Agència de Gestió d'Ajuts Universitaris i de Recerca

UNIÓ EUROPEA
Fons Social Europeu

Memòria justificativa de recerca de les beques predoctorals per a la formació de personal investigador (FI)

La memòria justificativa consta de les dues parts que venen a continuació:
1.- Dades bàsiques i resums
2.- Memòria del treball (informe científic)

Tots els camps són obligatoris

1.- Dades bàsiques i resums

Títol del projecte ha de sintetitzar la temàtica científica del vostre document.
Propietats protectores de la lipoproteïna de alta densitat (HDL) sobre l'acció inflamatoria de la lipoproteïna de baixa densitat electronegativa (LDL(-)) en monòcits humans.

Dades de l'investigador (benficiari de l'ajut)

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Universitat Pompeu Fabra

Número d'expedient

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Paraules clau: cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
LDL, HDL, monocits, inflamació, citoquinas.

Data de presentació de la justificació

13/10/2009

Resum en la llengua del projecte (màxim 300 paraules)
La llengua del projecte es anglès.

Resum en anglès(màxim 300 paraules)

Electronegative low-density lipoprotein (LDL(-)) is a modified fraction of LDL present in peripheral blood whose proportion is elevated in subjects with increased cardiovascular risk. LDL(-) has been shown to have an inflammatory effect on human endothelial cells and mononuclear blood cells. On the other hand, high-density lipoprotein (HDL) is known to have a protective effect against cardiovascular disease, partly mediated by its anti-inflammatory properties. The objective of the current work is to study the putative protective properties of HDL towards the inflammatory effect of LDL(-) in human monocytes, in order to elucidate the mechanisms behind their interaction.

Total LDL and HDL were isolated by ultracentrifugation and LDL(-) was obtained from total LDL by anion exchange chromatography. HDL and LDL(-) were incubated together and then re-isolated, and their characteristics were compared to those of untreated lipoproteins. The inflammatory activity of the lipoproteins was determined by incubating monocytes with lipoproteins and measuring cytokine release from the cultured monocytes. The biochemical composition and electrophoretic mobility of the lipoproteins were also determined before and after their interaction.

Incubation of HDL with LDL(-) reduced the inflammatory effect of LDL(-) and, in turn, HDL gained inflammatory properties. This indicates a transfer of inflammatory potential taking place during the interaction of LDL(-) and HDL. Additionally, LDL(-) lost non-esterified fatty acids (NEFAs) while HDL gained the same. We conclude that a transfer of NEFAs takes place between LDL(-) and HDL. These observations suggest that NEFAs play a role in the inflammatory effect mediated by LDL(-).

2.- Memòria del treball (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

Durante el periodo de la beca he realizado un Master en Investigación Biomédica en la Universitat Pompeu Fabra. He trabajado en el metabolismo de las lipoproteínas y su relación con la arteriosclerosis y el riesgo cardiovascular. Concretamente, se ha estudiado el efecto protector de la HDL (lipoproteína de alta densidad) sobre el efecto inflamatorio de la LDL(-) (lipoproteína de baja densidad electronegativa).

Se ha observado que HDL contraresta las propiedades inflamatorias de LDL(-), disminuyendo la liberación de citocinas en monocitos inducida por LDL(-). Se ha observado una transferencia de potencial inflamatorio de LDL(-) a HDL, en paralelo a una transferencia de ácidos grasos no esterificados (NEFAs) de LDL(-) a HDL. Al contrario, la apoA-1, la proteína mas abundante de HDL, reduce el potencial inflamatorio de la LDL(-) sin cambiar el contenido de NEFAs. La acción protectora de HDL sobre LDL(-) está parcialmente mediada por la transferencia de NEFAs entre las lipoproteínas, sin embargo, otros mecanismos probablemente están implicados.

Además de la realización del Master, he participado en un proyecto centrado en el estudio de la expresión genética inducida por LDL(-) en células mononucleares sanguineas mediante una aproximación genómica. Se ha observado la inducción por parte de la LDL(-) de genes implicados en los procesos inflamatorios asociados a la arteriosclerosis. Concretamente, la LDL(-) induce la expresión de Fas e inhibe la expresión de CD36, CSF1R y PPARG. Este efecto está mediado por la activación del factor de transcripción NF-kB y la inhibición del AP1. De este trabajo se ha obtenido una publicación científica en "Frontiers in Bioscience" (2009, en prensa, FBS/928).

Adjunto la tesina del Master (dos archivos llamados "Tesina Master Ragnhild Birkelund.doc" y "Figuras Master Ragnhild Birkelund.doc") y el articulo científico (un archivo llamado "FBS Birkelund.pdf").

Universitat Pompeu Fabra
Master of Biomedical Research
2008-2009

PROTECTIVE PROPERTIES OF HDL TOWARDS THE INFLAMMATORY EFFECT OF ELECTRONEGATIVE LDL IN HUMAN MONOCYTES

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Barcelona, June 2009

Summary

Electronegative low-density lipoprotein (LDL(-)) is a modified fraction of LDL present in peripheral blood whose proportion is elevated in subjects with increased cardiovascular risk. LDL(-) has been shown to have an inflammatory effect on human endothelial cells and mononuclear blood cells. On the other hand, high-density lipoprotein (HDL) is known to have a protective effect against cardiovascular disease, partly mediated by its anti-inflammatory properties. The objective of the current work is to study the putative protective properties of HDL towards the inflammatory effect of LDL(-) in human monocytes, in order to elucidate the mechanisms behind their interaction.

Total LDL and HDL were isolated by ultracentrifugation and LDL(-) was obtained from total LDL by anion exchange chromatography. HDL and LDL(-) were incubated together and then re-isolated, and their characteristics were compared to those of untreated lipoproteins. The inflammatory activity of the lipoproteins was determined by incubating monocytes with lipoproteins and measuring cytokine release from the cultured monocytes. The biochemical composition and electrophoretic mobility of the lipoproteins were also determined before and after their interaction.

Incubation of HDL with LDL(-) reduced the inflammatory effect of LDL(-) and, in turn, HDL gained inflammatory properties. This indicates a transfer of inflammatory potential taking place during the interaction of LDL(-) and HDL. Additionally, LDL(-) lost non-esterified fatty acids (NEFAs) while HDL gained the same. We conclude that a transfer of NEFAs takes place between LDL(-) and HDL. These observations suggest that NEFAs play a role in the inflammatory effect mediated by LDL(-).

INTRODUCTION

Atherosclerosis is a multifactorial disease characterized by the formation of atherosclerotic plaques in the blood vessel wall. The plaques occur principally in large and medium-sized elastic and muscular arteries, and can lead to ischemia of the heart, brain or extremities. The process of atherosclerosis has been considered by many to consist largely of the accumulation of lipids within the artery wall. However, recent research shows that the atherosclerotic lesions represent a series of cellular and molecular events that can be described as an inflammatory disease (Ross 1999).

Atherosclerosis is characterized by the accumulation of cholesterol in the arterial intima. Low-density lipoprotein (LDL) is the main transporter of cholesterol in the blood and a major player in the development of atherosclerosis. Indeed, high plasma concentration of LDL is one of the principal risk factors for atherosclerosis (Lusis 2000). On the other hand, high-density lipoprotein (HDL) is strongly protective against atherosclerosis (Lusis 2000), and an inverse relationship between plasma levels of HDL and cardiovascular risk has been widely described. It is therefore interesting to study the interaction between these particles and their role in atherosclerosis and inflammation.

LDL is a heterogeneous group of particles, varying in size, composition and structure. The particles have a range of density between 1,019 and 1,063 g/mL, and a diameter of 18-28 nm. They contain a hydrophobic core of non-polar lipids (cholesterol esters and triglycerides) surrounded by a layer of protein and polar lipids (phospholipids, free cholesterol and non-esterified fatty acids (NEFAs)). The protein content mainly consists of apolipoprotein apoB-100, which is present at one molecule per LDL particle (Hevonoja, Pentikainen et al. 2000).

An early event in the formation of atherosclerotic plaques is the trapping of LDL in the extracellular matrix of the arterial wall, mainly by binding to proteoglycans (Lusis 2000). It has been proposed that it here receives oxidized products of the endothelial cell metabolism, leading to the formation of mildly oxidized LDL, which is biologically active (Navab, Hama et al. 2000). However, other modifications may also take place in the trapped LDL, including lipolysis, proteolysis and aggregation (Lusis 2000). This modified LDL can cause a series of effects that are central to the development of atherosclerosis, including monocyte chemotaxis, transmigration across the endothelial monolayer into the intima, and differentiation into

macrophages (Murphy, Woollard et al. 2008). In turn, the macrophages can secrete reactive oxygen species that convert trapped, mildly oxidized LDL into highly oxidized LDL (Navab, Berliner et al. 1996). This highly oxidized LDL can be internalized by macrophages through scavenger receptors, resulting in intracellular accumulation of cholesterol and the formation of foam cells (Navab, Berliner et al. 1996; Murphy, Woollard et al. 2008). The foam cells, in turn, secrete cytokines that can cause smooth muscle cell migration and proliferation (Berliner and Haberland 1993). With time, the foam cells die, leaving behind a growing mass of extracellular lipids and other cell debris contributing to the necrotic core of the atherosclerotic lesion (Lusis 2000).

More than native LDL it is considered to be modified LDL that presents atherogenic properties. There is controversy whether modification of LDL occurs only in the intima or if it also can take place in circulation (Kovanen and Pentikainen 2003). However, several research groups have found a minor modified subfraction of LDL in peripheral blood (Avogaro, Bon et al. 1988; Sanchez-Quesada, Benitez et al. 2004). This modified form of LDL can be isolated from the total LDL on the basis of electric charge by anion exchange chromatography, and is therefore called electronegative LDL (LDL(-)). LDL(-) constitutes about 5% of the total LDL in normolipemic individuals (De Castellarnau, Sanchez-Quesada et al. 2000). Controversy exists as to whether LDL(-) is simply oxidized LDL, or whether the particles also contain additional, non-oxidative, modifications (Sanchez-Quesada, Benitez et al. 2004). However, our group has found no evidence of extensive oxidation in LDL(-); its antioxidant and thiobarbituric acid-reactive substances contents were similar to those of the remaining LDL fraction called electropositive LDL (LDL(+)) (De Castellarnau, Sanchez-Quesada et al. 2000). LDL(-) has also been shown to differ from LDL(+) in biochemical composition, for example having a higher content of triglycerides and NEFAs (De Castellarnau, Sanchez-Quesada et al. 2000).

LDL(-) has been shown to have an inflammatory effect on human endothelial cells (De Castellarnau, Sanchez-Quesada et al. 2000) and mononuclear circulating cells, inducing the release of cytokines (Benitez, Bancells et al. 2007). In addition, a subpopulation of LDL(-) shows an increased tendency for aggregation (Bancells, Benitez et al. 2008) and higher binding affinity to human aortic proteoglycans (Bancells, Benitez et al. 2009). The proportion of LDL(-) has been found to be increased in subjects with increased risk of cardiovascular disease, including diabetic and Familial Hypercholesterolemia patients (Sanchez-Quesada, Otal-Entraigas et al. 1999; Sanchez-Quesada, Perez et al. 2001). Together, these data

suggest that LDL(-) could be an important atherogenic factor; however, little is known about the components responsible for its effect or the molecular mechanisms behind it.

HDL particles are smaller and denser than LDL particles, with a range of density between 1,063 and 1,210 g/mL, and a diameter of 5-12 nm. Similarly to LDL, HDL contains a hydrophobic core of non-polar lipids surrounded by a layer of protein and polar lipids; however, in comparison with LDL, HDL has a lower lipid content compared to the protein content. The major protein component of HDL is apoA-I, but it also contains low levels of other proteins. The lipid content consists of free cholesterol and cholesterol esters, phospholipids, triglycerides and NEFAs.(Skinner 1994; Lund-Katz, Liu et al. 2003).

The atheroprotective role of HDL is widely recognized, and has often been attributed to the ability of HDL to promote reverse cholesterol transport (Lusis 2000), which entails removing cholesterol from peripheral tissues and transporting it to the liver for excretion (Tall 1998). However, HDL also presents additional anti-inflammatory properties. It can prevent the nonenzymatic oxidation of LDL phospolipids (Navab, Hama et al. 2000), it inhibits the production of inflammatory molecules by cells stimulated by modified LDL, and it possesses a direct anti-inflammatory effect on the endothelium (Navab, Imes et al. 1991; Calabresi, Gomaraschi et al. 2003).

Some protective effects of HDL have been attributed to apoA-I. For example, pre-incubation of LDL with apoA-I decreases the ability of LDL to generate lipid hydroperoxides and to induce monocyte adherence or monocyte chemotactic activity when added to human artery wall cell cocultures (Navab, Hama et al. 2000). In addition to apoA-I, lipid components (Navab, Imes et al. 1991) and enzymatic activities of HDL, such as paraoxonase (PON) (Mackness, Durrington et al. 2004) and platelet activator factor acetyl hydrolase (PAF-AH) (Watson, Navab et al. 1995) have been related to its atheroprotective action, although the mechanism is unclear.

Since the bioactivity of oxidized LDL particles can be regulated by HDL, it is interesting to study specifically whether HDL also can regulate the activity of LDL(-). The aim of this study was to assess the putative protective properties of HDL towards this inflammatory effect of LDL(-) in human monocytes. We also wanted to understand the mechanisms behind the inflammatory effect of LDL(-) and the protective effect of HDL.

METHODS

Isolation of total LDL and HDL

Whole blood samples from healthy normolipemic subjects were obtained in EDTA-containing Vacutainer tubes, and LDL and HDL were isolated from plasma by sequential flotation ultracentrifugation (Havel, Eder et al. 1955). Briefly, the density of plasma is adjusted using KBr, followed by centrifugation at 100000 g (36000 rpm) at 4°C for 20 hours. During this process, the lipoproteins with a density lower than that of the plasma float to the top and can thereby be removed from the solution. The remaining plasma is readjusted to a higher density, and the process is repeated, allowing the separation of particles of higher density. In addition to KBr salt, density solutions of the same density as the adjusted plasma were used, in order to increase the volume and thereby facilitate separation of the lipoproteins. Density solutions were made with KBr, and contained 1mM EDTA to avoid oxidative modification, as well as chloramphenicol and gentamycin to avoid bacterial contamination.

LDL particles have a density of 1,019-1,063 g/mL, but for all experiments LDL was isolated at the density range 1,019-1,050 g/mL, in order to avoid contamination with Lipoprotein(a) (density 1,050-1,100 g/mL). For the same reason, HDL was isolated in the density range 1,100-1,210 g/mL.

Lipoprotein dialysis

To conserve the lipoproteins after isolation they were dialysed in buffer A (Tris-HCl 10mM, EDTA 1mM, pH 7,4), in order to eliminate the KBr. The buffer contained EDTA which prevents oxidation of the lipoproteins. The dialysed samples were stored at 4°C.

Isolation of LDL fractions

Total LDL was subfractionated into LDL(+) and LDL(-) by preparative anion exchange chromatography in an Äkta-FPLC system (Amersham Pharmacia), using a HiLoad 26/10 Q-Sepharose column. The separation was carried out using a stepwise gradient of NaCl created by the use of two buffers, A and B, at varying concentrations. *Buffer A*: Tris-HCl 10mM, EDTA 1mM, pH 7,4. *Buffer B*: Tris-HCl 10mM, EDTA 1mM, NaCl 1M, pH 7,4. Total LDL was separated into two fractions, with LDL(+) (native unmodified LDL) eluting at 0.245M NaCl, and LDL(-) eluting at 0.6M NaCL.

Pre-incubation of lipoproteins

LDL(-) and HDL, LDL(-) and apoA-I, or LDL(+) and HDL, were incubated together at 1:1 or 2:1 proportion for 2 hours at 37°C with gentle agitation. Lipoprotein concentration was expressed as g/L of apoB for LDL and g/L of apoA-I for HDL. Just prior to pre-incubation, LDL(-), LDL(+) and HDL were dialysed in phosphate buffered saline (PBS) by gel filtration chromatography using PD-10 Desalting Columns containing Sephadex G-25 (GE Healthcare). The incubation was done in PBS in the presence of 20µM butylated hydroxytoluene (BHT) in order to avoid oxidation. After this pre-incubation, the lipoproteins were re-isolated by ultracentrifugation according to their density. LDL(-), LDL(+) and HDL were incubated alone under the same conditions for use as controls.

Lipid and apoprotein composition

Lipid and apoprotein content was determined by commercial methods using a Hitachi 917 autoanalyzer. Total cholesterol, triglycerides (Roche Diagnostics), phospholipids and NEFAs (Wako Chemicals) were measured by enzyme colorimetric methods, whereas apoA-I and apoB were quantified by immunoturbidimetric methods using antibodies to apoA-I or apoB (Roche Diagnostics).

LDL(+) and LDL(-) were examined for contamination by HDL by measuring apoA-I content before and after pre-incubation. Similarly, HDL was examined for contamination by LDL by measuring apoB content.

Electrophoretic analysis

The mobility of LDL and HDL was determined in commercial 0,5% agarose gels (Midigel lipo, Biomidi), following the manufacturer's instructions. In these gels, lipoproteins migrate according to electric charge.

The electrophoresis was carried out at 90V for 1 hour in barbital buffer supplied in the gel kits. After electrophoresis, the gel was fixated for 10 min in fresh fixative solution (distilled H_2O: glacial acetic acid: ethanol in proportion 90mL:30mL:180mL), rinsed in distilled H_2O and dried. The gel was stained with Sudan Black, a lipid specific stain (supplied with kit). Destaining was performed with a solution of 150 mL ethanol and 150 mL aqueous solution containing 20 g NaCl per liter, before the gel was rinsed with distilled H_2O and dried.

Isolation and seeding of human monocytes

Whole blood was collected from healthy blood donors in EDTA-containing Vacutainer tubes. The blood was centrifuged at 800 g for 10 min and the leukocytes, forming a white layer between the plasma and the erythrocytes, were collected. Mononuclear cells (monocytes and lymphocytes) were isolated from the leukocyte fraction by density gradient centrifugation using a commercially available density gradient solution (LinfoSep, Biomedics). After centrifugation at 800 g for 20 min, the mononuclear cells form a layer just above the density solution. The mononuclear cells were collected, washed twice in PBS at 250 g for 10 min to remove the density solution, and resuspended in complete cell culture medium (RPMI 1640 (Biowhittaker) containing 10% fetal calf serum, 0,1 IU/L penicillin, 100 mg/L streptomycin, 1mM Na-pyruvate, 1% non-essential amino acids). Cells were counted in a Neubauer chamber and seeded onto cell culture plates. After a minimum of four hours incubation monocytes were separated from lymphocytes on the basis of their different adhesive properties to the cell culture plate. Monocytes adhere to the plastic whereas lympocytes do not, and in this manner the lymphocytes can be removed by discarding the medium, leaving the monocytes adhered to the well. Fresh cell culture medium was then added to adhered monocytes.

Incubation of monocytes with lipoproteins

Monocytes were cultured at 37ºC and 5% CO_2 in 12-well plates, with 1 x 10^6 cells/well, in complete cell culture medium. Before addition of lipoproteins, complete medium was removed, the cells were washed twice in PBS and fresh deficient cell culture medium was added (RPMI 1640 (Biowhittaker) containing 1% fetal calf serum, 0,1 IU/L penicillin, 100 mg/L streptomycin, 1mM Na-pyruvate, 1% non-essential amino acids).

Immediately prior to incubation with monocytes, the lipoproteins were dialysed in cell culture medium by gel filtration chromatography using PD-10 Desalting Columns as described above.

Lipoproteins were added to the monocytes at a concentration of 150 µg apoA-I or apoB/mL medium. In pre-incubation conditions, LDL(+), LDL(-) or HDL incubated and re-isolated as described above was added alone to the cells. In co-incubation conditions previously untreated LDL(-) and HDL were added simultaneously to the monocytes. A well containing monocytes incubated without lipoproteins was included as a blank. The cells were then incubated at 37°C and 5% CO_2 for 20 hours.

Cytokine release quantification by enzyme-linked immunosorbent assay (ELISA)

Supernatants from monocytes incubated with or without lipoproteins were collected at 20 hours of incubation, centrifuged to eliminate cellular debris and stored at -80°C. The release of the cytokines interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), interleukin 8 (IL-8) and interleukin 10 (IL-10) were determined in the supernatant by ELISA, according to the manufacturer's instructions. ELISA kits were from Bender MedSystems (IL-6 and IL-10), BLK diagnostics (IL-8) and Bioscience (MCP-1).

Statistical analysis

Results were expressed as mean ± SD. Differences between groups were tested with the Wilcoxon t-test for paired data (lipoproteins isolated from the same plasma pool).

RESULTS

Cytokine release

Incubation of monocytes with LDL(-) alone induced the release of cytokines MCP-1, IL-6, IL-8 and IL-10 at a significantly higher level than did incubation of monocytes with LDL(+) (Figure 1, striped bars show LDL(+), grey bars show LDL(-)). This finding agrees with previous reports that LDL(-) is more inflammatory than LDL(+) (Benitez, Bancells et al. 2007). Co-incubation of HDL and LDL(-) with monocytes strongly decreased the release of cytokines with respect to incubation with control LDL(-) (Figure 1, dotted bars). This decrease was concentration-dependent, as the cytokine release decreased with increased ratio of HDL to LDL(-). Co-incubation of HDL and LDL(+) also decreased the release of cytokines induced by LDL(+), but to a lower degree than the reduction seen for LDL(-) (data not shown).

Pre-incubation of LDL(-) with HDL reduced the cytokine release from monocytes compared to the cytokine release induced by control LDL(-) (Figure 1, white bars). Again, this effect was concentration-dependent, as the cytokine release decreased with increased ratio of HDL to LDL(-). The cytokine release from cells incubated simultaneously with both LDL(-) and HDL (co-incubation condition) was lower than the release from cells incubated with LDL(-) that was pre-incubated with HDL (pre-incubation condition).

To investigate whether apoA-I could be involved in the anti-inflammatory effect of HDL, the experiment was performed pre-incubating LDL(-) with apoA-I, followed by incubation of re-isolated LDL(-) with monocytes. The results were similar to those obtained with HDL. The monocytes treated with LDL(-) that had been pre-incubated with apoA-I showed a lower cytokine release than monocytes treated with control LDL(-) (Figure 1, white bars, right). The concentration of MCP-1 and IL-6 were comparable to the levels released by LDL(-) pre-incubated with HDL, whereas the release of IL-8 and IL-10 was inhibited to a higher degree.

To further investigate the effect of the interaction between LDL(-) and HDL on their inflammatory properties, we tested the induction of cytokine release from monocytes by HDL that had been pre-incubated with LDL(-) and thereafter re-isolated. This pre-treated HDL showed an increased induction of cytokine release compared to control HDL (Figure 2, grey bars vs left white bars). Indeed, comparing figures 1 and 2, it can be observed that the release of IL-6 and IL-8 were similar to the levels induced by LDL(-), while the levels of MCP-1 and IL-10

were lower than those induced by LDL(-), although still significantly higher than those induced by control HDL.

On the other hand, HDL that was pre-incubated with LDL(+) followed by re-isolation showed similar levels of cytokine release as control HDL, although slightly higher (Figure 2, white bars, middle).

Lipid and apoprotein composition

In order to assess the alteration in biochemical composition caused by the interaction between LDL(+) or LDL(-) and HDL, the biochemical composition of the pre-incubated particles was measured and compared to the composition of control LDL(+), LDL(-) or HDL. Lipid and protein composition of LDL subfractions and HDL before and after pre-incubation is shown in Table 1 and Figure 3.

Differences between LDL(+) and LDL(-) were similar to those previously reported (De Castellarnau, Sanchez-Quesada et al. 2000); briefly, LDL(-) presented lower relative content of apoB and increased triglyceride and NEFA content compared to LDL(+) (Table 1 and Figure 3A, grey bar vs striped bar). After pre-incubation of LDL(+) and HDL, or LDL(-) and HDL, no changes in cholesterol, phospholipid, triglyceride, apoA-I or apoB were found (Table 1). However, after pre-incubation of LDL(-) and HDL the content of NEFAs was significantly altered in both particles. In LDL(-), the content of NEFAs relative to the content of apoB was significantly reduced (Figure 3A, white bar, left), whereas in HDL the NEFA content relative to the content of apoA-I was significantly increased (Figure 3B, grey bar). On the contrary, HDL incubated with LDL(+) and thereafter re-isolated did not show a significant increase in NEFA content relative to control HDL (Figure 3B, white bar, right).

In order to assess the role of apoA-I in the protective effect of HDL, the composition of LDL(-) was examined after pre-incubation with purified apoA-I. This pre-incubation did not change the NEFA content in LDL(-) (Figure 3A, white bar, right), nor did it change any other of the components measured (Table 1).

In all experiments, LDL was examined for contamination of HDL and vice versa. In all cases, the level of apoA-I in LDL and the level of apoB in HDL were undetectable (data not shown), indicating correct and complete separation of the lipoproteins.

Electrophoretic mobility

The electrophoretic mobility of LDL(+), LDL(-) and HDL before and after pre-incubation was also examined (Figure 4). LDL(-) pre-incubated with HDL followed by re-isolation had slightly decreased electrophoretic mobility compared to control LDL(-) (lanes 4 and 5), indicating a lower electronegative charge. On the contrary, HDL increased its electrophoretic mobility after incubation with LDL(-) (lanes 7 and 9). The electrophoretic mobility of LDL(-) was also examined after incubation with apoA-I. This LDL(-) had the same electrophoretic mobility as untreated LDL(-), indicating that the electric charge was unchanged (lanes 4 and 6).

Pre-incubation of LDL(+) with HDL or apoA-I had no effect on the electrophoretic mobility of LDL(+) (lanes 1, 2 and 3). Neither did pre-incubation with LDL(+) have any effect on HDL (lane 8).

DISCUSSION

The atheroprotective role of HDL is widely established, with one of its most recognized properties being the ability to inhibit the formation of oxidized LDL, which plays a central role in the atherosclerotic process. Here it is shown that HDL also can work by different mechanisms, as it can counteract the inflammatory effects of LDL(-). It is known that LDL(-) can induce cytokine release in monocytes, and in the current work we report that HDL can counteract this effect, as pre-incubation of LDL(-) with HDL reduces the cytokine release from human monocytes compared to untreated LDL(-).

Pre-incubating LDL(-) with apoA-I also reduces the cytokine release induction by LDL(-) compared to control LDL(-). This suggests that apoA-I may be the component of HDL responsible for its anti-inflammatory properties towards LDL(-).

Pre-incubation also has an effect on the inflammatory properties of HDL, as HDL pre-incubated with LDL(-) causes a higher cytokine release from monocytes than untreated HDL. As such, HDL gains inflammatory properties upon interaction with LDL(-).

When comparing the co-incubation and pre-incubation conditions, the cytokine release in the co-incubation condition is lower than the release in the pre-incubation condition. In the co-incubation condition LDL(-) and HDL are both incubated with the cells simultaneously, whereas in the pre-incubation condition LDL(-) is first allowed to interact with HDL and thereafter re-isolated. The additional decrease in cytokine release seen in the co-incubation condition might be due to the presence of HDL in the culture medium. This suggests that, in addition to its anti-inflammatory effect towards LDL(-) observed in the pre-incubation condition, HDL could have a direct anti-inflammatory effect on the cells, causing a further inhibition of cytokine release.

The inhibition of cytokine release induced by LDL(-) observed in the pre-incubation condition suggests that a modification of LDL(-) takes place during its interaction with HDL, promoting a decrease in its inflammatory effect. Similarly, HDL also seems to be modified during pre-incubation, increasing the induction of cytokine release from monocytes. In this manner, we see a transfer of inflammatory potential between LDL(-) and HDL during their interaction.

The observed transfer of inflammatory activity could be caused either by a transfer of some component between the two particles, by a conformational change or by a biochemical reaction taking place between the particles. Investigating the changes in composition of LDL(-) and HDL after their interaction can help us identify what components of LDL(-) are responsible for its inflammatory action. Therefore, we examined the biochemical composition of the LDL(+), LDL(-) and HDL particles before and after their pre-incubation.

We found that most of the components studied were unchanged; however, the content of NEFAs was significantly altered in LDL(-) and HDL after their interaction. HDL gained NEFAs while LDL(-) lost NEFAs. As NEFAs are negatively charged, this finding was supported by electrophoresis, as LDL(-) showed decreased electrophoretic mobility after incubation with HDL, indicating a reduced negative charge. On the contrary, HDL increased its electrophoretic mobility after incubation with LDL(-), indicating an increased negative charge. Since NEFA content is the main determinant of the electric charge of LDL(-) (Gaubatz, Gillard et al. 2007), these changes could be explained by a transfer of NEFAs from LDL(-) to HDL during co-incubation.

Several lines of evidence suggest a role of non-oxidized polar lipids in LDL(-)-induced chemokine release, among these NEFAs. For example, it has been reported that LDL enzymatically modified by cholesterol esterase and trypsin increases its NEFA content and induces IL-8 production by endothelial cells (Suriyaphol, Fenske et al. 2002). The NEFA content is approximately two- or threefold increased in LDL(-) compared to LDL(+) or total LDL (Benitez, Villegas et al. 2004). Our group has also observed that in vitro enrichment of LDL(+) with NEFAs to the same level as that observed in LDL(-) gives LDL(+) the same inflammatory characteristics as LDL(-) (Sanchez-Quesada, Benitez et al. 2004).

It could be tempting to suggest that the protection of HDL against the inflammatory action of LDL(-) is caused by a transfer of NEFAs, and that the NEFAs are, at least in part, responsible for the inflammatory action of LDL(-). However, this is inconsistent with the results obtained from the experiments with apoA-I. ApoA-I also reduced the inflammatory effect of LDL(-) as measured by the release of cytokines from monocytes, but it did not reduce the NEFA content of LDL(-). Again, this was supported by electrophoresis, since incubation of LDL(-) with apoA-I did not change its electrophoretic mobility. Therefore, diminution in NEFA content of LDL(-) does not seem to be the only factor involved in the inhibiton of cytokine release.

A possible explanation for the discrepancy is that apoA-I alone acts by a different mechanism than does the whole HDL particle. The involvement of other lipids apart from the ones evaluated by our methods is also feasible. Specifically, lysophospatidylcholine (LPC) has been suggested to play a role in the inflammatory properties of LDL(-) (Benítez, Camacho et al. 2004). The ability of apoA-I to bind highly polar phospholipids is well established, and it is known that oxidized lipids can be transferred from LDL to HDL or apoA-I when they are incubated together (Navab, Hama et al. 2000). Although we did not find evidence of oxidative modification in LDL(-) it cannot be ruled out that minor amounts of oxidized phospholipids could be transferred between LDL(-) and HDL/apoA-I.

Some groups have reported the detection of several additional, minor proteins in LDL (Karlsson, Leanderson et al. 2005). It is possible that one or more of these proteins can be transferred between the lipoprotein particles and thereby alter their inflammatory properties. The molecules being transferred could themselves have inflammatory characteristics or alter the conformation of another component, changing its inflammatory properties. Alternatively, the molecule being transferred could have enzymatic activity, causing the formation of an inflammatory molecule, or it could act as a cofactor for another such enzyme.

In summary, our results show that HDL counteracts the inflammatory properties of LDL(-) by diminishing the release of cytokines from monocytes induced by LDL(-). This protective action is partly mediated by NEFA transfer between the lipoprotein particles. However, other mechanisms are likely to be involved in this effect of HDL, since purified apoA-I also inhibits inflammation without promoting NEFA transfer. Further studies focused on changes in lipid and protein composition of the lipoproteins after their interaction would be necessary, in order to understand the mechanisms involved in the observed inflammatory and anti-inflammatory effects of LDL(-) and HDL.

BIBLIOGRAPHY

Avogaro, P., G. B. Bon, et al. (1988). "Presence of a modified low density lipoprotein in humans." Arteriosclerosis **8**(1): 79-87.

Bancells, C., S. Benitez, et al. (2009). "High binding affinity of electronegative LDL to human aortic proteoglycans depends on its aggregation level." J Lipid Res **50**(3): 446-55.

Bancells, C., S. Benitez, et al. (2008). "Novel phospholipolytic activities associated with electronegative low-density lipoprotein are involved in increased self-aggregation." Biochemistry **47**(31): 8186-94.

Benitez, S., C. Bancells, et al. (2007). "Pro-inflammatory action of LDL(-) on mononuclear cells is counteracted by increased IL10 production." Biochim Biophys Acta **1771**(5): 613-22.

Benitez, S., V. Villegas, et al. (2004). "Impaired binding affinity of electronegative low-density lipoprotein (LDL) to the LDL receptor is related to nonesterified fatty acids and lysophosphatidylcholine content." Biochemistry **43**(50): 15863-72.

Berliner, J. A. and M. E. Haberland (1993). "The role of oxidized low-density lipoprotein in atherogenesis." Curr Opin Lipidol **4**: 373-381.

Calabresi, L., M. Gomaraschi, et al. (2003). "Endothelial protection by high-density lipoproteins: from bench to bedside." Arterioscler Thromb Vasc Biol **23**(10): 1724-31.

De Castellarnau, C., J. L. Sanchez-Quesada, et al. (2000). "Electronegative LDL from normolipemic subjects induces IL-8 and monocyte chemotactic protein secretion by human endothelial cells." Arterioscler Thromb Vasc Biol **20**(10): 2281-7.

Gaubatz, J. W., B. K. Gillard, et al. (2007). "Dynamics of dense electronegative low density lipoproteins and their preferential association with lipoprotein phospholipase A(2)." J Lipid Res **48**(2): 348-57.

Havel, R. J., H. A. Eder, et al. (1955). "The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum." J Clin Invest **34**(9): 1345-53.

Hevonoja, T., M. O. Pentikainen, et al. (2000). "Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL." Biochim Biophys Acta **1488**(3): 189-210.

Karlsson, H., P. Leanderson, et al. (2005). "Lipoproteomics I: mapping of proteins in low-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry." Proteomics **5**(2): 551-65.

Kovanen, P. T. and M. O. Pentikainen (2003). "Circulating lipoproteins as proinflammatory and anti-inflammatory particles in atherogenesis." Curr Opin Lipidol **14**(5): 411-9.

Lund-Katz, S., L. Liu, et al. (2003). "High density lipoprotein structure." Front Biosci **8**: d1044-54.

Lusis, A. J. (2000). "Atherosclerosis." Nature **407**(6801): 233-41.

Mackness, M. I., P. N. Durrington, et al. (2004). "The role of paraoxonase 1 activity in cardiovascular disease: potential for therapeutic intervention." Am J Cardiovasc Drugs **4**(4): 211-7.

Murphy, A. J., K. J. Woollard, et al. (2008). "High-density lipoprotein reduces the human monocyte inflammatory response." Arterioscler Thromb Vasc Biol **28**(11): 2071-7.

Navab, M., J. A. Berliner, et al. (1996). "The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture." Arterioscler Thromb Vasc Biol **16**(7): 831-42.

Navab, M., S. Y. Hama, et al. (2000). "Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1." J Lipid Res **41**(9): 1481-94.

Navab, M., S. S. Imes, et al. (1991). "Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein." J Clin Invest **88**(6): 2039-46.

Ross, R. (1999). "Atherosclerosis--an inflammatory disease." N Engl J Med **340**(2): 115-26.

Sanchez-Quesada, J. L., S. Benitez, et al. (2004). "Electronegative low-density lipoprotein." Curr Opin Lipidol **15**(3): 329-35.

Sanchez-Quesada, J. L., C. Otal-Entraigas, et al. (1999). "Effect of simvastatin treatment on the electronegative low-density lipoprotein present in patients with heterozygous familial hypercholesterolemia." Am J Cardiol **84**(6): 655-9.

Sanchez-Quesada, J. L., A. Perez, et al. (2001). "Effect of glycemic optimization on electronegative low-density lipoprotein in diabetes: relation to nonenzymatic glycosylation and oxidative modification." J Clin Endocrinol Metab **86**(7): 3243-9.

Skinner, E. R. (1994). "High-density lipoprotein subclasses." Curr Opin Lipidol **5**(3): 241-7.

Suriyaphol, P., D. Fenske, et al. (2002). "Enzymatically modified nonoxidized low-density lipoprotein induces interleukin-8 in human endothelial cells: role of free fatty acids." Circulation **106**(20): 2581-7.

Tall, A. R. (1998). "An overview of reverse cholesterol transport." Eur Heart J **19 Suppl A**: A31-5.

Watson, A. D., M. Navab, et al. (1995). "Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein." J Clin Invest **95**(2): 774-82.

	LDL(+)		LDL(-)			HDL		
	Control	After HDL	Control	After HDL	After apoA-I	Control	After LDL(+)	After LDL(-)
% Cholesterol	41,5±1,8	41,9±1,9	42,1±1,7	41,8±2,0	42,3±2,5	18,5±2,2	18,7±1,7	18,4±1,1
% Triglycerides	6,2±1,0	6,1±1,0	9,4±1,2	9,4±1,3	9,6±1,1	4,6±3,3	3,8±0,6	4,1±0,8
% Phospholipids	28,7±2,8	28,6±3,0	27,1±2,9	27,5±3,2	27,8±3,5	34,8±3,6	35,5±4,7	34,5±3,8
% ApoA-I	N/A	N/A	N/A	N/A	N/A	42,1±4,6	42,0±4,3	43,0±3,6
% ApoB	23,6±1,3	23,5±1,3	21,3±1,1	21,3±1,0	20,3±1,6	N/A	N/A	N/A

Table 1. Composition of lipoprotein particles before and after pre-incubation. Content of cholesterol, triglycerides, phospholipids, apoA-I (HDL) and apoB (LDL) is shown. Data is expressed as mass of lipid or protein as a percentage of the total mass of the particle. Numbers are the mean of 9 experiments.

FIGURE LEGENDS

Figure 1

Cytokine release by human monocytes promoted by LDL(+), LDL(-), LDL(-) co-incubated with HDL and LDL(-) pre-incubated with HDL or apoA-I followed by re-isolation. Monocytes were incubated for 20 hours with LDL(+) or LDL(-). Afterwards, the release of MCP-1, IL-6, IL-8 and IL-10 was evaluated by ELISA. Cytokine release is expressed in ng/10^6 cells. Numbers are expressed as mean of 4 experiments. * indicates statistically significant difference respective to control LDL(+)); # indicates statistically significant difference respective to control LDL(-); $p<0.068$.

Figure 2

Cytokine release by human monocytes promoted by untreated HDL or HDL treated with LDL(+) or LDL(-) and subsequently re-isolated. Monocytes were incubated with HDL for 20 hours, and afterwards the release of MCP-1, IL-6, IL-8 and IL-10 was evaluated by ELISA. Cytokine release is expressed in ng/10^6 cells. Numbers are expressed as mean of 4 experiments. # indicates statistically significant difference respective to control HDL; $p<0.068$.

Figure 3

NEFA content of LDL(+), LDL(-) and HDL untreated and after indicated treatment. NEFA content is expressed as mol NEFA/mol apoB for LDL and mol NEFA/mol apoA-I for HDL. a) LDL(-) was incubated with HDL or apoA-I, followed by re-isolation and determination of NEFA content. b) HDL was incubated with LDL(+) or LDL(-), followed by re-isolation and determination of NEFA content. Data are the mean ± SD of 7 experiments. * indicates statistically significant difference respective to LDL(+); # indicates statistically significant difference respective to untreated LDL(-) (a) or untreated HDL (b); $p<0.05$.

Figure 4

Agarose electrophoresis of LDL(+), LDL(-) and HDL untreated and after indicated treatment.