



Vitamins C and E prevent endothelial VEGF and VEGFR-2 overexpression induced by porcine hypercholesterolemic LDL

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Abstract

Objective: Vascular endothelial growth factor (VEGF) is believed to play a role in the development of atherosclerosis and has been found to be increased in hypercholesterolemia. We examined the hypothesis that endothelial VEGF and VEGF receptor-2 (VEGFR-2) expression is upregulated by hypercholesterolemic low-density lipoprotein (LDL) and, because it could be driven by oxidative stress, we tested whether vitamin C and E supplementation could modulate it.

Methods: Native LDL were characterized after isolation from adult normal (C-LDL), hypercholesterolemic (HC-LDL) and hypercholesterolemic mini-pigs receiving vitamins C and E (HCV-LDL). VEGF, VEGFR-2, HIF-1 α and superoxide anion (O₂⁻) productions were measured in porcine coronary endothelial cells (ECs) incubated for 48 h with native LDL. The effect of exogenous ascorbic acid and α - or β -tocopherol was also studied.

Results: HC-LDL, with high cholesterol ($P < 0.05$) and reduced tocopherol/cholesterol ratio ($P < 0.05$), increased significantly VEGF and VEGFR-2 ($p < 0.001$) in EC, associated with higher O₂⁻ and HIF-1 α expression, in comparison with C-LDL and HCV-LDL. The addition of vitamin C and α - or β -tocopherol to the culture medium prevented the induction of VEGF and VEGFR-2 expression by HC-LDL, both at mRNA and protein levels.

Conclusions: Our data suggest HC-LDL induce endothelial VEGF and VEGFR-2 overexpression at least by increasing oxidative stress, and HIF-1 α is one of the signaling mechanisms involved. Prevention of VEGF and VEGFR-2 upregulation could help explain the beneficial effects of vitamins C and E in hypercholesterolemia-induced experimental atherosclerosis.

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1. Introduction

Coronary artery disease is a major cause of morbidity and mortality worldwide. Hypercholesterolemia, a major risk factor for coronary artery disease, is characterized by impaired endothelial function of the coronary arteries and has been used in experimental animals as a surrogate for

early coronary atherosclerosis. Even in the absence of overt atherosclerotic changes in the vascular wall, these functional abnormalities are associated with subtle morphological alterations, such as increased density of vasa vasorum in the coronary adventitia [1] and augmented vascular permeability [2]. It has been proposed that these vascular responses may be mediated by the release of growth factors, such as vascular endothelial growth factor-A (VEGF) [3].

VEGF, the main factor responsible for angiogenesis and vascular permeability, is a 45-kDa glycoprotein secreted in the vascular wall by endothelial cell (EC) and smooth muscle cells (VSMC). Its expression is regulated mainly by

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oxidative stress [4], hypoxia [5] and nitric oxide (NO) [6,7]. The biological effects of VEGF are mediated mainly by two tyrosine kinase receptors: VEGFR-1 (Flt-1) and VEGFR-2 (flk-1/KDR). Endothelial VEGFR-2 expression is essential for vasculogenesis, angiogenesis and hematopoiesis [8]. This proangiogenic cytokine has been used in clinical trials to stimulate collateral vessel formation for the treatment of ischemic heart disease [9], whereas experimental studies have shown that VEGF-mediated angiogenesis is not only necessary but sufficient for atherosclerotic plaque growth [10].

Hypercholesterolemia is also characterized by increased oxidative stress and low NO bioavailability, two mechanisms known to contribute to atherosclerotic plaque development that are also involved in the regulation of VEGF expression [4,6]. Increased VEGF levels have been reported in plasma from hypercholesterolemic individuals [11], in coronary arteries from hypercholesterolemic pigs [3] and in the vascular wall of apolipoprotein E-deficient (apoE^{-/-}) mice [12]. In this context, assuming that VEGF acting through VEGFR-2 may be one of the factors responsible for the increased permeability and adventitial angiogenesis observed in porcine hypercholesterolemia [3], we hypothesized that native LDL isolated from hypercholesterolemic animals (HC-LDL) could differentially upregulate either of these genes in porcine coronary endothelial cells.

Whereas there is still controversy as to the potential role of antioxidant strategies in cardiovascular disease prevention [13,14], we and others have convincingly shown the potential for different dietary antioxidants to help prevent the development and progression of atherosclerosis in certain experimental models, giving support to this hypothesis [15–17].

Considering that changes in endothelial expression of VEGF and/or VEGFR-2 induced by HC-LDL could be a consequence of increased oxidative stress, we examined the effect of LDL from hypercholesterolemic pigs supplemented with vitamins C and E on porcine coronary endothelial cell cultures. The effect of exogenous addition of vitamins C and α - or β -tocopherol was also investigated, to elucidate whether antioxidant mechanisms can fully account for the effects of vitamin supplementation.

2. Methods

2.1. Experimental protocol

A total of 18 male Yucatan miniature pigs (4-months old; mean weight, 33 ± 2 kg), were procured from our breeding center and maintained in the animal facilities of CIFA (Centro de Investigación en Fármaco-Biología Aplicada) a GLP-accredited center at the University of Navarra (Pamplona, Spain). The investigation conforms with the Guide for the Care and Use of Laboratory

Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Animal Research Ethics Committee of University of Navarra.

Animals were divided into three groups and fed for 12 weeks, using the following dietary formulations: standard porcine chow (Porcisanthers, Sanders, Spain; C, $n=6$); high-cholesterol diet (HC, $n=6$) containing 24.5% animal lard, 4% cholesterol (Roig Farma, Spain) and 1.5% biliar extract (Roig Farma); and a high-cholesterol diet plus vitamins C and E (HCV, $n=6$) given in the same way as HC for 3 weeks, and supplemented thereafter 9 weeks with 1 g ascorbic acid and 1000 IU D,L- α -tocopherol acetate (Roig Farma) per animal and day. This antioxidant supplementation proved to be efficient at preventing eNOS down-regulation in vivo [16].

2.2. Low-density lipoprotein isolation

Low-density lipoproteins (LDLs) in a density range of 1.019–1.063 g/ml were isolated from porcine plasma by density gradient ultracentrifugation as described [16]. Isolation of LDL from hypercholesterolemic plasmas required a second ultracentrifugation, to separate LDL from VLDL+IDL. EDTA (1 mmol/l, pH 7.4) and butylated-hydroxytoluene (BHT, 1 μ mol/l) were added to prevent oxidation of LDL throughout the isolation. LDL protein was determined by Lowry's method [18], and cholesterol was measured by standard enzymatic assay. LDL lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS) [12]. LDL characterization was completed by electrophoretic mobility on 3% SDS-polyacrylamide gels (SDS-PAGE) and measurement of tocopherol content. LDLs were dialyzed overnight against cell culture medium (10,000 MWCO Slide-A-Lyzer, Pierce, USA) before addition to cell cultures.

2.3. Cell culture

Coronary arteries from untreated pigs ($n=15$) were used to obtain normal EC cultures as previously described [16]. Experiments were carried out on subcultured passage 2 to 3 cells, after reaching confluency and 48 h of adaptation to serum-free endothelial medium. α -Tocopherol was from Merck, ascorbic acid was from Sigma, β -tocopherol was generously provided by Dr. Christine Gartner (Cognis, Germany); all the other cell culture reagents were from GIBCO (Invitrogen SA, Spain).

2.4. RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen) and reverse transcribed with oligo(dT) primers with M-MLV reverse transcriptase (Invitrogen), in the presence of SuperRNase In (Ambion, USA). About 300 ng reverse-transcribed RNA was primed with

oligonucleotides specific for VEGF (5'-GAGACCCTGG-TGGACATC-3' and 5'-TTTCTTTGGTCTGCATTC-3'), VEGFR-2 (5'-GACTGTGGCGAAGTGTTC-3' and 5'-GTGCAGGGGAGGGTTGGCGTAG-3') and β -actin (5'-GTGCGGGACATCAAGGAGAA-3' and 5'-AGGAAGGAGGGCTGGAAGAG-3'). Real-time PCR was performed as described previously (95 °C for 10 min and run for 40 cycles at 95 °C for 15 s and 60 °C for 1 min) with SYBR Green PCR Master Mix (Applied Biosystems, Spain) on the ABI PRISM 5700 Detection System (Applied Biosystems) [12]. Potential genomic DNA contamination was excluded by using intron-encompassing primers. All samples were assayed in triplicate and normalized based on their β -actin content.

2.5. Protein extraction, electrophoresis and Western blotting

Protein from cell lysates was extracted after RNA isolation, following TRIzol manufacturer's instructions. VEGF and HIF-1 α were quantified by Western blotting: conditioned medium or endothelial cell homogenates (10 μ g total protein) were subjected to 12% SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with anti-VEGF (1:1000; sc7269, Santa Cruz Biotechnologies, USA) or anti-HIF-1 α (1:1000; sc10790, Santa Cruz Biotechnologies), followed by a peroxidase-linked sheep anti-mouse or donkey anti-rabbit IgG (1:5000; Amersham Biosciences, Spain). Blots were developed by enhanced chemiluminescence according to the manufacturer's instructions (ECL, Amersham Biosciences). VEGFR-2 expression in cell lysates was similarly assessed, subjected to 6% SDS-PAGE, probed with anti-VEGFR-2 (1:1000; sc6251, Santa Cruz Biotechnologies) and developed by enhanced chemiluminescence (ECL Plus, Amersham Biosciences). Even loading was verified by Ponceau red staining.

2.6. Endothelial cell migration assay

This assay was performed in 24-well transwells (5- μ m pore; Corning, Spain) as described [19]. Briefly, 40,000 ECs resuspended in 100 μ l DMEM were seeded into the upper chamber, and the lower chamber was filled with 600 μ l conditioned medium from native LDL-treated ECs. These

chambers were incubated for 6 h at 37 °C with 5% CO₂ to allow cells to migrate through the membranes. The non-migrated cells were scraped from the upper surface of the membrane with cotton swabs. After a rinse with phosphate-buffered saline, the membrane was stained with Diff-Quick Solution (Dade Behring). Five representative fields in each well were counted under the microscope at 200 \times magnification to determine the number of migrated cells.

2.7. Measurement of superoxide radical (O₂⁻) formation

O₂⁻ production by endothelial cells was measured by lucigenin-enhanced (5 μ M) chemiluminescence, using a 96-well microplate luminometer (Luminoscan Ascent, Thermo Labsystems, Finland). Briefly, confluent EC cultures in serum-free medium were treated with native LDL for 48 h at 37 °C. After washing, EC were incubated in HEPES-modified Krebs buffer (pH 7.4) containing NADPH (100 μ M) for 30 min, and dark-adapted lucigenin (5 μ M) was added into the wells just before reading. Light emission was recorded and expressed as mean relative light units/s (RLU/s) over 30 min. The specificity of O₂⁻ thus measured was confirmed by adding superoxide dismutase (1000 units/ml). Each experiment was performed at least in triplicate.

2.8. Statistical methods

Data are presented as mean \pm standard deviation. Analysis of variance (ANOVA) or Kruskal–Wallis test was applied to assess differences between treatment groups. After a significant ANOVA or Kruskal–Wallis, comparisons were made with Bonferroni post hoc or Mann–Whitney test, respectively. A value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. LDL characterization

Analysis of LDL from the different experimental groups showed that HC-LDL lipid load was significantly increased ($P < 0.05$) in comparison with C-LDL, being HCV-LDL

Table 1
Cholesterol and tocopherol content in plasma and LDL

| Group | Plasma | | LDL | | | |
|-------|----------------------------|-------------------------------|----------------------------------|--|-------------------------------|--------------------------------|
| | Total cholesterol (mM) | Tocopherol (μ M) | μ mol cholesterol/mg protein | nmol tocopherol/ μ mol cholesterol | nmol tocopherol/mg protein | μ M MDA/protein |
| C | 1.6 \pm 0.1 | 1.9 \pm 0.9 | 4.16 \pm 0.81 | 1.08 \pm 0.16 | 4.50 \pm 1.99 | 0.82 \pm 0.19 |
| HC | 5.9 \pm 1.5 ^a | 1.8 \pm 0.5 | 8.58 \pm 1.34 ^b | 0.50 \pm 0.09 ^b | 4.29 \pm 2.13 | 0.88 \pm 0.37 |
| HCV | 6.3 \pm 2.1 ^a | 15.9 \pm 1.9 ^{a,c} | 12.99 \pm 1.34 ^{b,d} | 1.12 \pm 0.09 | 14.53 \pm 3.57 ^a | 0.26 \pm 0.15 ^{a,c} |

^a Statistical significant difference from C, ($P < 0.01$).

^b Statistical significant difference from C, ($P < 0.05$).

^c Statistical significant difference between HC and HCV, ($P < 0.01$).

^d Statistical significant difference between HC and HCV, ($P < 0.05$).

even higher ($P<0.05$; Table 1). Vitamin E content of LDL, expressed as tocopherol/protein ratio, was similar in C and HC groups while it was significantly higher in HCV ($p<0.01$), as expected from the observed plasmatic levels of tocopherol. Nevertheless, LDL from hypercholesterolemic animals exhibited a significantly decreased tocopherol/cholesterol ratio ($p<0.05$), as compared with C and HCV groups (Table 1).

As shown in Fig. 1, changes in lipid composition affected LDL density and size. LDL from hypercholesterolemic pigs migrated higher than controls in the density gradient after plasma ultracentrifugation (Fig. 1), indicating lower density. Native LDL from different groups also exhibited different electrophoretic mobilities: HC- and HCV-LDL migrated more slowly than C-LDL in the 3% SDS-polyacrylamide gels (Fig. 1).

3.2. Effect of native LDL on VEGF and VEGFR-2 expression

We consistently observed that incubation of normal porcine coronary EC in the presence of 100 $\mu\text{g/ml}$ C-LDL for 48 h did not modify cellular VEGF expression. However, as shown in Fig. 2A, the same concentration of HC-LDL induced a significant increase of endothelial VEGF mRNA ($p<0.001$), which paralleled a higher VEGF protein content in the cell lysates and culture supernatants ($p<0.001$, Fig. 2 B and C). Interestingly, conditioned medium from HC-LDL-treated ECs significantly enhanced endothelial cell migration in vitro ($p<0.05$, Fig 3).

Because VEGFR-2 mediates VEGF angiogenic activity, we further analyzed the endothelial VEGFR-2 expression in

the same cultures. As expected, incubation of porcine coronary ECs with HC-LDL increased significantly VEGFR-2 mRNA, as compared with either nonstimulated or C-LDL-treated cells ($p<0.001$; Fig. 4A). A significantly higher VEGFR-2 protein content could also be demonstrated in cell lysates ($p<0.001$; Fig. 4B).

HCV-LDL, even when it carried more cholesterol than HC-LDL, behaved like C-LDL and did not modify endothelial VEGF and VEGFR-2 expression, both at mRNA and protein level (Figs. 2 and 4). Moreover, chemotactic activity of conditioned medium from HCV-LDL-treated ECs was similar to the one from C-LDL-treated cells (Fig. 3).

3.3. Effect of native LDL on endothelial O_2^- production and HIF-1 α expression

The HC-LDL-increased endothelial VEGF expression and previous reports describing VEGF induction by oxidative stress [4] led us to study the effect of LDL on ECs superoxide anion production. In marked contrast with C- and HCV-LDL, HC-LDL induced a significant increase in endothelial O_2^- generation ($p<0.05$), as detected by lucigenin chemiluminescence (Fig. 5A). O_2^- production in ECs treated with C- and HCV-LDL did not differ from cells maintained in the absence of LDL.

Based on the recently reported link between NADPH oxidase, HIF-1 α , VEGF and angiogenesis in atheroma [20], we also measured endothelial HIF-1 α expression. HC-LDL-treated ECs showed increased expression of HIF-1 α protein ($p<0.05$), while C- and HCV-LDL-treated cells exhibited expression levels similar to basal (Fig. 5B).

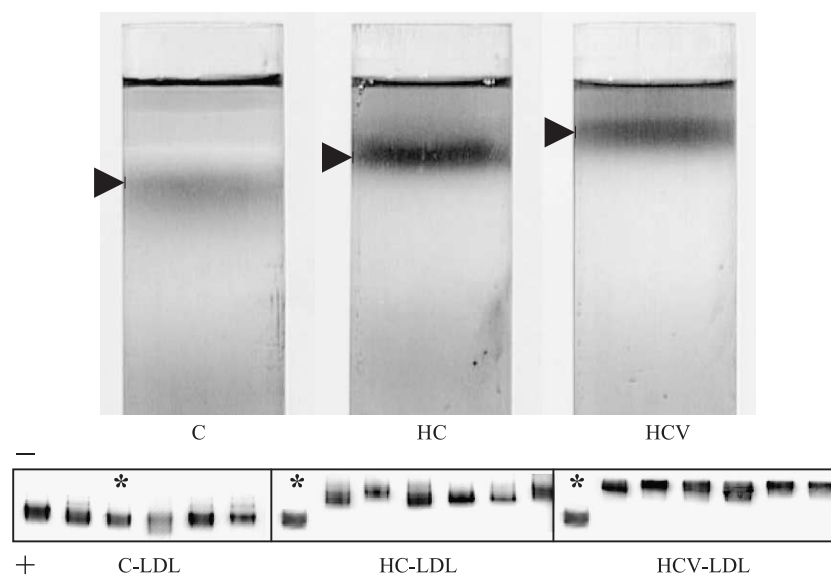


Fig. 1. Characterization of LDL from the different experimental groups. Upper panel: relative lipoprotein densities after density gradient ultracentrifugation of Coomassie blue and Sudan black-stained plasmas from control (C), hypercholesterolemic (HC) or vitamin-supplemented hypercholesterolemic pigs (HCV). Arrows indicate position of LDL band. Lower panel: LDL electrophoretic mobility in 3% polyacrylamide gel. A C-LDL sample (*) was included in each gel to be used as internal control. LDL from hypercholesterolemic pigs showed lower density and migrated slower than the control ones.

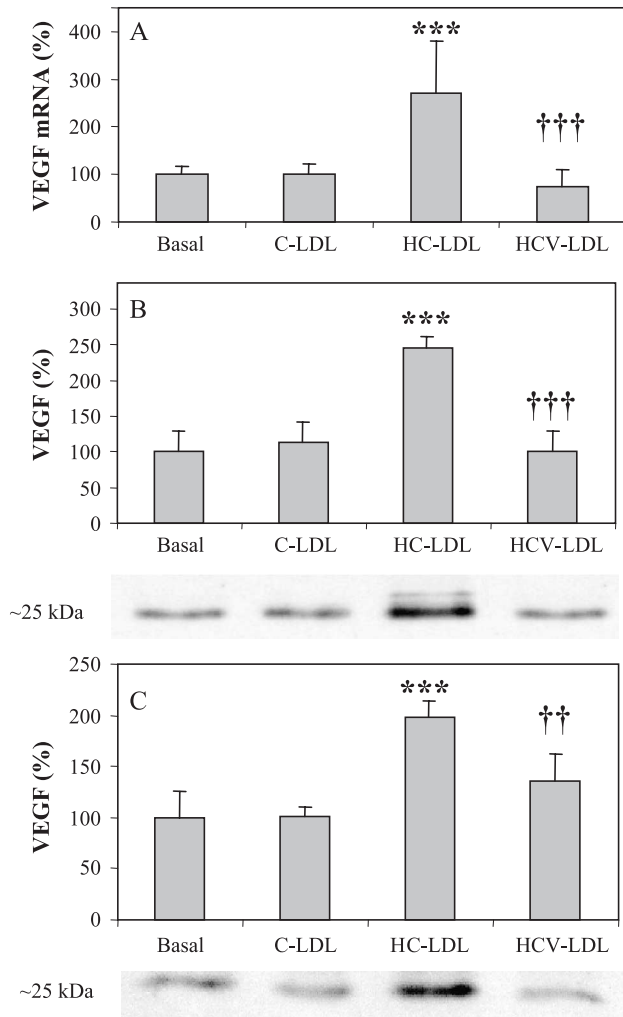


Fig. 2. Effect of native LDL on endothelial VEGF expression. Coronary endothelial cells were incubated for 48 h with 100 $\mu\text{g}(\text{prot})/\text{ml}$ native LDL from control (C-LDL), hypercholesterolemic (HC-LDL) or vitamin-supplemented hypercholesterolemic pigs (HCV-LDL). (A) VEGF mRNA expression, assessed by RT-qPCR and corrected by β -actin mRNA content, reported as percentage increase with respect to untreated cells (basal). VEGF protein, assessed by Western blot in cell homogenates (B) and conditioned culture medium (C), reported as percentage increase with respect to untreated cells. HC-LDL selectively induced VEGF mRNA and protein. Bars show the mean \pm S.D. for 10 independent experiments. Statistically significant differences from basal are marked as *** ($P < 0.001$), and from HC-LDL as ††† ($P < 0.001$).

3.4. VEGF and VEGFR-2 expression after addition of vitamins C and E

After observing that LDL from hypercholesterolemic pigs supplemented with vitamins C and E did not increase endothelial VEGF and VEGFR-2 expression, and because VEGF could be induced by oxidative stress, we further studied the effect of HC-LDL on ECs incubated with 15 μM α -tocopherol (similar to the plasmatic concentration observed in vitamin-supplemented pigs) and an equimolar amount of ascorbic acid.

The combination of ascorbic acid and α -tocopherol blocked the induction of VEGF and VEGFR-2 expression

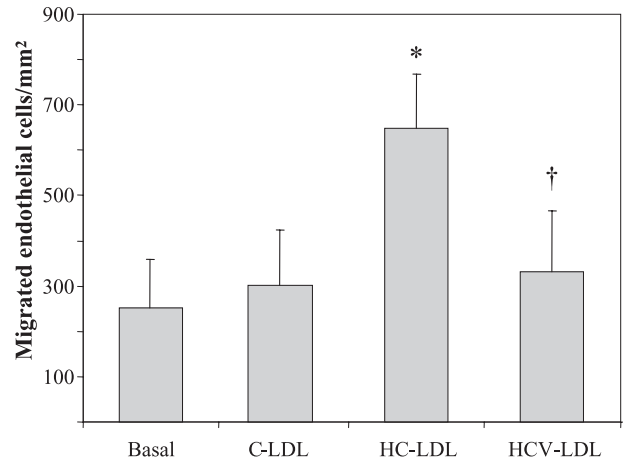


Fig. 3. Effect of native LDL on endothelial chemotaxis. As described in Methods, conditioned medium from coronary endothelial cells incubated for 48 h with 100 $\mu\text{g}(\text{prot})/\text{ml}$ native LDL from control (C-LDL), hypercholesterolemic (HC-LDL) or vitamin-supplemented hypercholesterolemic pigs (HCV-LDL) was placed in the bottom chambers. After 6-h incubation, the number of migrated cells was higher in the wells with HC-LDL supernatants. Bars show the mean \pm S.D. for five independent experiments. Statistically significant differences from basal are marked as * ($P < 0.05$), and from HC-LDL as † ($P < 0.05$).

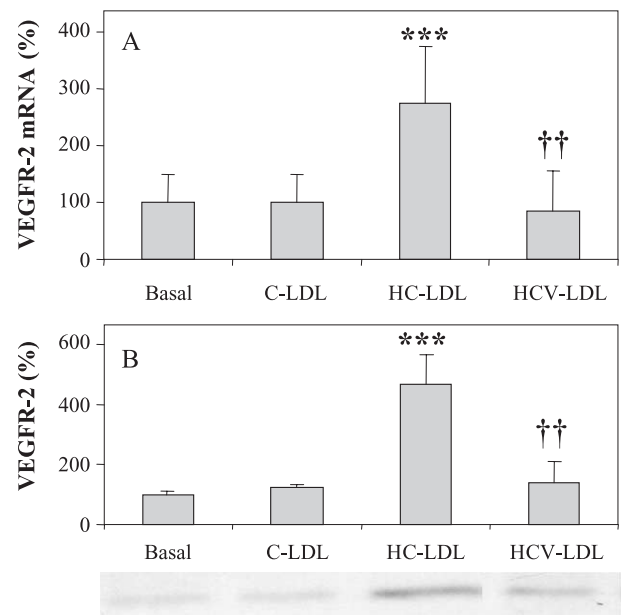


Fig. 4. Effect of native LDL on endothelial VEGFR-2 expression. Coronary endothelial cells were incubated for 48 h with 100 $\mu\text{g}(\text{prot})/\text{ml}$ native LDL from control (C-LDL), hypercholesterolemic (HC-LDL) or vitamin-supplemented hypercholesterolemic pigs (HCV-LDL). (A) VEGFR-2 mRNA expression, assessed by RT-qPCR and corrected by β -actin mRNA content, reported as percentage increase with respect to untreated cells (basal). (B) VEGFR-2 protein in cell homogenates was assessed by Western blot and reported as percentage increase with respect to untreated cells (basal). HC-LDL selectively induced VEGFR-2 mRNA and protein. Bars show the mean \pm S.D. for 10 independent experiments. Statistically significant differences from basal are marked as *** ($P < 0.001$), and from HC-LDL as †† ($P < 0.01$).

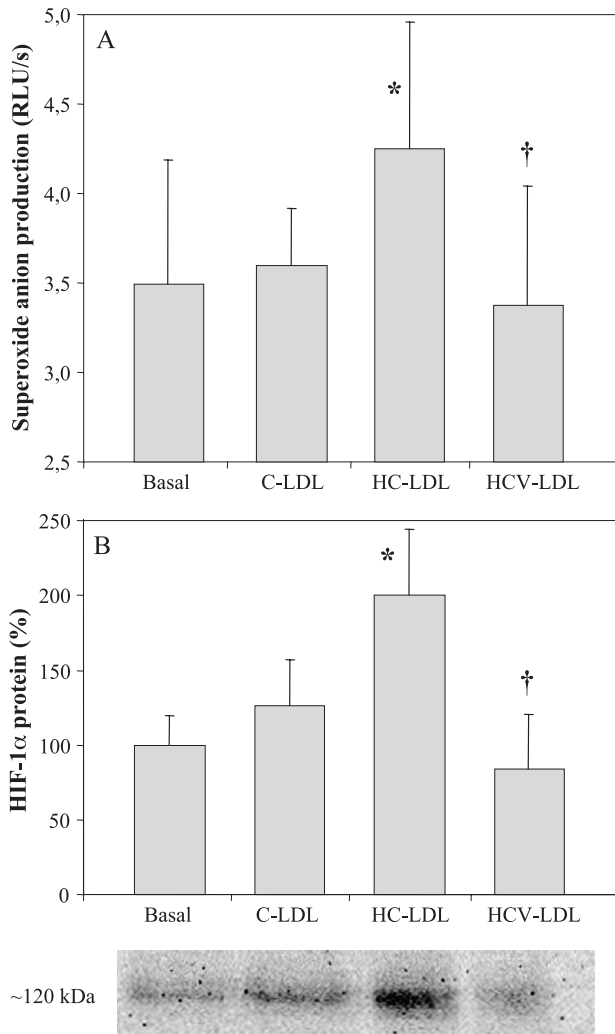


Fig. 5. Effect of native LDL on endothelial O_2^- synthesis and HIF-1 α expression. Coronary endothelial cells were incubated for 48 h with 100 μ g(prot)/ml native LDL from control (C-LDL), hypercholesterolemic (HC-LDL) or vitamin-supplemented hypercholesterolemic pigs (HCV-LDL). (A) O_2^- production, assessed by lucigenin chemiluminescence, reported as relative light units/s (RLU/s). Bars show the mean \pm S.D. for nine independent experiments. (B) HIF-1 α protein in cell homogenates was assessed by Western blot and reported as percentage increase with respect to untreated cells (basal). Bars show the mean \pm S.D. for three independent experiments. Statistically significant differences from basal are marked as * ($P < 0.05$), and from HC-LDL as † ($P < 0.05$).

by HC-LDL in porcine coronary ECs, both at mRNA and protein level (Figs. 6 and 7). Similarly, ascorbic acid and β -tocopherol also completely prevented VEGF and VEGFR-2 overexpression in HC-LDL-treated EC. Addition of ascorbic acid and α - or β -tocopherol per se, in the absence of LDL, did not modify endothelial VEGF or VEGFR-2 expression (Figs. 6 and 7).

4. Discussion

The present study is the first to show that native LDL isolated from hypercholesterolemic animals significantly

upregulated VEGF and VEGFR-2 expression in coronary EC in vitro, associated with higher O_2^- production and HIF-1 α expression, whereas LDL from hypercholesterolemic pigs treated with vitamins C and E did not show such a stimulatory effect. Moreover, exogenous addition of vitamins C and E to the culture medium completely prevented VEGF induction by HC-LDL, further suggesting an oxidative stress-mediated mechanism.

VEGF has a dual role in vascular homeostasis: it is not only the principal agent responsible for angiogenesis, vascular permeability and maintenance of endothelial

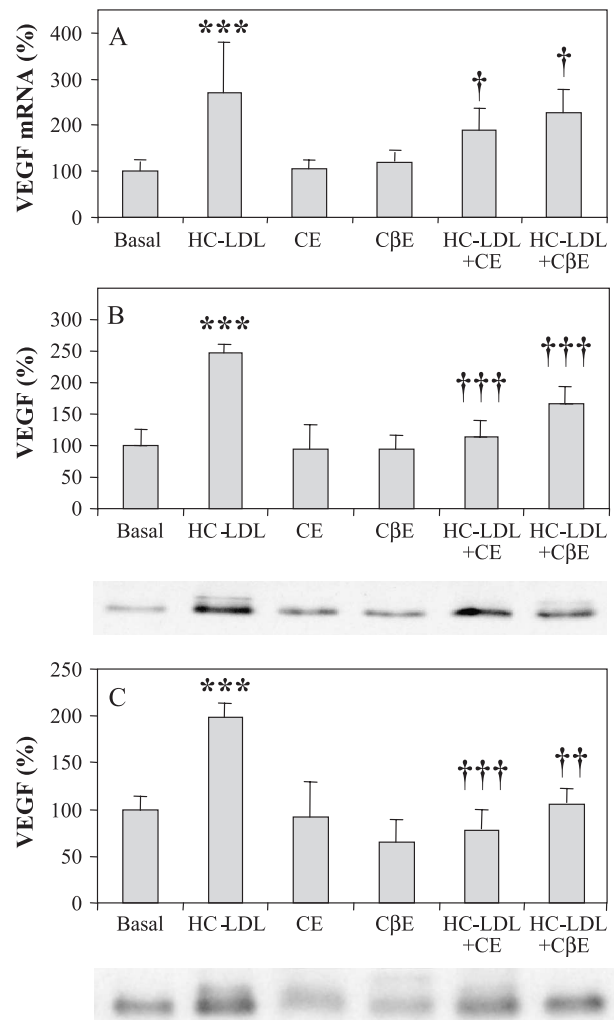


Fig. 6. Effect of vitamins C and E on endothelial VEGF expression. Coronary endothelial cells were incubated for 48 h with 100 μ g(prot)/ml HC-LDL in the presence of ascorbic acid (15 μ M)+ β -tocopherol (15 μ M; CE) or ascorbic acid (15 μ M)+ α -tocopherol (15 μ M; C β E). (A) mRNA expression, assessed by RT-qPCR and corrected by β -actin mRNA content, reported as percentage increase with respect to untreated cells (basal). VEGF protein, assessed by Western blot in cell homogenates (B) and conditioned culture medium (C), reported as percentage increase with respect to untreated cells. The combination of ascorbic acid and α - or β -tocopherol prevented HC-LDL-induced VEGF overexpression. Bars show the mean \pm S.D. for five independent experiments. Statistically significant differences from basal are marked as *** ($P < 0.001$), and from HC-LDL as † ($P < 0.05$), †† ($P < 0.01$) or ††† ($P < 0.001$).

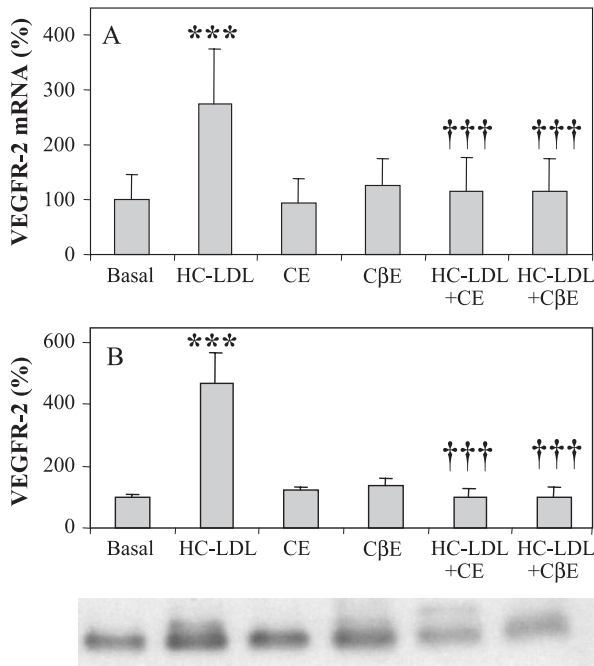


Fig. 7. Vitamin C and E effect on endothelial VEGFR-2 expression. Coronary endothelial cells were incubated for 48 h with 100 $\mu\text{g}(\text{prot})/\text{ml}$ HC-LDL in the presence of ascorbic acid (15 μM)+ β -tocopherol (15 μM ; CE) or ascorbic acid (15 μM)+ α -tocopherol (15 μM ; C β E). (A) VEGFR-2 mRNA expression, assessed by RT-qPCR and corrected by β -actin mRNA content, reported as percentage increase with respect to untreated cells (basal). (B) VEGFR-2 protein in cell homogenates was assessed by Western blot and reported as percentage increase with respect to untreated cells. The combination of ascorbic acid and α - or β -tocopherol prevented HC-LDL-induced VEGFR-2 overexpression. Bars show the mean \pm S.D. for five independent experiments. Statistically significant differences from basal are marked as *** ($P < 0.001$), and from HC-LDL as ††† ($P < 0.001$).

integrity but it also favors the progression and growth of the atherosclerotic plaque [21]. We have previously shown VEGF and VEGFR-2 overexpression in the aorta of apoE $^{-/-}$ mice [12], a well-established model of atherosclerosis showing high cholesterol concentration and increased lipid peroxidation [22], that could be prevented by vitamins C and E [12].

These observations led us to investigate the effect of hypercholesterolemia on endothelial VEGF expression; for this purpose, we incubated porcine coronary EC with native LDL isolated from hypercholesterolemic mini-pigs. We have previously shown in this animal model of hypercholesterolemia multiple manifestations of the vascular inflammatory process such as endothelial dysfunction, reduced endothelial eNOS expression, with increased vascular PAI-1 and MMP-1, that can be prevented or restored by vitamin C and E supplementation [16,17,23]. Aside from potential differences in lipid composition, HC-LDL distinguished from C-LDL mainly in their higher lipid load, a lower tocopherol/cholesterol ratio and reduced electrophoretic mobility. HC-LDL markedly increased endothelial VEGF expression, both at mRNA and at protein level, accompanied by a higher chemotactic activity of the

cell supernatants, in contrast with C-LDL that did not induce significant changes with respect to baseline conditions. In addition, HC-LDL also induced a marked increase in endothelial VEGFR-2, the major mediator of physiological and pathological effects of VEGF on ECs, at mRNA and protein levels, a novel finding not previously described. RT-PCR for measuring VEGF mRNA expression did not allow us to distinguish between the different VEGF isoforms (it amplifies a sequence from exon 3 to 5), but protein changes were evident in the culture medium and in cellular homogenates, reflecting an increase in secreted isoforms as well as in the extracellular matrix-binding ones. Besides an intense band at 25 kDa, protein homogenates from HC-LDL-treated EC showed a higher band that could correspond to a heparin-binding isoform induced by the treatment, although an artifact resulting from the increased amount of VEGF cannot be discarded. Our results confirm previous reports showing ox-LDL upregulation of VEGF expression in human coronary artery endothelial cells [24], although LDL modification by physiological oxidation is not directly comparable to that obtained in vitro with CuSO_4 .

Upregulation of VEGF and VEGFR-2 in the vessel wall could account for the increased vascular permeability [2] and enhanced vasa vasorum neovascularization observed in hypercholesterolemia [1]. Although VEGF upregulation could constitute a vascular homeostatic mechanism for compensating endothelial dysfunction [25], sustained vascular VEGF and VEGFR-2 overexpression could contribute to the atherosclerotic process by favoring lipoprotein infiltration through increased vascular permeability [2], recruiting and/or activating monocytes in early atherosclerosis [26] and inducing angiogenesis in advanced lesions [21]. This hypothesis is further supported by the antiatherosclerotic effect of the angiogenesis inhibitors endostatin and TNP-470 in the apoE $^{-/-}$ mouse [27], and recent reports demonstrating oxidative stress caused by p22phox overexpression induces vascular expression of VEGF, associated with angiogenesis and plaque progression [20].

Although the mechanism by which hypercholesterolemia may induce VEGF upregulation in the vessel wall remains unclear, among the possible pathophysiological stimuli are hypoxia [3], oxidative stress [4] and low NO bioavailability [6]. In our in vitro model, we have found VEGF overexpression to be associated with augmented O_2^- synthesis that could be leading to increased oxidative stress and higher levels of HIF-1 α capable of inducing VEGF gene transcription. Increased HIF-1 α does not reflect necessarily the existence of a hypoxic condition, difficult to explain as a result of HC-LDL treatment, but it could rather be part of the cellular response to the increased oxidative stress [20]. On the other hand, increased VEGF by itself could be enough to upregulate VEGFR-2 expression, as previously described [28]. Therefore, VEGFR-2 augmentation would be conceivable subsequent to VEGF induction by increased oxidative stress. However, further studies are required,

analyzing the balance between antioxidant (i.e., superoxide dismutase, catalase and glutathione peroxidase) and prooxidant genes (i.e., NADPH oxidase, xanthine oxidase), in order to properly characterize the details of this proposed mechanism.

Considering that increased reactive oxygen species, associated with hypercholesterolemia, could be at least one of the mechanisms responsible for the observed VEGF increase, we studied the effect of native LDL isolated from hypercholesterolemic mini-pigs treated with vitamins C and E. In spite of their relative similarity to HC-LDL, in terms of lipid load and electrophoretic mobility, HCV-LDL did not increase VEGF and VEGFR-2 either at mRNA or protein levels, following a pattern similar to C-LDL. The fact that HCV-LDL did not induced endothelial O_2^- synthesis or HIF-1 α protein could be the most likely explanation. However, the increased tocopherol/cholesterol and tocopherol/protein ratios exhibited by HCV-LDL, explaining their lower degree of lipid peroxidation, also suggest they can stand unmodified in the cellular environment for a longer time because of a lower susceptibility to endothelial oxidative modifications, thus avoiding VEGF upregulation due to a direct effect of oxidized LDL. Because changes in LDL lipid composition and/or physical characteristics could also be responsible for the absence of induction, we also studied the effect of treating ECs with vitamin C and α -tocopherol before incubating with HC-LDL. Vitamin C allows α -tocopherol regeneration from α -tocopheroxy radical, hence preventing vitamin E prooxidant activity and tocopherol-mediated peroxidation [29]. The fact that ascorbic acid and α -tocopherol supplementation completely prevented the effect of HC-LDL on VEGF and VEGFR-2 expression further supports our hypothesis.

Nevertheless, it can be speculated that the observed effect of vitamins C and E could not only be related to their antioxidant properties but also to the PKC inhibitory activity of α -tocopherol. The combination of ascorbic acid and β -tocopherol, an equally strong antioxidant in comparison to α -tocopherol but lacking other biological properties present in α -tocopherol (protein kinase C inhibitory and antiproliferative activities) [30], was equally effective at preventing VEGF and VEGFR-2 overexpression, suggesting that the reduction of oxidative stress is a crucial mechanism involved.

In conclusion, we demonstrate that hypercholesterolemic LDL induces endothelial expression of VEGF and VEGFR-2, and suggest that increased oxidative stress may be the main mechanism involved. It is tempting to speculate that this overexpression could be part of a vascular inflammatory process, thus favoring the development of atherosclerosis by facilitating lipoprotein infiltration and monocyte recruitment. However, further studies of the precise pathophysiological effects of vascular VEGF and VEGFR-2 upregulation in hypercholesterolemia, and its pharmacological modulation, are required to define its relationship with the atherosclerotic process.

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