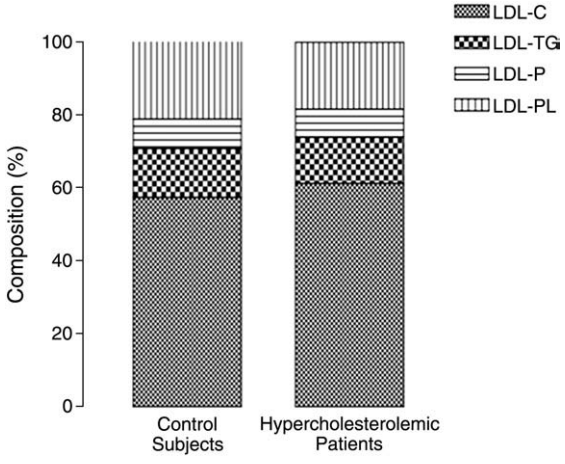

Fig. 4. Chemical composition of isolated LDL from hypercholesterolemic patients and control subjects. LDL, low-density lipoprotein; C, cholesterol; TG, triglycerides; P, proteins; PL, phospholipids.

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small and dense LDL particles, which are highly susceptible to oxidation by Lp-PLA_2. This concept is supported by the results shown in the present study which demonstrate an inverse association between NO synthesis and plasma Lp-PLA_2 activity and a direct association with LDL-C [22,24–26].

On the other hand, when the effect of isolated LDL fractions was tested on HUVECs, no differences were detected in NO synthesis or ROS formation. It is worthy to note that LDL concentration was exactly the same in all experiments carried out and that LDL chemical composition and Lp-PLA_2 activity measured in LDL fraction did not show any difference between both groups. Therefore, the effect of plasma on the synthesis of NO and ROS may be due to the concentration of LDL. In light of our results, it may be concluded that both LDL fractions were similar not only in their lipid and protein content but also in other factors that could influence NO synthesis and ROS formation by HUVECs. However, further experiments should be developed to deeply characterize LDL composition in hypercholesterolemic and control subjects (oxidation, subpopulation, etc.).

In conclusion, the present study showed that serum from hypercholesterolemic patients was able to induce *in vitro* endothelial dysfunction through a decrease in NO bioavailability and an increase in ROS formation. Experiments with isolated LDL fractions hypothesize that the reduction in NO synthesis and the increase in ROS formation were due to the increase in the number of LDL particles, main Lp-PLA_2 carrier. It cannot be discarded that other factors, such as cytokines, not determined in this study and not related to LDL particles, could exert an effect on NO synthesis and ROS formation by endothelial cells. Although the main limitation in this study was the small sample size, this work is the first to investigate the effects of hypercholesterolemic serum and isolated LDL fractions on endothelial cells. However, further studies are necessary to disclose the exact and complete mechanisms involved of Lp-PLA_2 in ROS and NO generation by endothelial cells and suggesting that this marker may be helpful in distinguishing individual with endothelial dysfunctions.

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