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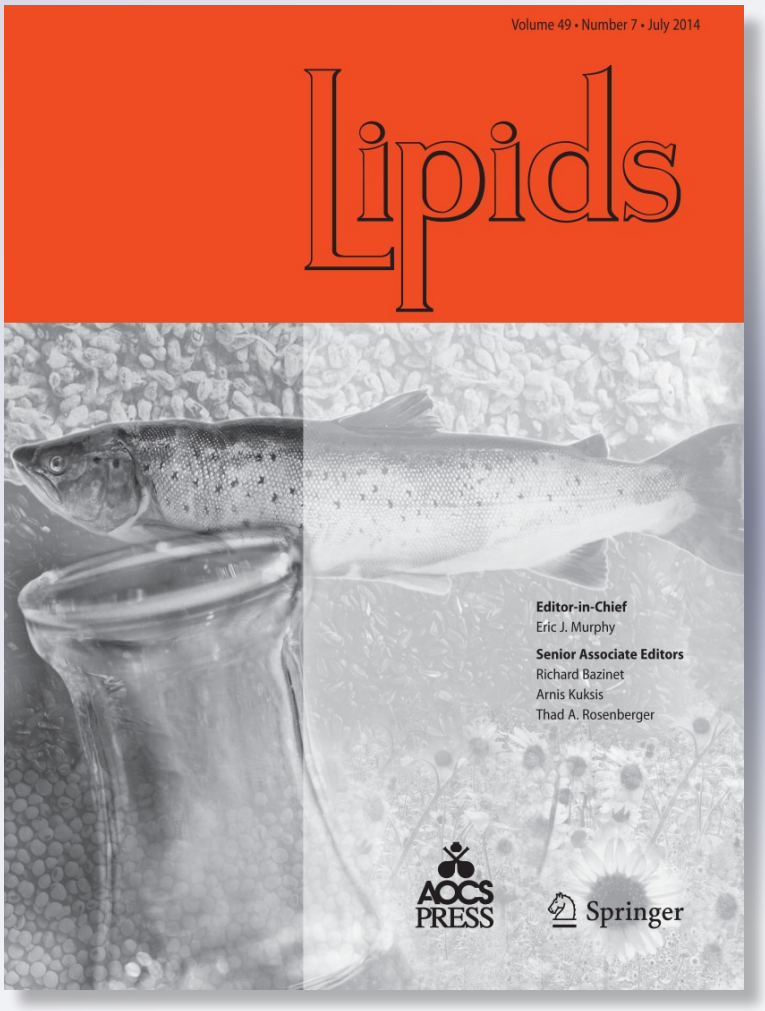

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ORIGINAL ARTICLE

Hypercholesterolemia Increases Plasma Saturated and n-6 Fatty Acids Altering Prostaglandin Homeostasis and Promotes Endothelial Dysfunction in Rabbits

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Abstract The present study evaluated the plasma fatty acid levels and the vascular prostaglandin (PG) release in a rabbit model of early hypercholesterolemia with endothelial dysfunction. Rabbits were fed either a control diet (CD) or a diet containing 1 % cholesterol (HD) for 5–6 weeks. The level of fatty acids was measured in plasma. The levels of PG and nitric oxide (NO) released from the aorta were also determined. Vascular morphology of the aorta was characterized by intima and media thickness measurements. The rabbits fed with HD had higher levels of arachidonic acid (ARA) and lower levels of oleic acid. The linoleic acid level was unchanged. PGI_2 and NO were diminished and $PGF_{2\alpha}$ levels, the PGI_2/TXA_2 ratio and the intima/media ratio were increased in rabbits fed with HD. In conclusion, feeding HD for a short period increased ARA plasma levels and unbalanced release of vasodilator/vasoconstrictor PG redirected the pathway to vasoconstrictor metabolite release. These lipid metabolism alterations in addition to the reduced NO levels and the moderate changes in the vascular morphology contributed to the endothelial dysfunction in this animal model. Therefore, the present findings support the importance of early correction or prevention of high cholesterol levels to disrupt the endothelial dysfunction process that leads to cardiovascular disease.

Keywords Hypercholesterolemia · Arachidonic acid · Prostaglandins · Rabbit aorta · Endothelial dysfunction · Fatty acids

Abbreviations

ARA	Arachidonic acid
CD	Control diet
COX	Cyclooxygenase
DGLA	Dihomo-γ-linolenic acid
EDHF	Endothelium derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
FAME	Fatty acid methyl esters
HD	High cholesterol diet
HDL-C	High density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
LNA	Linoleic acid
NO	Nitric oxide
OLA	Oleic acid
PAM	Palmitic acid
PG	Prostaglandin
PGE_2	Prostaglandin E 2
PGF_2	Prostaglandin F 2 alpha
PGI_2	Prostacyclin
PLA_2	Fosfolipase A2

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STA	Stearic acid
TC	Total cholesterol
TAG	Triacylglycerol
TXA_2	Tromboxane A2

Introduction

The fatty acid composition of the cell membrane is influenced by both dietary intake and metabolic pathways. A number of studies suggested a relationship between the essential fatty acids and the genesis of atherosclerosis [1]. The cellular cholesterol enrichment induces signaling pathways leading to phospholipase A2 (PLA_2) activation. [2]. PLA_2 releases arachidonic acid (ARA, 20:4n-6) from membrane phospholipids [3]. Therefore, an increase in the PLA_2 activity would increase the formation of ARA. ARA originates from both the diet and the elongation-desaturation process of its precursor, linoleic acid (LNA, 18:2n-6). The δ-5 and δ-6 desaturases are key enzymes of this pathway. The δ-6 catalyzes the conversion from LNA to dihomo-γ-linolenic acid (DGLA, 20:3n-6), which is desaturated to ARA by δ-5 [4]. ARA is the precursor of important molecules involved in inflammation, such as prostaglandins (PGs) and leukotrienes, and is thought to play an important role in the atherosclerotic process. Increased production of ARA from platelets of patients with type IIa hypercholesterolemia was reported [5].

The endothelium produces many vasoactive compounds to control the function of vascular smooth muscle cells and circulating blood cells. These complex systems determine a fine equilibrium, which regulates the vascular tone. Endothelial cells are the major source of vasodilator PGs, especially prostacyclin (PGI_2) [6, 7], and also produce other vasodilators, such as nitric oxide (NO) or the endothelium derived hyperpolarizing factor (EDHF). This ability is independent of their capacity to release vasoconstrictor PGs like thromboxane A2 (TXA_2), which is released from platelets as well as endothelial cells [8]. It is known that PGI_2 exerts the opposite effects to those of TXA_2. PGI_2 has potent antiaggregatory, vasodilatory and also cytoprotective effects [9]. Furthermore, TXA_2 and PGI_2 biosynthesis becomes relevant to atherosclerosis since PGI_2/TXA_2 balance may determine vascular tone and the risk for thrombosis [10]. Production of TXA_2 in the atherosclerotic aorta is increased [11, 12]. However, contradictory results were reported regarding the release of PGI_2 in hypercholesterolemia. Pfister et al. [13] found basal aortic production of PGI_2 reduced in cholesterol-fed rabbits compared with normal-fed rabbits. Mehta et al. [12] found increased PGI_2 synthesis in atherosclerosis. Fitzgerald et al. [14] demonstrated increased PGI_2 biosynthesis in patients with severe hypercholesterolemia.

Prostaglandins play a key role in the generation of the inflammatory response. Considerable data supports that atherosclerosis is a chronic inflammatory process that is induced and perturbed by lipid accumulation [15, 16]. Evolution of hypercholesterolemia is associated with endothelial cell dysfunction, which is characterized by reduced vascular NO bioavailability, elevated oxidative stress, and the creation of a strongly pro-inflammatory condition. The endothelial dysfunction may be considered an early stage in atherosclerosis and has a pivotal role in the atherogenic process [17]. These symptoms can culminate in profound impairments/alterations to vascular reactivity.

In atherogenesis, the morphological changes of the arterial wall occur during a presumably long subclinical lag phase characterized by gradual thickening of intima. The measurement of intima and media thickness has emerged as one of the methods chosen for determining the anatomical extent of arterial wall deterioration. Thus, the ratio of intima/media may be considered as a marker of vascular dysfunction [18].

The rabbit is the species perhaps more sensitive to dietary cholesterol overload [19]. A rabbit maintained on a high cholesterol diet rapidly develops hypercholesterolemia and accumulates lipids in the aortic intima [20, 21]. We have previously described a rabbit model of early hypercholesterolemia induced by feeding a high cholesterol diet during a short time period [22, 23]. In such conditions, we found endothelial dysfunction characterized by reduced relaxation to acetylcholine and increase of the angiotensin II-reactivity. Both effects were cyclooxygenase (COX)-dependent. Moreover, contradictory results were reported regarding the role of PGI_2 in endothelial dysfunction in atherosclerosis [24, 25].

As was stated in a previous paragraph, we found that early hypercholesterolemia induces endothelial dysfunction [22, 23]. Evidence indicates that the age-related changes in the endothelial eicosanoid profile contribute to endothelial dysfunction [26]. However, there is less information about how the plasma fatty acid levels and the profile of PGs released from the endothelium change with feeding a high cholesterol diet during a short-time period. Therefore, the aim of the present study was to analyze the plasma fatty acid levels and the vascular PG release in a rabbit model of early hypercholesterolemia with endothelial dysfunction.

Materials and Methods

Animals

The experimental protocols for this study were approved by our Institutional Animal Care and Use Committee. All

animal care and use programs were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Publication 86–23, revised 1985). Twenty-four male hybrid Flanders rabbits were acquired from two recognized local breeders (Cabaña "Los Prieto", Villa Mariano Moreno and Cabaña "Paz", San Miguel de Tucumán). These farms meet all conditions required by the Argentine authority to qualify as breeders of rabbits. Animals initially weighing 850–1,000 g were housed individually in gridded cages on a constant 12-h light/dark cycle under controlled temperature and conditions. They were fed standard rabbit chow 100 g/day. After a one week acclimation period, they were randomly divided into two groups. Control rabbits ($n = 12$) were fed with a standard chow diet (CD), which is an appropriate maintenance diet for a normal adult rabbit. Hypercholesterolemic rabbits ($n = 12$) were fed with a diet prepared by adding 1 % cholesterol (Sigma, St Louis, USA) to standard chow diet (HD). Animals were maintained for 5–6 weeks in their diets with free access to tap water. The fatty acid composition of standard rabbit chow was palmitic acid (PAM, 16:0) 19 %, stearic acid (STA, 18:0) 1.6 %, oleic acid (OLA, 18:1n-9) 17 %, palmitoleic acid (16:1) 0.13 %, LNA 50 %, linolenic acid (ALA, 18:3n-3) 6.3 %, ARA 0.37 %. Only male rabbits were used to avoid the secondary variability to sex differences in this experimental model. The animals were weighed before dietary manipulation and every day throughout the period of the experiment.

Plasma Collection and Lipids Measurement

At the end of the 5–6 weeks of dietary intervention, the animals were fasted overnight and anesthetized with Ketamin (75 mg/kg) and diazepam (0.5 mg/kg). Blood samples were collected by direct cardiac puncture into two polypropylene tubes, one for serum and one for plasma. The blood for plasma was collected in EDTA or citrate. Serum was separated by allowing the blood to clot at 37 °C and centrifuged at 3,000 rpm for 10 min. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triacylglycerol (TAG) were measured using colorimetric reactions with commercial kits (Wiener, Argentina).

Fatty Acid Determination

Total lipids were extracted from plasma using chloroform/methanol solution (2:1, v/v) according to Folch et al. [27]. They were derived from a HCl/methanol solution according to Van Nieuwenhove et al. [28]. One microliter of fatty acid methyl esters (FAME), dissolved in hexane, was injected into a gas chromatograph (GC, Model 6890N, Agilent Technologies, Wilmington, DE) equipped with a flame ionization detector (Agilent Technologies, Wilmington, DE) and an automatic injector (Model 7683, Agilent Technologies Shanghai, China) into an HP-88 capillary column (100 m × 0.25 mm × 0.20 μm, Agilent Technologies, Wilmington, DE). GC conditions involved an injector with a temperature of 255 °C. The initial oven temperature of 75 °C was increased to 165 °C at 8 °C/min and was held there for 35 min. It was then increased to 210 °C at 5.5 °C/min and maintained for 2 min, and afterwards to 240 °C at 15 °C/min and held for 3 min. The detector temperature was 280 °C. Nitrogen was used as a carrier gas at a flow rate of 18 ml/min at 38 psi. C17:0 was used as the internal standard. FAME were identified and quantified by comparison with the retention times and peak areas of standards (Sigma, St Louis, USA). Results were expressed as g/100 g of FAME.

Prostaglandin Release Measurement

The thoracic aorta was removed from CD and HD rabbits and sectioned into four rings (0.5 cm). The adhering perivascular tissue was carefully removed. Rings were transferred to a 4 ml organ bath containing Krebs solution with the following composition: 128 mM NaCl, 4.7 mM KCl, 14.4 mM NaHCO3, 1.2 mM NaH2PO4, 0.1 mM Na2-ethylenediaminetetraacetic acid, 2.5 mM CaCl2, and 11.1 mM glucose at pH 7.2. Krebs solution was kept at 37 °C and aerated with 95 % O2 and 5 % CO2. The endothelium was kept intact from two of the four rings (endothelium-intact arteries) and was removed by rubbing the luminal surface (endothelium with removed arteries) from the other two rings of each animal. Samples were collected 20 min after the equilibration period (Basal).The effect of the incubation with ARA 10^{-6} M in the profile of PGs released from the aorta was studied in endothelium intact arteries from rabbits fed the CD and rabbits fed the HD. Samples were collected 30 min after the equilibration period (ARA-stimulated). At the end of the incubation period, the media was acidified to pH 3.5 with 1 M formic acid. Prostanoids were extracted from the media by solid phase extraction using cartridges containing ODS-silica (Extract-clean SPE C18 500 mg/3 ml, Phenomenex). Methanol was added to the sample, which was adjusted to 15 %, then acidified to pH 3–3.5 with 1 N HCl. The sample was loaded onto the cartridge and then was washed with 15 % methanol, water and petroleum ether. Prostanoids were eluted with ethyl acetate and brought to dryness under N2. Then reversed-phase high performance liquid chromatography (RP-HPLC) was carried out. The HPLC system consisting of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, a manual injection valve with a 20 μl loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA)

was used to analyze the chromatograms. An XBridgeTM C18 column (4.6 mm × 150 mm, 5 μm; Waters Corporation, Milford, MA) with two gradient solvent systems was used. Data collection was carried out with EmpowerTM 2 software. The presence of PGs in the samples was confirmed by UV spectrometry (190–400 nm) in comparison with the standard compounds. The solvent system was 1.7 mM H_3PO_4: acetonitrile 67.2/32.8 (v/v). The flow rate was 1 ml/min and UV absorption was measured at 218 nm. Dried samples were reconstituted in 0.15 ml of the mobile phase and injected into the HPLC system. Authentic standards of PGs: 6-keto PG $F_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and TXB_2 were run along with the samples and a bracket assay was carried out to determine the amount of PGs in the samples. The prostanoid curves were linear in the range from 10 to 1,000 ng ($R2 = 0.998$). To assess the recovery, 10 μg/ml of standard solutions were added into 4 ml of Krebs solution. The sample was analyzed in triplicate including a blank test to evaluate the average of recoveries. The recovery level of the prostanoids ranged from 70 % to 80 %. Similar results were obtained by Hishinuma et al. [29]. All values were corrected for recovery. Results were expressed as nanograms of prostanoids per milligram of tissue weight (ng/mg).

Nitrite Measurement

Nitrite was measured with the use of Griess reagent as previously described [30]. Aortic rings from rabbits fed the CD or rabbits fed the HD were placed in a 1 ml organ bath containing Krebs solution and aerated with 95 % O2 and 5 % CO2. Samples (500 μl) were collected from the incubation medium 20 min after the equilibration period. Nitrite was analysed with Griess reagent [*N*-(50 μl 1-Naphthylethylenediamine 0.2 % and 450 μl sulfanilamide 0.1 %)]. The tubes were kept at room temperature for 10 min to 15 min until a full pink color developed. Absorbance was measured at 540 nm with a spectrophotometer (Metrolab 1000). Amount of nitrite was estimated from a standard curve of sodium nitrite by using regression analysis ($y = a+bx$). Only curves with a correlation coefficient > 0.95 were used. Nitrite was expressed as picomol/mg tissue and as wet weight/ml.

Vascular Morphology

Histological analysis of segments of the thoracic aorta (adjacent to the aortic arch) from CD and HD rabbits was performed. The aorta was rinsed with normal saline solution and preserved in 10 % formaldehyde buffered solution (pH 7.4) for the next step. From each sample, serial sections were made (3–5 sections/aorta). The 5 μm sections were stained by the Haematoxylin-Eosin method. Media and intima thickness were measured by image analysis with the software Media Cybernetics® Image-Pro PlusTM. The ratio between the tunica intima and the tunica media was calculated. A code number was assigned to each section observed.

Statistical Analysis

All data is expressed as mean ± SE. The differences in the mean values between the two diet groups were tested by the unpaired Student's *t* test. The differences in the mean values between PGs levels before and after the incubation with ARA were tested by the paired Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Rabbits fed the diet enriched with cholesterol showed higher plasma levels of TC, LDL-C, HDL-C and TAG than animals fed the CD. Basal glucose level was similar in both groups. No difference was found in body weight at the end of the experiment (Table 1).

Major fatty acids present in total fatty acids of the plasma are listed in Table 2. The total levels of saturated fatty acids were similar in both diet groups but significant differences in some fatty acids were determined. Relative to rabbits fed the CD, rabbits fed the HD had a higher level of PAM (24.5 %, $p < 0.05$), and a lower level of STA (45 %, $p < 0.05$). With respect to monounsaturated fatty acids, palmitoleic acid level was increased (66.7 %, $p < 0.05$) and OLA level was decreased (24.2 %, $p < 0.05$) in HD rabbits compare to CD rabbits. The level of ARA in hypercholesterolemic rabbits was two-fold higher than the control. The levels of LNA or the omega-6/

Table 1 Plasma levels of lipids, glucose and weight values from rabbits fed a control diet (CD) or a high cholesterol diet (HD)

	CD	HD
Weight (g)	2,190 ± 0,178	2,040 ± 0,180
Basal glucose (mg/dl)	98.5 ± 3.2	108.9 ± 4.7
Cholesterol (mg/dl)	61.8 ± 10	872.3 ± 114**
LDL-cholesterol (mg/dl)	26.8 ± 3.1	666 ± 99**
HDL-cholesterol (mg/dl)	45.5 ± 4.1	164 ± 45*
Triglycerides (mg/dl)	98 ± 14	222 ± 33*

Data is expressed as mean ± SE of 12 rabbits

LDL low density lipoproteins, *HDL* high density lipoproteins

* $p < 0.05$; ** $p < 0.01$ indicates statistically significant differences between rabbits fed on a CD and rabbits fed on a HD (unpaired Student's *t* test)

Table 2 Fatty acids from plasma

	CD	HD
Satured fatty acids		
Palmitic (16:0)	22.5 ± 0.9	29.8 ± 1.1*
Stearic (18:0)	15.1 ± 0.5	8.3 ± 0.4*
Total SFA	37.6 ± 0.8	38.1 ± 0.2
Monounsaturated fatty acids		
Palmitoleic (16:1n-7)	1.0 ± 0.3	3.0 ± 0.2*
Oleic (18:1n-9)	27.7 ± 1.8	21.0 ± 0.8*
Total MUFA	28.7 ± 0.1	24.0 ± 0.5
Polyunsaturated fatty acids		
n-3 Fatty acids		
Linolenic (18:3n-3)	1 ± 0.14	1.8 ± 0.3
Docosahexaenoic (DHA) (22:6n-3)	1.97 ± 0.07	2.1 ± 0.3
Eicosapentaenoic (EPA) (20:5n-3)	0.24 ± 0.02	0.3 ± 0.05
Total n-3	3.2 ± 0.2	4.2 ± 0.6
n-6 fatty acids		
Linoleic (18:2n-6)	26.4 ± 1.0	25.5 ± 2.0
Arachidonic (20:4n-6)	3.0 ± 0.2	6.3 ± 0.3*
Total n-6	29.4 ± 0.5	31.8 ± 1.0
PUFA	32.4 ± 0.5	36.0 ± 0.5
n-6/n-3 ratio	9.2 ± 1.1	7.6 ± 0.6

Data is expressed in percentage as mean ± SE

PUFA polyunsaturated fatty acids

* $p < 0.05$ indicates statistically significant differences between rabbits fed a control diet (CD) and rabbits fed a hypercholesterolemic diet (HD) (unpaired Student's *t* test)

omega-3 fatty acids ratios were similar in rabbits fed the CD and rabbits fed the HD (Table 2).

Prostaglandin release was not detectable in endothelium removed arteries from control or hypercholesterolemic rabbits. In basal conditions, 6-keto $PGF_{1\alpha}$ and TXB_2, stable metabolites of PGI_2 and TXA_2 ,respectively, were the major COX products identified in endothelium intact arteries from rabbits fed a CD. In segments from rabbits fed the HD, $PGF_{2\alpha}$ and PGE_2 were also detected and 6-keto $PGF_{1\alpha}$ release was significantly lower as compared with rabbits fed the CD (Fig. 1). PGI_2/TXA_2 ratio was lower in rabbits fed the HD than in rabbits fed the CD (Fig. 2).

Incubation of aortic segments with ARA increased the basal release of 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in hypercholesterolemic rabbits and had no effect on rabbits fed a CD (Fig. 3). TXB_2 and PGE_2 levels were not significantly modified in both diet groups.

Basal NO release was lower in hypercholesterolemic rabbits as compared with control rabbits (Fig. 4).

Histological examination showed moderate thickening of the tunica intima in arteries from rabbits fed the HD (Figs. 5a, b, c and d).

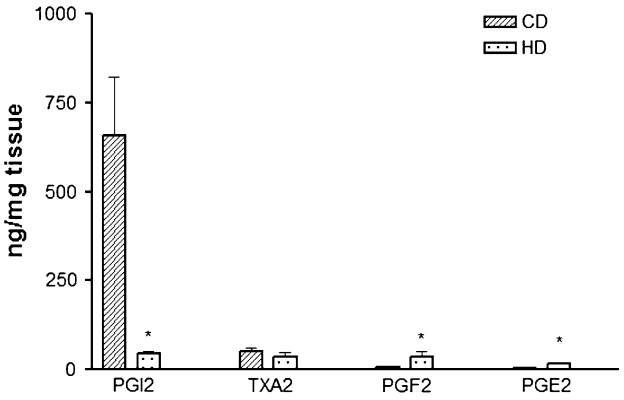

Fig. 1 Release of PGs by the aorta with intact-endothelium from rabbits fed a control diet (CD) or a high cholesterol diet (HD). Values are shown as mean ± SE. A value of $*p < 0.05$ indicates statistically significant differences between rabbits fed a CD and rabbits fed a HD (unpaired Student's *t* test)

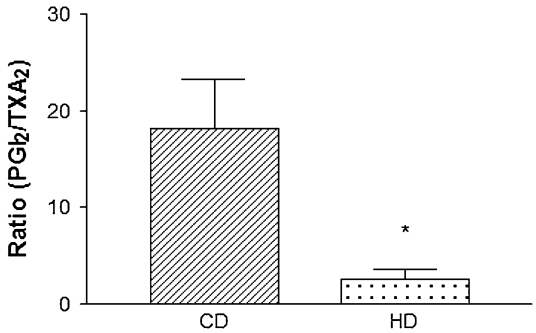

Fig. 2 PGI_2/TXA_2 ratio from the aorta of rabbits fed a control diet (CD) or a high cholesterol diet (HD). A value of $*p < 0.05$ indicates statistically significant differences between rabbits fed a CD and rabbits fed a HD (unpaired Student's *t* test)

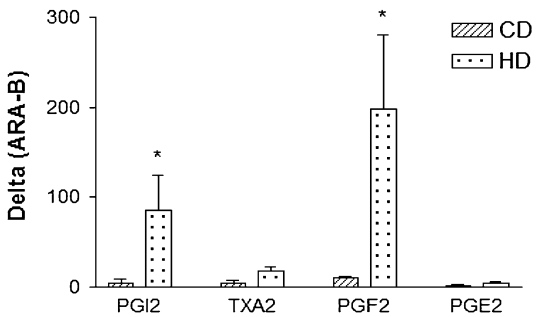

Fig. 3 Differences between PG levels post arachidonic acid 10^{-6} M (ARA) incubation and PG basal levels (DeltaARA-B). The release of PGs by the aorta was measured in endothelium intact arteries from rabbits fed a control diet (CD) or a high cholesterol diet (HD). Values are shown as mean ± SE. A value of $*p < 0.05$ indicates statistically significant differences between the ARA-stimulated condition and the basal condition (paired Student's *t* test)

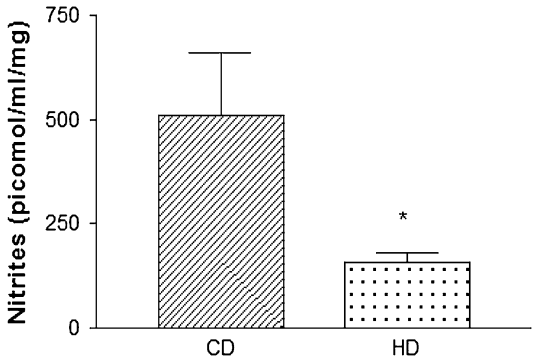

Fig. 4 Release of nitrites by endothelium intact aortas from rabbits fed a control diet (CD) or a high cholesterol diet (HD). Values are shown as mean ± SE. A value of $*p < 0.05$ indicates statistically significant differences between rabbits fed a CD and rabbits fed a HD (unpaired Student's *t* test)

Discussion

At present, no information about the lipid metabolism involving fatty acid levels and vascular PGs production in early hypercholesterolemia has been found in the literature. Our study demonstrated that a diet high in cholesterol for a short-time period modifies two endothelial dysfunction markers: reduction of NO release and increase of the aorta thickness were found. In such conditions, we observed increase of ARA plasma levels and a shift of the vascular PG profile released from rabbit aorta.

According to our search results, Risé et al. [31] found in plasma from hypercholesterolemic rabbits increase of PAM and palmitoleic acid and decrease of STA and OLA with respect to control rabbits. However, these authors reported an increase of LNA and a decrease of ARA in such conditions. In the current study, we encountered that the levels of LNA remained unchanged and the levels of ARA were increased in rabbits fed the HD as compared to rabbits fed the CD. One possible explanation for this disagreement could be that the short period of feeding a diet supplemented only with 1 % cholesterol is not enough to induce atherosclerosis. In support of this view, the rabbit aorta showed a moderate increase of intima and media thicknesses. This would be a marker of an early stage of atherosclerosis [18]. The endothelial dysfunction is a key early event preceding formation of plaques. Numerous studies in animal models demonstrated the relationship between endothelial dysfunction and hypercholesterolemia. However, these animals models were fed on a high cholesterol diet supplemented with other lipids as coconut oil for eight or more weeks. The animals develop atherosclerosis in such conditions [32]. Celermajer et al. [33] found endothelial dysfunction in children and adults with risk factors for atherosclerosis such as smoking and familiar hypercholesterolemia before anatomical evidence of plaque

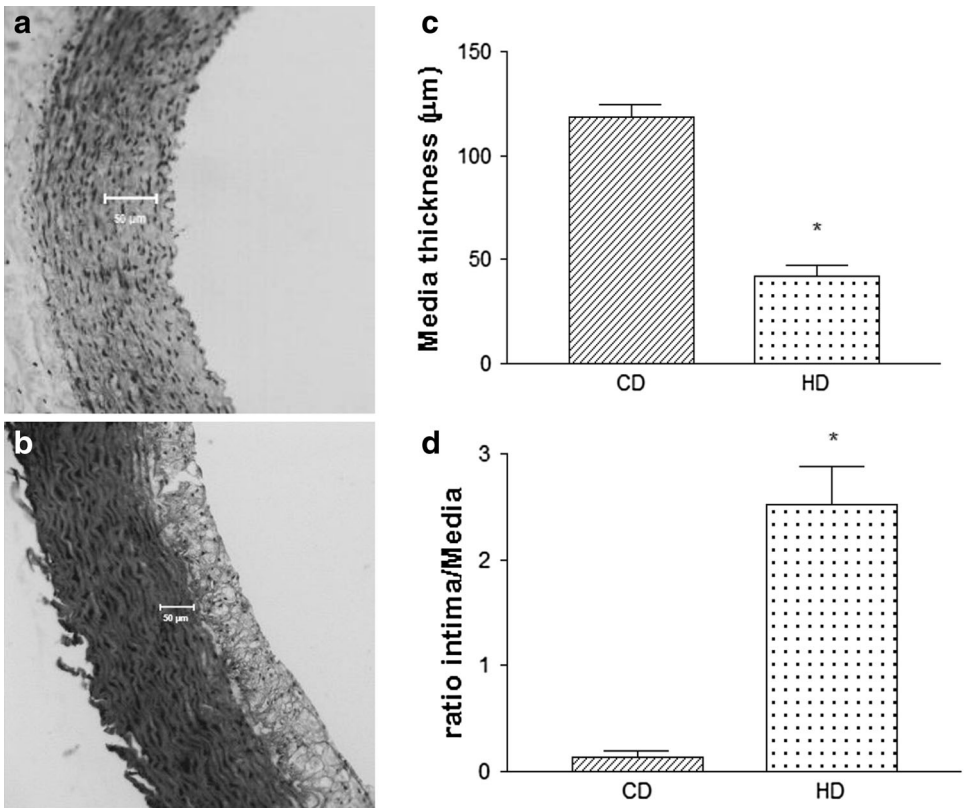

Fig. 5 Representative microphotographs of aorta sections from control (**a**) and hypercholesterolemic (**b**) rabbits. Sections were stained with haematoxylin/eosin after isolation from rabbits fed control (CD) or high cholesterol diets (HD) during 5–6 weeks, as described in the "Materials and Methods" section. Media thickness (**c**) was measured by image analysis with the software Media Cybernetics® Image-Pro PlusTM and the ratio intima/media (**d**) was calculated. A value of $*p < 0.05$ indicates statistically significant differences between rabbits fed a CD and rabbits fed a HD (unpaired Student's *t* test). Magnification × 10

formation. Thus, an altered LNA/ARA ratio could be an indicator of abnormal lipid metabolism related to a degree of atherosclerosis. Considering that LNA is a precursor of ARA, the association between normal levels of LNA and high levels of ARA may be an indicator of changes in the desaturase activity [5].

The PG release from rabbit aorta was endothelium dependent in both diet groups. The major metabolite of ARA released by the rabbit aorta was PGI_2, which was measured as its stable degradation product, 6-keto $PGF1_{\alpha}$. Smaller amounts of TXA_2 (measured as thromboxane B_2) and traces of $PGF_{2\alpha}$ and PGE_2 were also measured. Forstersmann et al. [24] found similar results with respect to PGI_2 levels. However, they found higher levels of PGE_2 than TXA_2. Levels of PGI_2 synthesized by the aorta were significantly lowered by feeding the HD. This is in agreement with some authors [10, 25], who reported that the first stage of experimental atherosclerosis may be related to strong suppression of PGI_2 synthesis by arteries. The second stage would be more dangerous since an increased generation of TXA_2 is combined with the decreased generation of PGI_2. Wang et al. [34] found no changes in PGI_2 levels and significant increase in TXA_2 release from the aortas of rabbits fed a HD for 15 weeks. Mehta et al. [12] demonstrated that aortic rings from rabbits fed a HD generate more PGI_2 and TXA_2 than normal aortic rings do. We found that the basal release of TXA_2 from arteries of rabbits fed the CD was about 10-fold lower than the release of PGI_2. In addition, no difference was found in TXA_2 levels between both diet groups. The decrease of PGI_2 levels with no changes in TXA_2 levels supports the view that the intake of a HD during a short time period induces early changes compatible with the first stage of experimental atherosclerosis. Csányi et al. [35] found in ApoE/LDLR-/- mice that impairment of NO-dependent relaxation precedes the development of atherosclerosis in the aorta. The upregulation of COX-2/PGI_2 and EDHF pathways that occurs early along the progress of atherosclerosis may compensate for the loss of the biological activity of NO. In the present model of hypercholesterolemia induced by the HD, we found that the release of both vasodilators NO and PGI_2 was reduced. These findings would mean differences between atherosclerosis induced by genetic manipulation and atherosclerosis induced by the diet.

The increased $PGF_{2\alpha}$ production was the most notable change observed in the PG profile. $PGF_{2\alpha}$ was almost undetectable in the aortas from control rabbits. In aortas from hypercholesterolemic rabbits $PGF_{2\alpha}$ levels were similar to TXA_2 levels. As far as we know this finding has not been reported previously and may be an original contribution to the knowledge of the ARA metabolism in early hypercholesterolemia. Conversion of PGE_2 to $PGF_{2\alpha}$ by the PGE_2 9 ketoreductase has been described [36]. Farina et al. [37] found that NO inhibited PGE_2 9 ketoreductase. Considering that in our model the basal production of NO was reduced, an increased PGE_2 9 ketoreductase activity might account for the higher levels of $PGF_{2\alpha}$ in rabbits fed the HD as compared with rabbits fed the CD.

Our studies demonstrated that the ARA plasmatic levels are increased in hypercholesterolemia. It has been shown that various phospholipids of the atherosclerotic aorta turn over more rapidly than those of the normal artery. A comparison of cholesterol esters in plasma and intima of rabbits fed a HD showed a remarkable similarity in composition [1, 2]. As the ARA is a precursor of PG synthesis, we investigated its effect on the profile of PG released by rabbit aorta in vitro. The incubation of aortic rings with ARA did not modify the PGs levels in rabbits fed the CD and increased twofold the release of PGI_2 and fivefold the release of $PGF_{2\alpha}$ in rabbits fed the HD. However, PGI_2 levels did not reach control levels. Since the conversion of ARA into the major eicosanoids is similar in hypercholesterolemic and normal aortic rings, it is likely that the enhanced release of ARA is responsible for increased biosynthesis of eicosanoids. Stuart et al. [38] observed that cholesterol induces an increase of the arachidonate release from human platelets in vitro. The increased arachidonate release from the phospholipid pool of aortic wall cells could be related to an effect of cholesterol on membrane fluidity [39, 40]. Since the diacylglycerol lipase or phospholipase activities are very susceptible to alterations in the lipid-water interphase, it can be hypothesized that hyperlipidemia affects the lipases involved in the release of arachidonate, which is a substrate for eicosanoid formation by endothelial and smooth muscle cells. The release of arachidonate eventually results in increase of the PGE_2 levels, which is converted to $PGF_{2\alpha}$, in hypercholesterolemia despite the similar conversion of arachidonate to its major metabolites. Accordingly, these data indicate a differential improvement of the aortic PG release redirecting the pathway to a vasoconstrictor metabolite release.

Accumulating evidence suggest that alterations in the production/release of PGs by the endothelium directly contribute to the endothelial dysfunction in vascular diseases [41–43]. In fact, increased vasoconstriction and reduced vasodilatation observed in aortas from hypercholesterolemic rabbits was reversed by treatment with COX inhibitors [22]. Our results showed that hypercholesterolemic rabbits displayed a decreased PGI_2/TXA_2 ratio and an increase in the $PGF_{2\alpha}$ release from the aortic rings. Therefore, the unbalanced release of vasodilator/vasoconstrictor PGs together with the reduction of NO release may contribute to the endothelial dysfunction observed in our model of early hypercholesterolemia.

In conclusion, feeding HD for a short period altered the LNA/ARA ratio, increased ARA plasmatic levels, reduced

PGI_2/TXA_2 ratio and increased $PGF_{2\alpha}$ production. These lipid metabolism alterations in addition to the reduced NO levels and the moderate changes in the vascular morphology could contribute to the endothelial dysfunction in this animal model. All these changes occur early and would be responsible for the progression of vascular diseases. Therefore, the present findings support the importance of early correction or prevention of high cholesterol levels disrupting the endothelial dysfunction process that leads to cardiovascular disease.

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References

1. Swell L, Law MD, Schools PE, Treadwell CR (1963) Dynamic aspects of cholesterol ester metabolism in rabbits with atherosclerosis. J Nutr 81:263–270
2. Pomerantz KB, Lander HM, Summers B, Hajjar DP (1997) G-protein-mediated signaling in cholesterol-enriched arterial smooth muscle cells. Biochemistry 36:9532–9539
3. Vansden BH (1980) Intracellular phospholipases A. Biochem Biophys Acta 604:191–246
4. Martinelli N, Girelli D, Malerba G et al (2008) FADS genotypes and desaturase activity estimated by the ratio of arachidonic acid to linoleic acid are associated with inflammation and coronary artery disease. Am J Clin Nutr 88:941–949
5. Mosconi C, Colli S, Tremoli E, Galli C (1988) Phosphatidylinositol (PI) and PI-associated arachidonate are elevated in platelet total membranes of type IIa hypercholesterolemic subjects. Atheroclerosis 72:129–134
6. Weksler BB, Marcus AJ, Jaffe EA (1977) Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. Proc Natl Acad Sci USA 74:3922–3926
7. DeWitt DL, Day JS, Sonnenburg WK, Smith WL (1983) Concentrations of prostaglandin endoperoxide synthase and prostaglandin I2 synthase in the endothelium and smooth muscle of bovine aorta. J Clin Investig 72:1882–1888
8. Ingerman-Wojenski C, Silver MJ, Smith JB, Macarak E (1981) Bovine endothelial cells in culture produce thromboxane as well as prostacyclin. J Clin Investig 67:1292–1296
9. Gryglewski RJ, Dembínska-Kieć A, Korbut R (1978) A possible role of thromboxane A2 (TXA2) and prostacyclin (PGI2) in circulation. Acta Biol Med Ger 37:715–723
10. Gryglewski RJ, Dembinska-Kiec A, Zmuda A, Gryglewska T (1978) Prostacyclin and thromboxane A2 biosynthesis capacities of heart, arteries and platelets at various stages of experimental atherosclerosis in rabbits. Atherosclerosis 31:385–394
11. Henriksson P, Stamberger M, Diczfalusy U (1987) Increased aortic thromboxane production in experimental atherosclerosis. Prostaglandins Leukot Med 29:71–77
12. Mehta JL, Lawson D, Mehta P, Saldeen T (1988) Increased prostacyclin and thromboxane A2 biosynthesis in atherosclerosis. Proc Natl Acad Sci USA 85:4511–4515
13. Pfister SL, Falck JR, Campbell WB (1991) Enhanced synthesis of epoxyeicosatrienoic acids by cholesterol-fed rabbit aorta. Am J Physiol 261:H843–H852
14. FitzGerald GA, Smith B, Pedersen AK, Brash AR (1984) Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N Engl J Med 310:1065–1068
15. Glass CK, Witztum JL (2001) Atherosclerosis: the road ahead. Cell 104:503–516
16. Ross R (1999) Atherosclerosis: an inflammatory disease. N Engl J Med 340:115–126
17. Hadi HAR, Carr CS, Al Suwaidi J (2005) Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. Vasc Health Risk Manag 1:183–198
18. Poredos P (2011) Markers of preclinical ateherosclerosis and their clinical relevance. Open Atheroscler Thromb J 4:1–10
19. Getz GS, Reardon CA (2012) Animal models of atherosclerosis. Arterioscler Thromb Vasc Biol 32:1104–1115
20. Yanni AE (2004) The laboratory rabbit: an animal model of atherosclerosis research. Lab Anim 38:246–256
21. Zilversmit DB, Sweeley CC, Newman HAI (1961) Fatty acid composition of serum and aortic intimal lipids in rabbits fed low- and high cholesterol diets. Circ Res 9:235–241
22. Jerez S, Sierra L, Coviello A, de Bruno MP (2008) Endothelial dysfunction and improvement of the angiotensin II reactivity in hypercholesterolemic rabbits: role of cyclooxygenase metabolites. Eur J Pharmacol 580:182–189
23. Jerez S, Sierra L, Scacchi F, de Bruno MP (2010) Hypercholesterolemia modifies angiotensin II desensitization and cross talk between alpha 1-adrenoceptor and angiotensin AT1 receptor in rabbit aorta. Eur J Pharmacol 635:149–155
24. Forstermann U, Hertting G, Neufang B (1984) The importance of endogenous prostaglandins other than prostacyclin for the modulation of contractility of some rabbit blood vessels. Br J Pharmacol 81:623–630
25. Dembinska-Kiec A, Gryglewska T, Zmuda A, Gryglewski RJ (1977) The generation of prostacyclin by arteries and by the coronary vascular bed is reduced in experimental atherosclerosis in rabbits. Prostaglandins 14:1025–1034
26. Qian H, Luo N, Chi Y (2012) Aging-shifted prostaglandin profile in endothelium as a factor in cardiovascular disorders. J Aging Res. doi:10.1155/2012/121390
27. Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
28. Van Nieuwenhove C, Cano PG, Chaia AP, González S (2007) Chemical composition and fatty acids content of buffalo cheese from northwest Argentina: effect on lipid composition of mice tissues. J Food Lipids 14:232–242
29. Hishinuma T, Suzuki K, Saito M, Yamaguchi H, Suzuki N, Tomioka Y, Kaneko I, Ono M, Goto J (2007) Simultaneous quantification of seven prostanoids using liquid chromatography/tandem mass spectrometry: the effects of arachidonic acid on prostanoid production in mouse bone marrow-derived mast cells. Prostaglandins Leukot Essent Fatty Acids 76:321–329
30. Jerez S, Peral de Bruno M, Coviello A (2001) Efecto de antagonistas de los receptores de angiotensina II y α2 adrenérgicos sobre la liberación de NO inducida por angiotensina II. Medicina 61:431–436
31. Risé P, Camera M, Caruso D, Ghezzi S, Vissiolo F, Galli D (2004) Synthesis of long-chain polyunsatured fatty acids is inhibited in vivo in hypercholesterolemic rabbits and in vitro by oxysterols. Prostaglandins Leukot Essent Fatty Acids 71:79–86
32. Kamimura R, Suzuki S, Sakamoto H, Miura N, Misumi K, Miyahara K (1999) Development of atherosclerotic lesions in cholesterol loaded rabbits. Exp Anim 48:1–7

33. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE (1992) Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 340:1111–1115
34. Wang T, Falardeau P, Powell WS (1991) Synthesis of prostaglandins and thromboxane B2 by cholesterol-fed rabbits. Arterioscler Thromb 11:501–508
35. Csányia G, Gajdab M, Franczyk-Zarowc M et al (2012) Functional alterations in endothelial NO, PGI2 and EDHF pathways in aorta in ApoE/LDLR −/− mice. Prostaglandins Other Lipid Mediat 98:107–115
36. Hoult JR, Bacon KB, Osborne DJ, Robinson C (1988) Organ selective conversion of prostaglandin D2 to 9 alpha, 11 beta-prostaglandin F2 and its subsequent metabolism in rat, rabbit and guinea pig. Biochem Pharmacol 37:3591–3599
37. Farina MG, Billi S, Sordelli MS et al (2006) Nitric oxide (NO) inhibits prostaglandin E2 9-ketoreductase (9-KPR) activity in human fetal membranes. Prostaglandins Other Lipid Mediat 79:260–270
38. Stuart MJ, Gerrard JM, White JG (1980) Effect of cholesterol on production of thromboxane B2 by platelets in vitro. N Engl J Med 302:6–10
39. Shattil SJ, Cooper RA (1978) Role of membrane lipid composition, organization, and fluidity in human platelet function. Prog Hemost Thromb 4:59–86
40. Beck R, Bertolino S, Abbot SE, Aaronson PI, Smirnov SV (1998) Modulation of arachidonic acid release and membrane fluidity by albumin in vascular smooth muscle and endothelial cells. Circ Res 83:923–931
41. Chan PS, Cervoni P (1986) Prostaglandins, prostacyclin, and thromboxane in cardiovascular diseases. Drug Develop Res 7:341–359
42. Iñiguez MA, Cacheiro-Llaguno C, Cuesta N, Díaz-Muñoz MD, Fresno M (2008) Prostanoid function and cardiovascular disease. Arch Physiol Biochem 114:201–209
43. Yuhki K, Kashiwagi H, Kojima F, Kawabe J, Ushikubi F (2010) Roles of prostanoids in the pathogenesis of cardiovascular diseases. Int Angiol 29:19–27