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Increased oxidative stress levels and normal antioxidant enzyme activity in circulating mononuclear cells from patients of familial hypercholesterolemia

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Abstract

Familial hypercholesterolemia (FH) is a clinical condition with high risk for developing atherosclerosis. Increased oxidative stress (OS) and FH have been related to atherosclerosis, but no data are available on levels of OS and antioxidant enzyme activity in circulating mononuclear cells (CMCs) from FH patients. Circulating mononuclear cells are important mediators in atherosclerosis development, and chronically increased blood OS present in FH can induce modification in CMC activity. The objective of the study was to analyze the OS levels in CMCs from FH patients and controls. We have selected 30 nonrelated FH index patients and 30 normoglycemic and normocholesterolemic controls matched by age, sex, body mass index, abdominal circumference, and homeostasis model assessment index. Production of free radicals was analyzed by measurement of xanthine oxidase activity in plasma, reduced and oxidized glutathione (GSH and GSSG, respectively), and malonyldialdehyde in levels CMCs. Antioxidant status was analyzed by measuring antioxidant enzyme activity as superoxide dismutase, catalase, and glutathione peroxidase. We have found that FH patients showed significantly higher xanthine oxidase and malonyldialdehyde enzyme activities, as well as increased GSSG and lower GSH values resulting in a higher GSSG/GSH ratio. These data indicate a higher free radical production in plasma and increased OS levels in CMCs from patients than from controls. No significant differences were found in superoxide dismutase, catalase, and glutathione peroxidase activities between both groups. These data show an important alteration of OS regulation in FH and the absence of antioxidant response in CMCs mediated by some of the major antioxidant enzymes.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by markedly elevated low-density lipoprotein cholesterol (LDL-C) levels, tendon xanthomata, and increased risk of premature coronary heart disease (CHD) [1]. Familial hypercholesterolemia shows great variability in the risk of premature CHD, which may be influenced by factors such as age, sex, diet, body mass index

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(BMI), abdominal circumference, LDL-C and high-density lipoprotein cholesterol (HDL-C) plasma values, and type of LDL receptor mutations or other genes; but they do not explain all the situations [2-4]. The potential role of other factors in the risk of developing atherosclerosis may help to understand the variability of this risk.

Atherosclerosis is characterized by a combination of both inflammatory and oxidative stress (OS) processes on the arterial wall [5,6]. Different evidences suggest that OS is implicated in the pathogenesis of endothelial dysfunction and in the progression of atherosclerosis, independently of the disease stage [7]. Oxidative stress results from the imbalance between prooxidant and antioxidant mechanisms present on the arterial wall as well as circulating cells. Recently, it has been shown that one of the principal enzymes in the vasculature (which contributes to reactive oxygen species [ROS] production) is xanthine oxidase (XO). This enzyme influences lymphocyte activity and cellular response [7-9].

The inactivation of ROS is mainly done by antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and the glutathione system, including the final products reduced and oxidized glutathione (GSH and GSSG, respectively), and different enzymes that control their levels such as glutathione peroxidase (GPx), glutathione reductase, glutathione synthetase, and so on. An increment in prooxidant enzyme activity increases ROS production, may saturate the capacity of antioxidant enzymes, and leads to the generation of OS [7,8]. These changes may induce alterations in the structure and function of cells involved in cardiovascular system and contribute to the initiation and progression of the atherosclerotic plaque [6-8]. A schematic metabolic pathway is shown in Fig. 1

There are evidences that other diseases with increased risk for developing atherosclerosis have also increased OS levels [10,11]. In hypertension, we have recently shown alterations of antioxidant enzymes activity in circulating mononuclear cells (CMCs) [12,13] and that XO could be related to OS

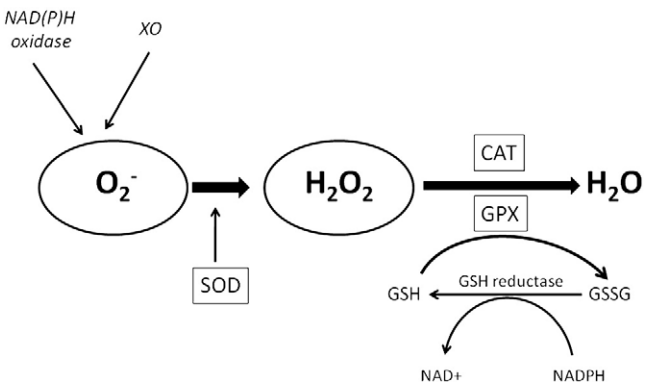

Fig. 1. Schematic representation of oxidant and antioxidant enzymes. Enzymes producing free radicals are in italics. Antioxidant enzymes are in squared boxes. Schematic representation of oxidant and antioxidant enzymes. Enzymes producing free radicals are in italics. Antioxidant enzymes are in squared boxes.

levels in these patients [14]. Few studies have evaluated OS levels in FH [15]; and little is known about XO, antioxidant enzymes, and OS status in plasma.

Therefore, the present study was undertaken to analyze the OS levels in CMC by measuring GSH; GSSG; malonyldialdehyde (MDA, a major product of lipid peroxidation); and the activity of XO, SOD, CAT, and GPx in FH patients. These data could show possible new risk factors that could be involved in the increased CHD risk that these patients suffer.

2. Subjects and methods

2.1. Subjects

The study population consisted of 30 nonrelated FH subjects (13 men) and 30 nonrelated normocholesterolemic normoglycemic controls (13 men). There were 6 menopausal women in each group, which was strictly matched by age. All subjects were white and lived in the Valencia region.

The institutional ethics committee from our institution approved the protocol, and all subjects gave written informed consent to enter the study.

The inclusion criteria for the control group were as follows: concentration of plasma total cholesterol (TC) less than 200 mg/dL, triglycerides (TGs) less than 150 mg/dL, and apolipoprotein (apo) B less than 1.2 g/L; fasting plasma glucose less than 110 mg/dL; and absence of personal or family history of dyslipidemia, cardiovascular disease, or diabetes. Homeostasis model assessment (HOMA) was calculated as indicated by Matthews et al [16]. Diagnostic criteria for FH included plasma levels of total and LDL-C above the 95th percentile corrected for age and sex, presence of tendon xanthomata, CHD in the index patient or in a first-degree relative, and bimodal distribution of total and LDL-C levels in the family, indicating an autosomal dominant pattern of phenotype IIa and genetic diagnosis of FH [17].

Exclusion criteria were clinical manifestations of CHD (see later), diabetes, hypertension, smoking habit, statin treatment (during the last 2 months), consumption of more than 30 g alcohol per day, participation in intense physical fitness or weight loss programs, body weight fluctuation greater than 10% in the previous 3 months, other chronic diseases, other secondary hyperlipemias, renal or hepatic insufficiency and hypothyroidism, infection or inflammatory disease in the 6 weeks before the study, and the use of drugs capable of modifying the lipid profile or OS that could not be withdrawn 6 weeks before starting the study.

A complete medical history and physical examination were carried out in all participants. Body mass index was calculated as the weight in kilograms divided by the square of height in meters. The CHD was diagnosed by the presence of angina pectoris and alterations in the stress electrocardiogram, history of myocardial infarction, or coronary revascularization procedures.

2.2. Measurement of lipids and lipoproteins

Samples were collected in basal state (4 weeks without treatment drugs that could affect lipid metabolism) after 12 to 14 hours of fasting. Blood samples were drawn from an antecubital vein in tubes containing EDTA (BD Vacutainer, Plymouth, UK) and were centrifuged within 4 hours. Plasma was stored at 4°C for a maximum of 3 days. Cholesterol and TG levels were measured by enzymatic techniques [18,19]. The HDL-C was measured after precipitation of apo B–containing lipoproteins with polyanions [20]. The LDL-C was calculated by subtraction of very low-density lipoprotein and HDL cholesterol from TC. Total plasma apo B was measured by immunoturbidimetry [21]. The coefficients of variation for lipids and lipoproteins were less than 5%.

2.3. Oxidative stress assays

Markers of OS were measured in CMC isolated by Ficoll-Hypaque methods as previously reported [22] except for XO. Oxidized glutathione and GSH were analyzed by high-performance liquid columns and UV detection [23]. Superoxide dismutase, CAT, and GPx activities and MDA were measured as previously described by spectrophotometry and high-performance liquid columns [24-26]. Xanthine oxidase activity was determined in plasma obtained by decantation of blood, using Amplex Red Xanthine/Xanthine oxidase assay kit (Molecular Probes, Eugene, OR). Oxidized LDL (oxLDL) was measured by commercial enzyme-linked immunosorbent assay (Biomedica, Wien, Austria).

2.4. Statistical analysis

Data were analyzed with the Statistical Package for the Social Sciences (SPSS 6.1.3 for Windows; SPSS, Chicago, IL) and expressed as mean ± SD. Mean values of quantitative variables were compared with Student t or Mann-Whitney test, depending on the size of the groups under comparison. The Student t test was used when comparing patients with controls (n = 30), and the nonparametric Mann-Whitney test was used to compare male or female patients with their corresponding controls (n = 13 for men and n = 17 for women). Proportions were compared with contingency tables and the χ^2 test or the Fisher exact test (n <5). Correlations were calculated using Pearson formula. Analysis of covariance was used to estimate the independent contributions of the age, sex, and BMI to the mean baseline oxidant and antioxidant enzyme activities.

3. Results

Clinical and biochemical characteristics and OS parameters of the study groups (FH and control subjects divided according to sex) are shown in Tables 1 and 2.

There were no differences in age, sex distribution, BMI, waist circumference, and HOMA values between groups. As expected, TC, LDL-C, and apo B concentrations in plasma were significantly higher in FH subjects.

Results can be observed in Table 2: OS status, assessed by the GSSG/GSH ratio, was significantly higher in FH patients compared with controls. This increased ratio results from both higher levels of GSSG and lower levels of GSH in FH patients. The levels of MDA, a byproduct of lipid peroxidation and marker of OS levels, were significantly higher in FH patients. On the other hand, oxLDL levels were higher in patients than controls. In contrast, activities of the main cytoplasm-antioxidant enzymes were not different between cases and controls; and we have only found lower levels of CAT in FH compared with controls in women.

Xanthine oxidase activity was significantly increased in FH subjects compared with controls, suggesting increased production of free radicals and favoring increased OS. In

Table 1
Age, anthropometric parameters, HOMA index, lipids, and apo B plasma values in FH patients and controls divided according to sex

	FH			Control		
	All (n = 30)	Male (n = 13)	Female (n = 17)	All (n = 30)	Male (n = 13)	Female (n = 17)
Age (y)	39.1 (11.9)	37.1 (8.7)	40.5 (13.8)	40.1 (13.9)	41.6 (14.6)	38.4 (13.6)
BMI (kg/m^2)	26.7 (3.6)	27.5 (2.1)	26.1 (4.4)	26.9 (4.9)	26.8 (3.2)	27.1 (6.5)
Waist circumference (cm)	85.3 (7.4)	89.6 (9.0)	82.1 (9.7)	85.6 (10.6)	91.3 (7.6)	79.4 (9.9)
HOMA	1.74 (0.90)	1.59 (0.80)	1.86 (0.90)	1.71 (1.01)	1.67 (0.90)	1.74 (1.10)
TC (mg/dL)	300.1 (56.7)*	305.3 (51.6)†	296.2 (61.5)‡	188.2 (26.5)	190 (32.6)	186.3 (35.7)
HDL-C (mg/dL)	45.3 (13.1)	45.9 (7.1)	52.5 (12.2)	48.1 (9.1)	45.8 (8.1)	50.5 (9.6)
LDL-C (mg/dL)	216.7 (55.6)*	226.6 (49.6)†	209.1 (60.1)‡	121.1 (26.5)	122.1 (26.6)	119.8 (27.3)
TGs (mg/dL)	133.2 (63.2)	145.6 (73.8)	123.6 (54.1)‡	95.4 (42.2)	110.0 (47.6)	79.9 (29.8)
Apo B (mg/dL)	135.7 (27.7)*	138.8 (25.4)†	133.4 (29.7)‡	85.7 (16.5)	88.8 (16.0)	82.2 (16.9)
Uric acid (mg/dL)	4.6 (1.2)	5.3 (1.7)§	4.0 (1.0)	4.4 (1.3)	5.4 (1.0)‖	3.5 (0.7)

All values are indicated as mean (SD). All differences found are significant after adjustment by age, sex, and BMI.

* $P < .001$; FH vs controls (Student t).

† $P < .001$; FH men vs control men (Mann-Whitney test).

‡ $P < .01$; FH women vs control women (Mann-Whitney test).

§ $P < .1$; FH women vs FH men (Mann-Whitney test).

‖ $P < .001$; control women vs control men (Mann-Whitney test).

Table 2
Prooxidant and antioxidant parameters in FH and control subjects

	FH			Control		
	All (n = 30)	Male (n = 13)	Female (n = 17)	All (n = 30)	Male (n = 13)	Female (n = 17)
XO (mU/mL)	0.44 (0.13)‡	0.43 (0.05)§	0.44 (0.17)‖	0.32 (0.09)	0.36 (0.05)¶	0.29 (0.11)
MDA (U/mg protein)	0.28 (0.11)*	0.24 (0.07)	0.31 (0.13)‖	0.22 (0.1)	0.23 (0.11)	0.21 (0.09)
GSH (nmol/mg protein)	17.74 (3.2)‡	17.6 (3.4)§	17.7 (3.1)‖	22.56 (3.5)	22.7 (3.13)	22.4 (3.9)
GSSG (nmol/mg protein)	0.36 (0.14)*	0.32 (0.13)	0.40 (0.14)‖	0.28 (0.13)	0.30 (0.14)	0.25 (0.11)
GSSG/GSH	2.15 (0.95)‡	1.92 (0.8)	2.3 (1.0)‖	1.32 (0.73)	1.41 (0.8)	1.2 (0.16)
SOD (U/mg protein)	5.73 (2.2)	6.2 (2.7)	5.3 (1.8)	6.14 (3.5)	5.38 (3.2)	6.9 (3.8)
CAT (U/g protein)	191.1 (78.4)	182.1 (93.7)	150.4 (43.8)‖	188.2 (54.8)	176.6 (48.1)	200.6 (60.5)
GPx (U/g protein)	55.1 (6.1)	55.35 (6.1)	54.8 (6.4)	55.8 (7.6)	55.06 (8.15)	56.7 (7.2)
oxLDL	564.7 (399.7)*	668.3 (466.2)	527.6 (385.6)	337.3 (274.2)	385.9 (323.1)	279.8 (202.5)

All values are indicated as means (SD). All differences found are significant after adjustment by age, sex, and BMI.

* $P < .05$; FH vs controls (Student *t*).

‡ $P < .0001$; FH vs controls (Student *t*).

§ $P < .05$; FH men vs control men (Mann-Whitney test).

‖ $P < .05$; FH women vs control women (Mann-Whitney test).

¶ $P < .05$; control women vs control men (Mann-Whitney test).

controls, there was a significant increase of XO activity in men when compared with women, indicating a possible sex protective effect, which was not found in FH patients. Results were maintained after adjusting for age, BMI, and HOMA index. It is important to note that, although uric acid levels were not different between patients and controls, they were higher in men than women.

In the control group, we found a significant association of MDA with waist circumference ($r = 0.42$, $P = .019$) and XO with GSSG/GSH ($r = 0.57$, $P = .001$). No significant associations were found between OS and anthropometric parameters (BMI, waist circumference) or between OS and metabolic parameters (TC, LDL-C, HDL-C, apo B, and HOMA index) in FH patients.

On the other hand, we have found an inverse correlation in the total group between XO activity and GSH level ($r = -0.360$, $P = .004$) and a direct correlation between GSH level and GPx activity ($r = 0.390$, $P = .002$).

4. Discussion

This study, conducted in FH patients, demonstrates an increase in OS parameters in these subjects. Few studies have previously analyzed OS in FH subjects and measured other markers from those analyzed in the present work [15].

Our results showed that OS was increased in FH patients and particularly increased in FH women, indicating the absence of sex compensation in these patients. In contrast, previous studies in normocholesterolemic normoglycemic female subjects have shown increased OS levels due probably to hormonal effects [27].

Different studies have demonstrated that hypercholesterolemia is associated with enhanced cellular superoxide anion production [28]. In this work, we have shown that there is an increase in XO activity, which means an increased production of superoxide anion and, therefore, higher levels of OS. In addition, increased activity of XO is important in OS generation in blood and can influence lymphocyte activity and cellular response [29].

We have also found an inverse correlation between XO activity and GSH level in the total group and a direct correlation between GSH level and GPx activity. An increase in XO activity would generate an increment of ROS and a decrease of GSH due to antioxidant enzymes from glutathione system. An increment in GSH levels could activate the expression of enzymes that use this product as substrate and would explain the increment in GPx activity.

There is little information about cellular response against chronic OS produced in diseases with increased risk of cardiovascular disease regarding antioxidant enzyme activity. Usually, the response of cells under increased OS levels or free radicals is an increase in antioxidant enzymes activity. We have previously shown that hypertensive patients present altered response leading to a reduction in antioxidant enzyme activity in the presence of increased OS levels and an inadequate cellular response to increased OS levels [12,13]. In the present study, we have also shown that there is an altered response to chronic OS in FH; but this alteration is lower than the one found in hypertension.

There is experimental evidence suggesting that OS plays an important role in the development of atherosclerosis [7]. Increased activity of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase in vascular endothelial cells has been related to the development of atherosclerosis in both humans and animal models [8]. In plasma and coronary vessels of patients with CHD, an increase of NAD(P)H oxidase and XO, together with a decrease of SOD activities, has also been reported [9,30]. Moreover, low levels of red blood cells GPx activity have been proposed as an independent risk factor in patients with

coronary artery disease [31]; and some antioxidant enzymes may have prognostic value in addition to that of traditional risk factors [30].

Based on the established relation of increased OS with atherogenesis and the pathogenicity of cardiovascular processes, our results suggest that an increased oxidation status would be able to contribute, at least in part, to the increased risk of cardiovascular disease in FH patients beyond LDL-C plasma values and other traditional risk factors (it must be emphasized that diabetes, hypertension, obesity, and cigarette smoking were exclusion criteria in our study). This fact needs further studies.

In conclusion, in FH subjects (who are a genetic model of hypercholesterolemia and premature CHD), there is an increase in OS in CMCs and an altered response due to the absence of increased activity of antioxidant enzymes. These data could be important for understanding the alterations presented by FH and could be related to their increased atherosclerosis development risk. However, further prospective and intervention studies are necessary to evaluate the impact of OS in the pathogenesis of cardiovascular disease in FH population.

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