

Dietary supplementation with vitamins C and E prevents downregulation of endothelial NOS expression in hypercholesterolemia in vivo and in vitro

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Abstract

Impaired endothelium-dependent vasodilation has been associated with decreased NO bioavailability in hypercholesterolemia. This study aimed to determine whether antioxidant vitamins C and E could improve hypercholesterolemia-derived endothelial dysfunction in the porcine model, and whether observed in vivo results could be reproduced in vitro by incubation of coronary endothelial cells (EC) in the presence of native low-density lipoproteins (LDL). Adult mini-pigs were fed standard (C), cholesterol rich (HC) or cholesterol rich diet supplemented with vitamins C and E (HCV). Endothelium-dependent blood flow increase in response to acetylcholine was determined. Endothelial nitric oxide synthase (eNOS) expression was measured in arterial samples and in EC incubated with LDL isolated from porcine plasma. Vasomotor response to acetylcholine in HC was significantly lower ($P < 0.05$) than control and HCV. There was a significant ($P < 0.05$) decrease in eNOS immunoreactivity in HC, compared with HCV and control. Native LDL from HC, but not from HCV, induced a significant decrease in eNOS expression. Vitamins C and E treatment improved the endothelium-dependent vasomotor capacity and prevented decreased expression of eNOS in hypercholesterolemic pigs. A similar effect could be demonstrated in vitro, by incubation of EC with native LDL, suggesting that the effect of physiologically-modified LDL on eNOS could have a role in recovering vascular function. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

The vascular endothelium plays an important role in the regulation of the arterial vasomotor tone, mainly by the synthesis and release of vasoactive substances such as endothelium-derived relaxing factor, identified as nitric oxide (NO) or a closely related compound [1], endothelium-derived hyperpolarizing factor and prostacyclin. NO reduces vessel tone, interferes with monocyte

and leukocyte adhesion to vascular endothelium, decreases platelet aggregation and adhesion, and inhibits smooth muscle proliferation and migration. In vivo, the activity of the L-arginine-NO pathway results from a balance between the synthesis and breakdown of NO [2].

Hyperlipidemia and atherosclerosis are associated with abnormalities of the vascular function characterized both by an increase in the response to specific vasoconstrictor agents and marked attenuation of endothelium-dependent relaxation, although the exact mechanisms still remain unknown [3]. There is a large body of evidence supporting the notion that oxidation of lipids and lipoproteins may contribute to the patho-

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genesis of atherosclerosis, suggesting a number of potential mechanisms by which intra-arterial oxidation of lipoproteins might initiate and promote atherogenesis [4,5]. It has been shown *in vitro* that impairment of NO production in hypercholesterolemia can be due to reduced transcription and enhanced breakdown of endothelial constitutive NO synthase (eNOS) transcripts [6], or modulation of caveolin abundance in endothelial cells (EC) [7]. Alternatively, diminished NO activity could be due to a decrease in NO production, by reduced availability of L-arginine or tetrahydrobiopterin, or caused by enhanced catabolism: increased endothelial superoxide production may inactivate endothelium-derived NO and provide a source for other oxygen radicals, contributing to the early atherosclerotic process [8].

A number of studies in different animal models have considered the potential for different dietary antioxidants to help prevent development and progression of atherosclerosis. Vitamin C, vitamin E, β -carotene, probucol and selenium, alone or combined, have been evaluated in several studies and most of the experimental evidence helps substantiate this hypothesis [5,9], but is not yet conclusive [10]. Vascular dysfunction associated with hypercholesterolemia has been shown to be prevented in experimental animals and human individuals by dietary supplementation with vitamin C and/or E [11,12].

In the present study we investigated whether antioxidant vitamins C and E could improve hypercholesterolemia-derived endothelial dysfunction in the porcine model, and whether observed *in vivo* results could be reproduced *in vitro* by incubation of coronary EC in the presence of native low-density lipoproteins (LDL). Treatment with vitamins was initiated 3 weeks after the induction of hypercholesterolemia, to more closely mimic a potential clinical situation.

2. Material and methods

2.1. Experimental design

Thirty-six male Yucatan miniature pigs (4 months old; mean weight, 33.3 ± 1.69 kg), procured from our breeding center, were maintained in a humidity- and temperature-controlled room. The investigation was performed in accordance with the European Community guidelines for animal ethical care and use of laboratory animals (Directive 86/609), and was approved by the Animal Research Ethics Committee of University of Navarra.

Animals were divided into three groups and fed during 5, 6 or 8 weeks, to assess the potential impact of time on the effect of vitamin supplementation, using the following dietary formulations: standard porcine

chow (Porcisanthers, Sanders, Spain) (C, $n = 12$); high-cholesterol diet (HC, $n = 12$) containing 24.5% animal lard, 4% cholesterol (Roig Farma, Spain) and 1.5% biliar extract (Roig Farma); and a HC plus vitamins C and E (HCV, $n = 12$) fed in the same way as HC for 3 weeks, and supplemented thereafter (up to 5, 6 or 8 weeks) with 1 g ascorbic acid and 1000 IU D,L- α -tocopherol acetate (Roig Farma) per animal and day.

2.2. Animal experiments

Each animal was fasted overnight before the day of the study, but allowed *ad libitum* access to tap water. Animals were sedated, intubated and ventilated through an endotracheal tube. Anesthetic induction was completed by *i.v.* injection of metomidate 2 mg/kg (Esteve Veterinaria, Spain), 0.2 mg/kg pancuronium bromide (Organon, The Netherlands) and 0.05 mg fentanyl (Roche, Switzerland) were given to maintain a constant level of anesthesia. The right internal jugular vein and carotid artery were exposed by cutdown and cannulated. Arterial blood samples for blood cell count and lipid profiles were collected in tubes containing EDTA. After *i.v.* injection of a bolus of 10000 U heparin, a 7F Judkins coronary guiding catheter was used to engage the left internal iliac artery (LIIA). A flexible angioplasty guidewire of 0.35 mm (0.014 in.) was advanced into the lumen of a 3F Doppler catheter (NuVel, NuMed Inc, USA) connected to a 20 MHz pulsed Doppler velocimeter (MDV-20, Millar Instruments, USA), and positioned into the non-branching medial section of the LIIA; the guiding catheter was then retracted to abdominal aorta. Doppler flow velocity (maximum velocity and average peak velocity) was recorded continuously in a chart recorder (Hewlett-Packard, USA).

Animals were allowed a 15 min equilibration period to ensure stability, and 4.8 ml of 0.28 mol/l glucose was selectively infused for 2 min into the LIIA via the infusion lumen of the Doppler catheter. In a similar fashion, 2.4 ml of acetylcholine (Sigma, USA) at concentrations of 10^{-6} , 10^{-5} and 10^{-4} mol/l (to achieve estimated final blood concentrations of 10^{-8} , 10^{-7} and 10^{-6} mol/l, assuming iliac blood flow of approximately 80 ml/min) was selectively infused into the LIIA, lasting 1 min for each concentration. The endothelium-independent vasodilator capacity was assessed by injecting 1 mg nitroglycerin (NTG) into the LIIA. A 5 min recovery period was allowed between each injection, until blood flow returned to basal values.

At the end of the procedure, iliac angiographic images were recorded and animals were sacrificed by an *i.v.* dose of 65 mg/kg pentobarbital (Sigma) followed by a bolus of KCl.

2.3. Blood analysis

Platelet, red and white blood cell count, platelet size, were determined in blood anticoagulated with EDTA, using a Coulter SKTS counter (Coulter Electronics, USA).

Serum total cholesterol (TC), triglycerides (TG) and high density lipoprotein (HDL) cholesterol were measured by an enzymatic method, using a fully automated clinical analyzer (Hitachi 717, Roche). Low-density lipoprotein (LDL) cholesterol was calculated with Friedewald formula [13], modified for the ratio cholesterol/TG in very low-density porcine lipoproteins (8:1).

2.4. Vitamin E content

α -Tocopherol (AT) was measured in plasma and LDL by normal-phase high-performance liquid chromatography [14]. After addition of AT acetate as internal standard to each sample, vitamin E was extracted from 200 μ l of plasma or 50 μ l of LDL (4 μ g cholesterol/ μ l) with hexane (800 μ l), following the addition of methanol (200 μ l). The samples were centrifuged at $1500 \times g$ for 15 min, evaporated under nitrogen and separated on a μ Bondapak C18 column (3.9×300 mm², Waters, USA). The mobile phase consisted of acetonitrile/methanol/H₂O (49:48:3) pumped at a flow rate of 1 ml/min. AT was quantified spectrophotometrically at 292 nm.

2.5. Low-density lipoprotein isolation

LDLs in density range of 1.019–1.063 g/ml were isolated from porcine plasma by density gradient ultracentrifugation [15]. Addition of EDTA (1 mmol/l, pH 7.4) and butylated-hydroxytoluene (BHT, 1 μ mol/l) prevented oxidation of LDL throughout the isolation. The LDL fraction was extensively dialyzed against 0.15 M NaCl, 1 mmol/l EDTA (pH 7.4) and 1 μ mol/l BHT, sterile filtered (0.2- μ m pore size Syringe-Pre-filter Plus, Nalgene, USA), and stored at 4 °C. LDL protein was determined by Lowry's method [16], and cholesterol was measured enzymatically as described [17]. LDL characterization was completed by electrophoretic mobility on 3% SDS-polyacrylamide gels and measurement of AT content. LDLs were dialyzed overnight against cell culture medium (10 000 MWCO Slyde-A-Lyzer, Pierce, USA) before addition to cell cultures.

2.6. Cell culture

Coronary arteries from untreated pigs were used to obtain normal EC cultures by enzymatic dispersion [18]. Arteries were cut longitudinally and intima was digested with 0.2 mg/ml chymotrypsin (Sigma) in PBS for 10 min. EC were gently scraped off with a cotton swab and

transferred to gelatin-coated culture dishes containing M199 culture medium and 20% pig serum (Linus, Spain), supplemented with antibiotic/antimycotic solution. Experiments were carried out on subcultured passage 3–4 cells, after reaching confluency and 48 h of adaptation to serum-free endothelial medium. The purity of cultures was determined on the basis of their characteristic cobblestone morphology and absence of smooth muscle- α -actin staining. AT was from Merck, ascorbic acid was from Sigma, all the other cell culture reagents were from GIBCO-BRL (Invitrogen SA, Spain).

2.7. Protein extraction, electrophoresis and western blotting

After stimulation, EC were washed and disrupted with homogenization buffer containing 1.8 mM NaH₂PO₄, 1.6 mM Na₂HPO₄, 50 mM NaCl, 0.1% SDS, 0.5% sodium azide, 1% Triton X-100, and a protease-inhibitor cocktail (Complete EDTA-free, Roche). Protein concentration was determined according to Bradford [19].

Cell lysates (15 μ g total protein) were subjected to 12% SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and probed with anti-eNOS (1:2000, N30020, Transduction Laboratories, USA), followed by a peroxidase-linked sheep anti-mouse IgG (1:5000, Amersham-Pharmacia Biotech Europe, Germany). Blots were developed by enhanced chemiluminescence according to the manufacturer's instructions. Even loading was verified by Ponceau red staining.

2.8. Tissue processing and eNOS immunostaining

The iliac arterial tree was dissected, perfused with 4% paraformaldehyde at 4 °C for 15 min, and left in 4% paraformaldehyde at 4 °C for 4 h. Arteries were cut to 1 cm long pieces and paraffin embedded (up to 8 pieces per block).

A standard avidin–biotin peroxidase complex method was used for immunostaining [5]. Three-micrometer sections were deparaffinized and rehydrated, endogenous peroxidase was inhibited with 3% H₂O₂/Tris-buffered saline and nonspecific binding was blocked by incubation with 5% normal rabbit serum. Specimens were incubated with a monoclonal antibody recognizing eNOS (1:300, N30020, Transduction Laboratories) at 4 °C overnight. Sections were incubated with biotinylated secondary rabbit anti-mouse antibodies (1:200, Dako, Denmark), and developed with avidin–biotin complexes (1:100, ABC, Dako), according to the manufacturer's instructions.

Immunohistochemical processing was done in batches of 12 slides, including internal control sections (scored

'0' and '3'). At least 2 histological slides from each animal, containing up to 5 sections of the LIIA, were processed as described before and reviewed by five independent observers in a blinded fashion. Arterial sections with atherosclerotic lesions or intimal thickening were excluded from the study. A visual grading score system to quantify endothelial staining for eNOS was established as follows: 0, no staining; 1, positive staining in <25% endothelium; 2, positive staining in >25% but <75%; 3, positive staining in >75%. The score for each slide was calculated as the median score between observers.

2.9. Data analysis

All data are presented as mean value \pm S.E.M. Analysis of variance (ANOVA) or Kruskal–Wallis test were applied to assess differences between treatment groups. After a significant ANOVA or Kruskal–Wallis, comparisons were made with Bonferroni posthoc or Mann–Whitney test, respectively. Statistical significance was accepted at the 95% confidence level ($P < 0.05$). Analysis was performed by using the computer program SIGMASTAT (SPSS Science, USA).

3. Results

3.1. Animal model characteristics

The comparison among animals from the same group sacrificed at different times (5, 6 or 8 weeks) showed no statistical differences in any of the studied parameters, and therefore they are presented together. Animals from all groups had similar body weight at the beginning of the experiment. After treatment, as shown in Table 1, weight gain in HC (89%) and HCV (84%) was higher ($P < 0.05$) than in the control (44%).

There were no differences in baseline values of hematocrit, white blood cell count and platelet count between groups. These values did not change significantly after diet and were similar in all groups.

3.2. Plasma lipids and vitamin E

Baseline concentrations of TC, HDL and LDL were similar in all groups. The hypercholesterolemic diet substantially increased (up to 250%) TC levels in HC and HCV at the time of sacrifice (Table 1). The increase in TC levels could be attributed primarily to elevated LDL levels, although HDL levels were also higher. Treatment with vitamins C and E had no effect on TC and LDL concentration at the time of sacrifice, but HDL concentration in this group was 20% higher than in the HC. None of the experimental diets influenced TG concentration. AT plasma levels in HCV were significantly higher than C and HC ($P < 0.05$), but no differences were observed between the three time periods.

3.3. LDL characterization

Vitamin E content of LDL, expressed as AT/protein ratio, was similar in C and HC groups while it was significantly higher in HCV, as expected from the observed plasmatic levels of AT. Nevertheless, hypercholesterolemic animals exhibited a significantly decreased AT/cholesterol ratio in LDL, as compared with C and HCV groups (Table 2).

HC–LDL lipid load was significantly higher ($P < 0.05$) than C–LDL, being HCV–LDL even higher ($P < 0.05$; Table 2). These changes in lipid composition affected LDL size, as reflected by the different electrophoretic mobilities: HC– and HCV–LDL migrated more slowly than C–LDL on 3% SDS-polyacrylamide gels (data not shown).

Table 1

Animal weight, plasma TC (mmol/l), HDL (mmol/l), LDL (mmol/l), TG (mmol/l) and α -tocopherol (μ mol/l), at baseline (A) and at the end of the experiment (B)

Group	Weight (kg)	Total cholesterol	LDL	HDL	TG	α -tocopherol
<i>A. Before treatment</i>						
C	32 \pm 3	1.64 \pm 0.11	0.60 \pm 0.08	0.90 \pm 0.05	0.47 \pm 0.04	
HC	31 \pm 3	1.56 \pm 0.05	0.54 \pm 0.06	0.85 \pm 0.05	0.49 \pm 0.04	
HCV	31 \pm 3	1.53 \pm 0.06	0.51 \pm 0.03	0.91 \pm 0.05	0.53 \pm 0.03	
<i>B. Sacrifice</i>						
C	46 \pm 1	1.61 \pm 0.08	0.65 \pm 0.06	0.80 \pm 0.04	0.47 \pm 0.05	2.00 \pm 0.41
HC	55 \pm 2	5.02 \pm 0.62**	3.49 \pm 0.53**	1.23 \pm 0.07**	0.43 \pm 0.07	1.67 \pm 0.22
HCV	53 \pm 3	5.29 \pm 0.65**	3.26 \pm 0.45**	1.57 \pm 0.12**†	0.35 \pm 0.05	14.04 \pm 3.66*

Values are expressed as mean \pm S.E.M. Statistically significant differences from control group are marked as * ($P < 0.05$) or ** ($P < 0.01$), and between HC and HCV as † ($P < 0.05$).

Table 2

LDL characteristics: AT content of LDL, expressed in relation to LDL cholesterol or protein, and cholesterol/protein ratio

Group	nmol α -Tocopherol/ μ mol cholesterol	nmol α -Tocopherol/mg protein	μ mol Cholesterol/mg protein
C	1.14 \pm 0.18	4.76 \pm 0.74	4.19 \pm 0.20
HC	0.53 \pm 0.11*	4.52 \pm 0.72	9.52 \pm 0.65*
HCV	1.19 \pm 0.10	13.77 \pm 1.58**	13.11 \pm 0.67*†

HC–LDL show a significant reduction in AT/cholesterol content, while in HCV–LDL this ratio is restored to control levels. C–LDL and HC–LDL carry similar amounts of AT, while HCV–LDL transport a significantly higher amount of AT. Statistically significant differences from control group are marked as *($P < 0.05$) or **($P < 0.01$), and between HC and HCV as †($P < 0.05$).

3.4. Hemodynamics

At the time of sacrifice, increases in blood flow velocity in the LIIA of control animals ranged from 75 to 200%, in response to increasing concentrations (10^{-6} , 10^{-5} and 10^{-4} mol/l) of acetylcholine (Table 3). Infusion with the same volume of saline did not affect blood flow velocity, showing that flow changes were specifically induced by acetylcholine. HC pigs, but not HCV animals, exhibited a significant reduction in the blood flow velocity increase (40–50%) in response to acetylcholine when compared with controls ($P < 0.05$). The endothelium-independent arterial relaxation, in response to NTG perfusion, was similar (about 120%) in all groups.

No significant correlation was found between duration of treatment and increase of blood flow velocity. Since no differences between animals within the same group and sacrificed at different times were observed, data are presented together.

3.5. eNOS expression in vivo

Hematoxylin-eosin staining of arterial sections revealed intact endothelial structure in all groups, with occasional areas of intimal thickening more frequently in the HC animals. Staining for eNOS revealed an overall decrease in the presence of enzyme immunoreactivity after 5–8 weeks of cholesterol-rich diet, in comparison with normal animals (Fig. 1; C, HC). This reduction could be reversed by supplementing the hypercholesterolemic diet with vitamins C and E (Fig.

1; HCV). There were no noticeable differences between animals within the same group and sacrificed at different time points (5, 6 and 8 weeks of diet). Results from the scoring system for constitutive eNOS showing statistically significant differences between HC and control ($P < 0.05$), and HC and HCV ($P < 0.05$) can be observed in Fig. 1 (upper panel). No significant correlation was found between treatment length and eNOS score.

3.6. eNOS expression in vitro

Incubation of normal porcine coronary EC with 100 μ g (protein)/ml LDL induced a time-dependent reduction in eNOS expression in all cases. LDL from HC animals markedly inhibited eNOS expression at 48 and 96 h (60 and 80% reduction in respect to basal value), as compared both with baseline and EC incubated with LDL from control group. However, native LDL from HCV did not exert that inhibitory effect on EC (Fig. 2). Additional experiments were performed (in triplicate), and the addition of ascorbic acid (14 μ M) and AT (14 μ M) to the culture medium completely prevented the inhibitory effect of HC-LDL on eNOS expression ($92 \pm 6\%$ with respect to non-treated cells at 48 h).

4. Discussion

The present study is the first to show that native LDL isolated from hypercholesterolemic animals downregulate eNOS expression in coronary EC in vitro, whereas

Table 3

Changes in iliac artery blood flow velocity (reported in cm/s), in response to increasing doses of acetylcholine and nitroglycerin (1 mg) in the three groups of animals

Group	B	Saline	Acetylcholine			Nitroglycerin
			10^{-6} mol/l	10^{-5} mol/l	10^{-4} mol/l	
C	5.05 \pm 0.93	5.16 \pm 0.98	8.61 \pm 2.29	11.49 \pm 2.32	14.22 \pm 3.34	11.53 \pm 2.14
HC	5.30 \pm 1.47	5.47 \pm 1.61	7.07 \pm 2.5*	9.02 \pm 3.4*	11.16 \pm 3.94*	10.14 \pm 3.82
HCV	5.25 \pm 1.15	5.28 \pm 1.31	8.14 \pm 2.8	11.78 \pm 3.28	15.78 \pm 4.94	10.99 \pm 3.98

Vascular response to acetyl choline was significantly reduced (* $P < 0.05$) in hypercholesterolemic pigs (HC), as compared with controls (C) and vitamin C+E treated hypercholesterolemic (HCV) group.

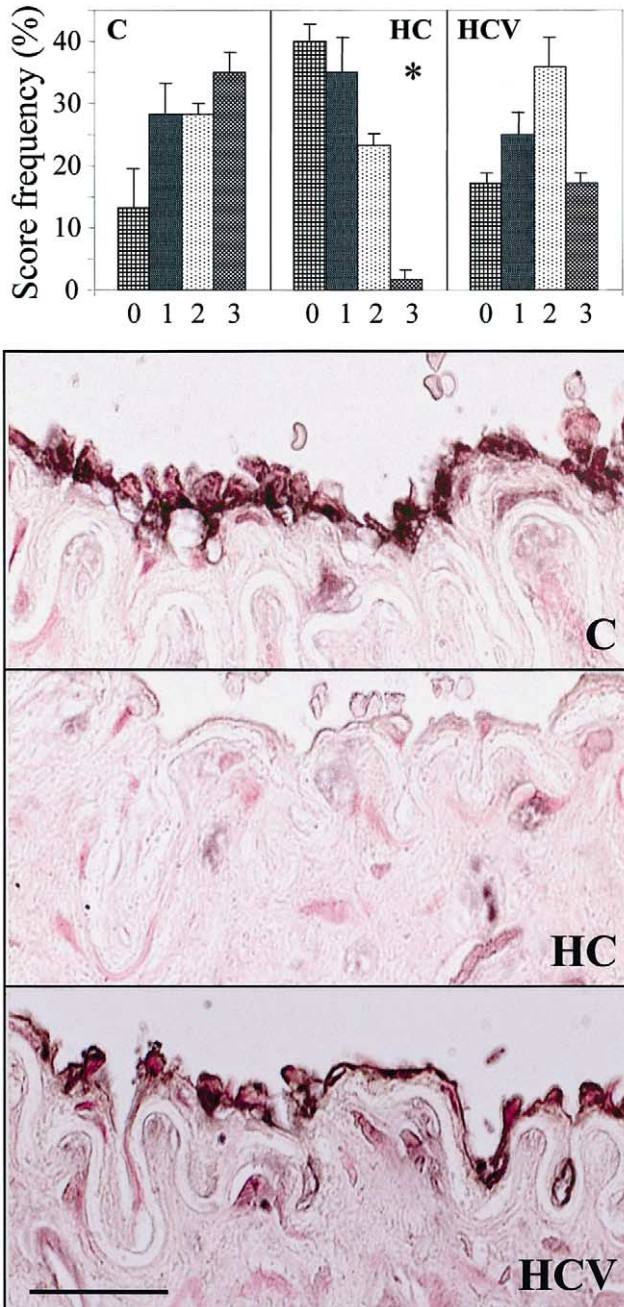


Fig. 1. Upper panel: semi-quantitative analysis of eNOS immunostaining in LIIA. Score frequency is represented on the Y-axis and the different scores for the three experimental groups are on the X-axis (explanations are given in the text). C, control; HC, hypercholesterolemic; HCV hypercholesterolemic treated with vitamins C and E. (* $P < 0.05$; Kruskal–Wallis, Mann–Whitney). Lower panel: representative photomicrographs of LIIA from control animals (C) showing strong endothelial immunostaining signal for eNOS, which is practically absent in HC, whereas HCV animals exhibited a significant recovery in eNOS expression. Scale bar: 10 μ m.

dietary treatment with vitamins C and E prevents this inhibitory effect through LDL modification. These results confirm our in vivo observations: reduced eNOS expression in porcine arteries and impaired

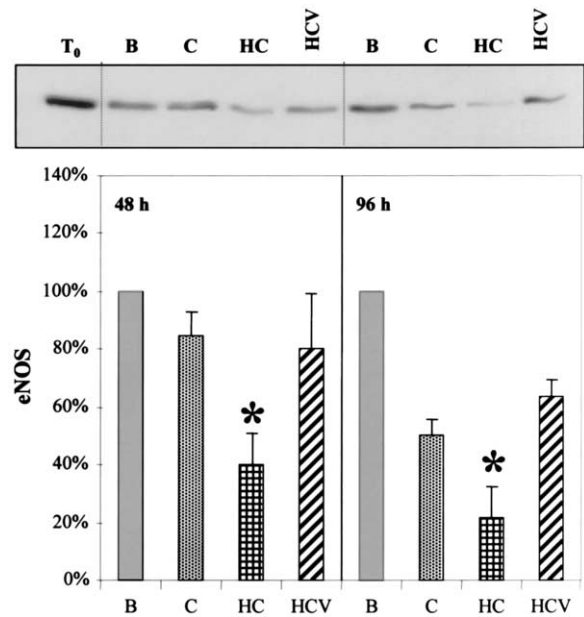


Fig. 2. Coronary porcine EC eNOS expression after incubation with 100 μ g (protein)/ml native LDL from C, HC or HCV pigs, at 48 and 96 h. Upper panel: representative western blot, including samples corresponding to cells before (T_0) and after switching to serum-free media (B). Lower panel: densitometric analysis of five different experiments, eNOS expression referred to basal (B). Incubation of coronary EC with native LDL from hypercholesterolemic animals significantly reduced eNOS expression at 48 and 96 h (* $P < 0.05$), but native LDL from C and HCV did not exert this inhibitory effect. EC were maintained under serum free conditions.

endothelium-dependent vasodilatory function induced by hypercholesterolemia, can be prevented by dietary supplementation with vitamins C and E.

Characteristics of the animal model were similar to previous studies [20]: animals fed a hypercholesterolemic diet showed higher weight gain and plasma cholesterol levels. Dosage of antioxidants was chosen from a similar model demonstrating that animals treated with vitamins C and E have smaller degree of LDL oxidation and best response to vascular injury [21]. Treatment with vitamins C and E increased significantly plasma and LDL concentration of AT. However, it did not modify TG, total and LDL cholesterol levels, but increased significantly the HDL level. We did not find significant differences in hematologic parameters.

Endothelial dysfunction in pigs fed a cholesterol-rich diet was demonstrated by the marked increase of hindlimb vascular resistance, and a fainter and less extended expression of eNOS as previously reported [3]. Observed endothelial dysfunction cannot be attributed exclusively to reduced NO bioavailability, since most of agonist-induced endothelium-dependent dilatation in peripheral circulation seems to be mediated by EDHF [22].

Incubation of coronary EC with HC–LDL induced a marked downregulation of eNOS expression, starting at 48 h and more evident at 96 h, similarly to previous

findings for native and oxidized LDL [23,24]. Interestingly, there is an apparent contradiction with recent reports showing an increase in caveolin, without changes in eNOS expression in bovine aortic EC in the presence of human LDL from subjects with hypercholesterolemia [7]. The different origin (vascular bed and animal) of the cells and the heterologous nature of the stimulus (human LDL vs bovine cells) could account for some of the differences. In vitro effects of LDL could be attenuated by the presence of AT in the culture medium (18 nmol/l), so that differences in eNOS expression could be enhanced in its absence. Our observations suggest that regulation of eNOS expression could be one of the key players in the vascular dysfunction in this model of porcine hypercholesterolemia, although it does not discard other factors acting and performing an important role in the onset and maintenance of the dysfunction.

The observed negative effect of HC-LDL on eNOS expression could be due to their higher susceptibility to oxidation, as reflected by their reduced AT/cholesterol ratio. Although EC can oxidize LDL in vitro, differences in lipid composition between C- and HC-LDL and in LDL aggregability, and the possible higher lipid uptake by EC in the presence of HC-LDL, carrying three times more fat than C-LDL, should be taken into consideration.

Dietary supplementation of hypercholesterolemic animals with vitamins C and E increased significantly AT level in plasma and LDL AT/protein ratio, as expected from the smaller extent of LDL oxidation observed in a similar model [21], and it also brought LDL AT/cholesterol ratio back to normal levels. Vitamin treatment was accompanied by the restoration of vasodilator activity, as had been reported in a rabbit model [25]. This functional improvement was accompanied by an increase in eNOS expression, as immunohistochemically shown in the LIIA of HCV pigs, reaching similar extension and intensity to those observed in controls.

HCV-LDL, in spite of their relative similarity to HC-LDL in size and lipid load, exhibited different effects in vitro, reproducing our in vivo observations. Coronary EC incubated in presence of HCV-LDL for 48 h showed similar eNOS expression to the C-LDL-treated ones, and slightly higher at 96 h. This observation, together with the increased AT/cholesterol and AT/protein ratio for the HCV-LDL, seems to indicate that HCV-LDL can stand unmodified in the cellular environment for a longer time, because of a lesser susceptibility to endothelial oxidative modification, thus avoiding eNOS downregulation due to a direct effect of oxidized LDL. The fact that the simultaneous addition of ascorbic acid and AT prevented completely the inhibitory effect of HC-LDL on eNOS expression further supports this hypothesis. Vitamin E in LDL particles acts as a chain-breaking antioxidant, prevent-

ing lipid peroxidation of polyunsaturated fatty acids and modification of proteins by reactive oxygen species [26]. However, LDL is a major vehicle for vitamin E delivery to peripheral tissues [27] and AT availability for EC could differ according to in vivo experimental conditions, indicating that a direct action of AT on EC metabolism cannot be ruled out [28].

The absence of differences between animals sacrificed at different time periods should be interpreted cautiously: it seems to indicate effects of hypercholesterolemia are maximal at 5 weeks and benefits of vitamin supplementation are maximal after 1 week of treatment. In this sense, recent reports in a porcine model have shown vascular reactivity in animals fed a hypercholesterolemic diet for 2 and 4 weeks was similar to normal animals, but it was significantly attenuated in pigs receiving the same diet for 6 and 12 weeks [29]. Moreover, it has been described in a similar model that serum vitamin E levels remained unchanged after 1 or 3 weeks of dietary supplements [21]. However, in our experimental design time periods could be too short to find significant differences, and small size of experimental groups ($n=4$) compromises statistical sensitivity. Further experimentation should be carried out, in larger sized groups, to precisely define the effect of time on studied parameters.

In our animal model, vitamin C and E supplementation have a clear beneficial effect in hypercholesterolemia-derived vascular dysfunction, in agreement with most of the experimental research work and some clinical trials (Nurses' Health Study, CHAOS) [30]. Their effects have been explained mainly through their antioxidant properties, superoxide anion scavenging activity or direct effect on the vascular wall. In contrast, more recent studies (GISSI and HOPE trials) reported that vitamin E treatment had no positive effects on cardiovascular disease patients [30]. Aside from the intrinsic differences between the human and the various animal models, the higher homogeneity between experimental conditions, together with the exhaustive control of housing conditions and dietary intake, can reasonably explain why results obtained in experimental studies are more consistent, emphasizing that the vitamin E status should be properly assessed before antioxidant treatment in patients with hypercholesterolemia. Defining the most susceptible patient populations will be necessary for the development of specific and efficacious approaches to antioxidant therapy.

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