Postprandial antioxidant gene expression is modified by Mediterranean diet supplemented with coenzyme Q₁₀ in elderly men and women

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Abstract Postprandial oxidative stress is characterized by an increased susceptibility of the organism towards oxidative damage after consumption of a meal rich in lipids and/or carbohydrates. We have investigated whether the quality of dietary fat alters postprandial gene expression and protein levels involved in oxidative

stress and whether the supplementation with coenzyme Q₁₀ (CoQ) improves this situation in an elderly population. Twenty participants were randomized to receive three isocaloric diets each for 4 weeks: Mediterranean diet supplemented with CoQ (Med+ CoQ diet), Mediterranean diet (Med diet), saturated

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fatty acid-rich diet (SFA diet). After 12-h fast, volunteers consumed a breakfast with a fat composition similar to that consumed in each of the diets. Nrf2, p22^{phox} and p47^{phox}, superoxide dismutase 1 and 2 (SOD1 and SOD2), glutathione peroxidase 1 (GPx1), thioredoxin reductase (TrxR) gene expression and Kelch-like ECH associating protein 1 (Keap-1) and cytoplasmic and nuclear Nrf2 protein levels were determined. Med and Med+CoQ diets induced lower Nrf2, p22^{phox}, p47^{phox}, SOD1, SOD2 and TrxR gene expression and higher cytoplasmic Nrf2 and Keap-1 protein levels compared to the SFA diet. Moreover, Med+CoQ diet produced lower postprandial Nrf2 gene expression and lower nuclear Nrf2 protein levels compared to the other diets and lower GPx1 gene expression than the SFA diet. Our results support the antioxidant effect of a Med diet and that exogenous CoQ supplementation has a protective effects against free radical over-generation through the lowering of postprandial oxidative stress modifying the postprandial antioxidant protein levels and reducing the postprandial expression of antioxidant genes in peripheral blood mononuclear cells.

Keywords CoQ10 · Mediterranean diet · Oxidative stress · Gene expression

Abbreviations

Apo	Apolipoprotein
CAT	Catalase
CoQ	Coenzyme Q10
GPx	Glutathione peroxidase
H ₂ O ₂	Hydrogen peroxide
Keap-1	Kelch-like ECH associating protein 1
Med diet	Mediterranean diet
Med+CoQ diet	Mediterranean supplemented with CoQ
MUFA	Monounsaturated fatty acid
PBMCs	Peripheral blood mononuclear cells
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SFA diet	Saturated fatty acid-rich diet
SOD	Superoxide dismutase
TG	Triacylglycerol
TrxR	Thioredoxin reductase

Introduction

Aging may be defined as an inherently complex process that is manifested within an organism at genetic, molecular, cellular, organ, and system levels (Harman 2009). The aging process induces age-related changes and leads to increased occurrence of many diseases. Although the fundamental mechanisms are still poorly understood, a growing body of evidence points toward the oxidative damage caused by reactive oxygen species (ROS) as one of the primary determinant of aging (Droge 2002). A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during the pathological conditions like diabetes, cardiovascular diseases, cancer, and aging-related diseases (Elahi et al. 2009). Although aging is an inevitable event, linked to the pass of time, nutritional intervention may influence the intrinsic rate of aging as well as the incidence of these age-associated diseases.

Extensive prospective studies suggest that a high degree of adherence to a Mediterranean diet (Med diet) is associated with reduced mortality, prevents the onset and progression of coronary heart disease (Covas 2007) and other aging-related diseases (Trichopoulou et al. 2003; Estruch et al. 2006). Common components of this diet include monounsaturated fatty acids (MUFA), α -tocopherol, phenolic compounds, phytoesters, and other antioxidants so the leading hypothesis on the mechanism of this association is a decrease of oxidative stress due to the antioxidant capacity of this diet (Visioli and Galli 2001).

Fasting is not the typical physiological state of the modern human being, which spends most the time in the postprandial state. In line with this notion, oxidative stress has received considerable attention over the past several years in the fasting state; however, there is a paucity of data on postprandial oxidative stress. With regard to the postprandial state, we have recently demonstrated the antioxidant effect of Med diet rich in olive oil and that exogenous coenzyme Q₁₀ (CoQ: 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone) supplementation in synergy with a Med diet (Med+CoQ diet) improves the postprandial oxidative stress in elderly men and women, with a higher increase in capillary flow, a lower plasma biomarker of oxidative stress levels and a greater postprandial decrease in plasma antioxidant enzymatic activities with respect to a Western diet

rich in saturated fatty acid (SFA diet; Yubero-Serrano et al. 2010).

Interest in CoQ comes from the fact that it is an important mitochondrial redox component (Battino et al. 2001) and endogenously produced lipid-soluble antioxidant of the human organism. CoQ plays a crucial role in the generation of cellular energy, enhances the immune system, and acts as a free radical scavenger with antioxidant properties (Quiles et al. 2005). Aging, poor eating habits, and stress affects the organism's ability to provide adequate amounts of CoQ.

Many research suggest that using CoQ supplements may help maintain health of elderly people or treat some of the health problems or diseases such as adjunctive therapy in the treatment of congestive heart failure (Kaikkonen et al. 2002), as an anticancerogenic and immune-stimulating agent (Folkers et al. 1993) and for slowing down the progression of Parkinson's disease in the early stage (Muller et al. 2003).

As discussed above, one of the causes of aging is the change at the molecular level, which may cause alterations in the expression levels of genes and protein involved in oxidative stress. Peripheral blood mononuclear cells (PBMCs) are a subset of white blood cells, which include lymphocytes and monocytes, and play a critical role in the immune system. Various studies showed disease-characteristic gene expression patterns in PBMCs (Mass et al. 2002; Burczynski and Dörner 2006) and they can be easily and repeatedly collected in sufficient quantities in contrast to the more invasive sampling of adipose, muscle and liver tissues, among others (de Mello et al. 2008). However, little is known of nutritional effects on PBMCs gene expression patterns.

According to these premises, the aim of this study was to determine whether diets with different fat quality influence on the postprandial gene expression and protein levels involved in oxidative stress and that this hypothetical improvement could be boosted by supplementation with a natural antioxidant, like CoQ, a natural antioxidant agent in PBMCs of healthy elderly people.

Experimental procedures

Participants and recruitment

Volunteers were recruited using various methods including the use of general practitioner databases, and poster and newspaper advertisements. A total of

63 persons were contacted among those willing to enter the study. All participants underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrolment and gave their informed consent before joining the study. Inclusion and exclusion criteria were fulfilled by 20 patients (age ≥ 65 years; 10 men and 10 women). Power analysis was set a 90% to detect variations equal or larger than 20% in the mRNAs gene expression (Jimenez-Gomez et al. 2009). The sample size calculation indicated that we needed at least 18 participants. Clinical inclusion criteria were: age ≥ 65 years, body mass index 20–40 kg/m², total cholesterol concentration equal to or < 8.0 mmol/L and nonsmokers. Clinical exclusion criteria were: age < 65 years, diabetes or other endocrine disorders, chronic inflammatory conditions, kidney or liver dysfunction, iron deficiency anemia (hemoglobin < 12 g/dL men, < 11 g/dL women), prescribed hypolipidaemic and anti-inflammatory medication, fatty acid supplements including fish oil, consumers of high doses of antioxidant vitamins (A, C, E, β -carotene), highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week, weight change equal or > 3 kg within the last 3 months, smokers, alcohol, or drug abuse (based on clinical judgment). The study protocol was approved by the Human Investigation Review Committee of the Reina Sofia University Hospital according to institutional and Good Clinical Practice guidelines.

Study design

Participants were randomly assigned to receive, in a crossover design, three isocaloric diets for 4-week periods each (Supporting Fig. S1). The three diets were as follows: (1) Mediterranean diet supplemented with CoQ (Med+CoQ diet; 200 mg/day in capsules), containing 15% of energy as protein, 47% of energy as carbohydrate and 38% of total energy as fat [24% MUFA (provided by virgin olive oil), 10% SFA, 4% polyunsaturated fatty acid (PUFA)]. (2) Mediterranean diet not supplemented with CoQ (Med diet), with the same composition of the first diet but supplemented by placebo capsules and (3) Western diet rich in saturated fat (SFA diet) with 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat (12% MUFA, 22% SFA, 4% PUFA).

The cholesterol intake was kept constant (< 300 mg/day) during the three periods. Both the CoQ and the

placebo capsules were specially produced by the same company (Kaneka Corporation, Osaka, Japan) and were identical in weight and external aspect. Patients taking capsules were unaware whether they were in the Med+CoQ or Med dietary period (Supporting Table S1). The composition of the experimental diets was calculated by using the US Department of Agriculture (1987) food tables and Spanish food composition tables for local foodstuffs (Varela 1980).

Before the start of the intervention period, volunteers completed a 3-day weighed food diary and an extensive Food Frequency Questionnaires, which allowed identification of foods to be modified. At the start of the intervention period, each patient was provided with a handbook for the diet to which they had been randomized. Advice was given on foods to choose and those to avoid if eating outside home. They were also instructed to write down in the diary about any menu eaten out of home and to call the monitoring study nurse reporting such event. At baseline, volunteers were provided with a supply of study foods to last for 2 weeks. They collected additional study foods every fortnight or when required. At these times, a 24-h recall of the previous day's food intake and a short food use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A points system was used to assess the number of food exchanges achieved in the 24-h recall and additional advice was given if either the 24-h recall or food use questionnaire showed inadequate intake of food exchange options. Volunteers were asked to complete 3-day weighed food diaries at baseline, weeks 2 and 4. Weighed food intake over two weekdays and one weekend day was obtained using scales provided by the investigators. Fat foods were administered by dietitians in the intervention study. The dietary analysis software Dietsource version 2.0 was used. (Novartis S.A., Barcelona, Spain).

At the end of the dietary intervention period, the subjects were given a fatty breakfast with the same fat composition as consumed in each of the diets. Patients presented at the clinical centers at 8 h following a 12-h fast (time 0), abstained from alcohol intake during the preceding 7 days. After cannulation of a blood vessel, a fasting blood sample was taken before the test meal, which was then ingested within 20 min under supervision. The test meal reflected fatty acid composition of each subject after the chronic dietary intervention. Subsequent blood samples were drawn at 2 and 4 h.

Test meals provided an equal amount of fat (0.7 g/kg body weight), cholesterol (5 mg/kg of body weight), and vitamin A (60,000 IU/m² body surface area). The test meal provided 65% of energy as fat, 10% as protein, and 25% as carbohydrates. The composition of the breakfasts was as follows: Med with CoQ (400 mg in capsules) breakfast (12% SFA, 43% MUFA, and 10% PUFA), Med with placebo capsules breakfast (12% SFA, 43% MUFA, and 10% PUFA), and SFA-rich breakfast (38% SFA, 21% MUFA, and 6% PUFA).

Biochemical determinations

Plasma samples

Venous blood samples were obtained at the end of the each dietary intervention period on fasting state after a 12-h fast, before breakfast ingestion and at 2 and 4 h after ingestion of breakfast. Samples from the fasting and postprandial states were collected in tubes containing 1 g ethylenediaminetetraacetic acid (EDTA)/L and were stored in containers with ice and kept in the dark. Particular care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from whole blood by low-speed centrifugation at 1,500×g for 15 min at 4°C within 1 h of extraction.

Isolation of PBMCs

PBMCs were isolated from 20 mL of venous blood in tubes containing 1 mg/mL of EDTA. The blood samples were diluted 1:1 in PBS, and cells were separated in Ficoll gradient by centrifugation at 800×g for 25 min at 20°C. The cells were collected and washed with cold PBS two times and finally resuspended in buffer A. This buffer contained 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mM KCl, 2 mM MgCl₂ and 1 mM EDTA and, at the time of use, 1 mM PMSF and 1 mM DTT were added. The cells thus obtained were stored at −80°C for further analysis.

RNA extraction and qRT-PCR analysis

RNA extraction

Total RNA from PBMCs was extracted using the trizol method according to the recommendations of the manufacturer (Tri Reagent®, Sigma, St Louis, MO, USA) and quantified in a NanoDrop 1000A

Spectrophotometer. RNA integrity was verified on agarose gel electrophoresis and stored at -80°C . Next, since polymerase chain reaction (PCR) can detect even a single molecule of DNA, RNA samples were digested with DNase I (AMPD-1 KT, Sigma) before RT-PCR.

Biotrove open arrayTM real-time PCR

Each reaction was performed with 1 μL of a 1:5 (v/v) dilution of the first cDNA strand, synthesized from 1 μg of total RNA using the commercial kit iScript cDNA Synthesis Kit (Bio Rad) according to the manufacturer's instruction.

The reaction of real-time PCR was carried out using the platform OpenArrayTM NT Cyclor (Applied Biosystems). This system of analysis of gene expression used as a method for quantification TaqMan probes. OpenArrayTM subarrays were preloaded by Biotrove with the selected primer pairs. The individual primer pairs (synthesized by Sigma-Aldrich, St. Louis, MO, USA) were preloaded into Bio Trove OpenArrayTM plates. Each primer pair was spotted in duplicate. The primers that amplify genes of interest were selected from the database TaqMan Gene Expression Assays (Applied Biosystems; <https://products.appliedbiosystems.com/ab/en/US/adirect/ab?Cmd=catNavigate2&catID=601267>) in Assays search tab taking as search criteria: selection and *Homo sapiens* Gene Expression Assays for each of the genes of interest.

Samples were loaded into OpenArray plates with the OpenArray NT Autoloader according to the manufacturer's protocols. Each subarray was loaded with 5.0 μL of master mix consisted of 1 $\times$ LighCycler FastStart DNA Master SYBR Green Kit (Roche Applied Sciences, Indianapolis, IN, USA), 1 $\times$ SYBR Green I 80 $\times$, 0.5% glycerol, 0.2% Pluronic F-68, 1 mg/mL bovine serum albumin (New England Biolaboratories, Beverly, MA, USA), 1 mM MgCl_2 , 400 nM FP, 400 nM RP, 8% Formamide, 0.25 $\times$ Rox, 1 $\times$ TIR amplicon, and cDNA samples. The PCR OpenArray thermal cycling protocol consisted of 95°C for 10 min, followed by cycles of 10 s at 95°C , 10 s at 53°C , and 10 s at 72°C . All samples were tested in duplicate. The Biotrove OpenArrayTM NT Cyclor System Software (version 1.0.2) uses a proprietary calling algorithm that estimates the quality of each individual C_T value by calculating a C_T confidence value for de amplification reaction.

In our assay, C_T values with C_T confidence values below 700 were regarded as background signals. The remaining positive amplification reactions were analyzed for amplicon specificity by studying the individual melting curves.

The same program allowed the selection of the most stable housekeeping gene in the samples processed for the relativization of the expression of genes of interest. Following this methodology, we analyzed the relative gene expression of these genes: Nrf2, p47^{phox}, and p22^{phox} (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits), superoxide dismutase 1 and 2 (SOD1 and SOD2), glutathione peroxidase 1 (GPx1), and thioredoxin reductase (TrxR).

Western blot analysis

Protein extraction

PMBCs were thawed on ice and buffer A was supplemented with 5 μg Aprotinin, 10 μg Leupeptin and 0.8% Nonidet NP-40. Cells were incubated on ice for 5 min, subjected to gentle agitation for 20 s in the vortex and then centrifuged at 13,000 rpm for 5 min at 4°C . The supernatant containing cytoplasmic proteins was distributed in aliquots that were stored at -80°C . The pellet was treated with 100 μL of lysis buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 20 μg Aprotinin, and 40 μg Leupeptin). The sample was incubated on ice for 20 min with periodic mixing with a vortex by stirring for 30 s every 5 min of incubation. Cells were then centrifuged at 13,000 rpm for 5 min at 4°C . The supernatant thus obtained, containing the nuclear proteins, was distributed in aliquots, and stored at -80°C . The extracted proteins were quantified using the method of Bradford (1976).

Western blot

Electrophoretic separation was carried out with 50 μg of protein for both cytoplasmic and nuclear fractions. After separation in sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (11% polyacrylamide), proteins were transferred to nitrocellulose membranes (BioTrace NT Membrane; PALL Gelman Laboratory). The following proteins were detected using their corresponding antibodies: Nrf2 (C-20, sc-722: mouse monoclonal; Santa Cruz Biotechnology, Inc.), Kelch-like ECH

associating protein 1 (Keap-1; H-190, sc-33569; rabbit polyclonal; Santa Cruz Biotechnology, Inc.); Actin (C-2, sc-8432; mouse monoclonal; Santa Cruz Biotechnology, Inc.). After incubation with these primary antibodies, samples were incubated with respective secondary antibodies (goat anti-mouse or anti-rabbit HRP-conjugate; Santa Cruz Biotechnology, Inc.). The development process was carried out with ECL-Plus Western Blotting Detection System (Amersham™) and used for autoradiography Hyperfilm MP high-performance autoradiography film (Amersham™).

Quantification of protein bands

The proteins were identified in the autoradiography by its position relative to molecular weight markers: 57 kDa for Nrf2, 69 kDa for Keap-1, and 40 kDa for actin. The relative amount of each was quantified by densitometry using the software WIN1D.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, IL, USA) was used for the statistical comparisons. The Kolmogorov–Smirnov test did not show a significant departure from normality in the distribution of variance values. In order to evaluate data variation, Student's *t* test and an analysis of variance for repeated measures was performed, followed by Bonferroni's correction for multiple comparisons. We studied the statistical effects of the type of fat meal ingested, independent of time (represented by *p*1), the effect of time (represented by *p*2), and the interaction of both factors, indicative of the degree of the postprandial response in each group of subjects with each fat meal (represented by *p*3). Differences were considered to be significant when *p*<0.05. All data presented in text and tables are expressed as means±standard error (±SE).

Results

Metabolic parameters levels

The baseline characteristics of the 20 participants who completed the three dietary intervention periods showed that males had higher height, waist circumference, TG

and Apo B than females. We did not find any other differences by gender (Supporting Table S2).

Moreover, we previously observed higher fasting plasma CoQ concentration (*p*<0.001) after the intake of the Med+CoQ diet compared with the Med and SFA diets. At 2 and 4 h after consumption of the Med+CoQ diet we observed a greater postprandial increase in plasma CoQ levels compared with the Med and SFA diets (*p*=0.018 and *p*=0.032, respectively; Supporting Fig. S2; Yubero-Serrano et al. 2010).

Diet intake- and expression genes-related oxidative stress in PBMCs

Nrf2

Nrf2 is the central transcription factor, essential for the coordinated induction of those genes encoding many stress-responsive or cytoprotective enzymes and related protein, such as SOD, GPx, glutathione S-transferase, and Thioredoxin (Dinkova-Kostova and Talalay 2008; Chen and Kong 2004). At 4 h after intake of the Med+CoQ diet, we observed a greater postprandial decrease in Nrf2 mRNA levels compared with the other diets (*p*=0.029; Fig. 1a). Furthermore, Nrf2 mRNA levels were lower after consumption of the Med diet compared with the SFA diet (*p*=0.039). No significant differences were detected in Nrf2 mRNA levels in fasting and at 2 h after intake of the three diets (Fig. 1a).

NADPH oxidase

NADPH oxidases are likely to be the predominant source of ROS in the vasculature (Brandes and Kreuzer 2005; Forstermann 2008; Grienling 2004). Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits (p47^{phox}, p67^{phox}, p40^{phox}, and Rac) with the membrane-bound cytochrome b558 (subunits gp91^{phox} and p22^{phox}; Brandes and Kreuzer 2005).

We observed a decrease in fasting p22^{phox} mRNA levels after intake of the Med and Med+CoQ diets compared to the SFA diet (*p*=0.032; Fig. 1b). At 2 h and 4 h after the SFA diet, we found higher postprandial p22^{phox} and p47^{phox} mRNA levels compared with the Med and Med+CoQ diets (*p*=0.038 and *p*=0.003, respectively; Fig. 1b,c). No significant differences were detected in p47^{phox}

Fig. 1 Fasting and postprandial levels of Nrf2 mRNA (a), p47^{phox} mRNA (b), and p22^{phox} mRNA (c) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for repeated measures. All values represent the mean $\pm$ standard errors (SE). Bars with different superscript letters depict statistically significant differences ($p < 0.05$). $p1$ diet effect, $p2$ time effect, $p3$ diet $\times$ time interaction

mRNA levels in fasting after intake of any of the three diets (Fig. 1c).

SODs

SOD is a key antioxidant enzyme, scavenging the superoxide radical (O_2^-). SOD form the first line of defence against oxidative stress. We studied the expression of the two isoforms of human SOD: cytoplasmic SOD gene (SOD1 gene) and mitochondrial

SOD gene (SOD2 gene). Fasting SOD2 mRNA levels were lower after participants consumed the Med and Med+CoQ diets than when they consumed the SFA diet ($p=0.007$; Fig. 2a). At 2 and 4 h after the SFA diet, we found higher postprandial SOD2 mRNA levels compared with the Med and Med+CoQ diets ($p=0.008$ and $p=0.003$, respectively). At 4 h after intake of the Med and Med+CoQ diets, we observed a greater postprandial decrease in SOD1 mRNA levels with respect to the SFA diet ($p=0.011$). However, no

significant differences were detected in SOD1 mRNA levels in fasting and at 2 h after intake of the three diets (Fig. 2b).

Gpx1

GPx enzyme catalyzes the reduction of H_2O_2 and scavenges organic hydroperoxides (de Haan et al. 1998). Fasting Gpx1 mRNA levels were greater after participants consumed the SFA diet than when they consumed the other two diet period ($p=0.013$; Fig. 2c). At 2 and 4 h after the Med+CoQ diet, we found lower postprandial Gpx1 mRNA levels compared with the SFA diet ($p=0.013$; Fig. 2c).

TrxR

TrxR is a ubiquitous oxidoreductase enzyme with antioxidant and redox regulatory roles. It also contributes to redox homeostasis and is involved in prevention, intervention, and repair of damage caused by H_2O_2 -based oxidative stress (Nordberg and Arner 2001).

At 4 h after intake of the SFA diet, we found a greater postprandial increase in TrxR mRNA levels compared to the other diets ($p=0.023$). No significant differences were detected in TrxR mRNA levels in fasting and at 2 h after intake of the three diets (Fig. 2d).

Diet intake and cellular protein levels related to oxidative stress

Cytoplasmic and nuclear Nrf2 in PBMCs

In fasting and at 4 h after intake of the Med+CoQ diet, we observed higher cytoplasmic Nrf2 protein levels compared to the other diets ($p=0.033$ and $p=0.011$, respectively; Fig. 3a). In addition, at 4-h Med diet induced a significant increase of cytoplasmic Nrf2 protein levels compared to the SFA diet ($p=0.026$). However, both in fasting and at 4 h after intake of the Med+CoQ diet, we found lower nuclear Nrf2 protein levels compared to the SFA diet ($p=0.001$ and $p=0.019$, respectively), with an intermediate effect for the Med diet (Fig. 3b).

Fig. 2 Fasting and postprandial levels of SOD1 mRNA (a), SOD2 mRNA (b), Gpx1 mRNA (c), and TrxR mRNA (d) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for

repeated measures. All values represent the mean ± standard errors (SE). Bars with different superscript letters depict statistically significant differences ($p < 0.05$). p_1 diet effect, p_2 time effect, p_3 diet × time interaction

Fig. 3 Fasting and postprandial levels of cytoplasmic Nrf2 (a), nuclear Nrf2 (b), and Keap-1 (c) in peripheral mononuclear cells according to the type of fat consumed. Representative

immunoblot of nuclear and cytoplasmic Nrf2, Keap-1, and Actin. Depicted bands are different parts of the same blot stained for each antigen (d)

Keap-1 in PBMCs

Nrf2 activity is repressed by the cytosolic protein Keap-1, which has been proposed to act by sequestering and tethering the transcription factor in the cytoplasm (Nguyen et al. 2005). In fasting and at 4 h after intake of the Med+CoQ diet, we observed higher Keap-1 protein levels compared to the other diets ($p=0.035$ and $p=0.003$, respectively) (Fig. 3c). In addition, at 4 h Med diet induced a significant increase of postprandial Keap-1 protein levels compared to the SFA diet ($p=0.017$).

Discussion

The present study demonstrates that the consumption of a Med diet reduces the postprandial expression of genes that encoded proteins related to oxidative stress such as SOD (SOD1 and SOD2), TrxR, and NADPH-oxidase (p22^{phox} and p47^{phox} subunits) and increases the cytoplasmic Nrf2 protein levels in PBMCs. Moreover, the addition of CoQ had an additive effect on the Med diet since the participants that consumed this diet showed a greater postprandial decrease in gene expression of Nrf2 and GPx1 and a greater postprandial increase in cytoplasmic

Nrf2 and Keap-1 protein levels in PBMCs with respect to the other diets.

In the same population of this study, we previously demonstrated that the Med diet improves the postprandial oxidative stress with a higher increase in capillary flow and plasma nitric oxide levels, a lower plasma lipid peroxidation products, nitrotyrosine and protein carbonyl levels, lower plasma antioxidant enzyme activities (GPx, catalase (CAT) and SOD), (Yubero-Serrano et al. 2010) and lower DNA damage in PBMCs (Gutierrez-Mariscal et al. 2011). Addition of exogenous CoQ in synergy with a Med diet had an additive effect reducing the postprandial oxidative stress in elderly men and women.

Moreover, few studies explored into the molecular effects that diet has on antioxidant gene expression and levels of proteins related with oxidative stress as Nrf2 and Keap-1 during the postprandial state, or into the effect of CoQ supplements in humans (Wang et al. 2010). While it is now widely accepted that oxidative damage plays an important role in the aging process (Lombard et al. 2005), the influence of age on the gene expression of antioxidant enzymes has not been widely studied; moreover, the few studies that have been performed have yielded conflicting results (Rao et al. 1990).

A central role in the defense against oxidative stress has been attributed to the transcription factor Nrf2. Nrf2 is localized in the cytoplasm where it interacts with the Keap-1, and is rapidly degraded by the ubiquitin–proteasome pathway (Zhang 2006). When the cell is exposed to oxidative stress, induction of Nrf2 occurs which dissociates Nrf2 from Keap-1. Stabilized Nrf2 then translocates to the nucleus and promotes the expression of genes encoding many antioxidant proteins and phase-II detoxifying enzymes such as CAT, SOD, GPx, and heme oxygenase-1 through binding specifically to the antioxidant-response element (ARE) found in the gene promoters (Xu et al. 2008; Kobayashi and Yamamoto 2005).

Our results show a greater increase in the cytoplasmic Nrf2 protein levels after intake of the Med+CoQ diet compared with the SFA diet, with an intermediate effect for the Med diet. These finding could be explained by the fact that the increase of Keap-1 protein levels obtained after intake of the Med+CoQ diet promotes its interaction with Nrf2 in the cytoplasm, which prevents the translocation of Nrf2 to the nucleus and does not allow the antioxidant genes expression. This hypothesis is corroborated with a greater postprandial decrease in the nuclear Nrf2 protein levels after intake of the Med+CoQ diet compared with the SFA diet, with an intermediate effect for the Med diet. Thus, after intake of the SFA diet, lower levels of Keap-1 in the cytoplasm and higher levels of nuclear Nrf2 protein may have favored the binding of Nrf2 to the ARE found in the antioxidant gene promoters and subsequent transcriptional gene activation. In fact, a postprandial rise in Nrf2, GPx1, SOD1, SOD2, and TrxR mRNA levels were observed for the SFA diet in our study.

SOD, GPx, and CAT are regarded as the first line of the antioxidant defense system enzymes against ROS. They protect cells against ROS produced during normal metabolism and after an oxidative insult. SOD metabolizes and protects the cells against O_2^- -mediated lipid peroxidation, while CAT and GPx acts on H_2O_2 and/or ROOH by decomposing them, thereby neutralizing their toxicity. Also, peroxides, including lipid hydroperoxides and hydrogen peroxide can directly be reduced by TrxR (Zhong and Holmgren 2000; Bjornstedt et al. 1995). By this mechanism, TrxR could function as an alternative enzymatic pathway for the detoxification of lipid hydroperoxides, otherwise mainly managed by GPx. Any changes in one of these systems may break the equilibrium and cause

cellular damage (Arsova-Sarafinovska et al. 2009). Previous studies have shown that the expression of SOD is upregulated by ROS (Mates et al. 1999). Thus, the decrease in SOD1 and SOD2 mRNA levels observed along the postprandial state of the Med and Med+CoQ diets with respect to the SFA diet could be explained on the basis of lower generation of superoxide in these diets. In addition, GPx1 mRNA levels showed the lowest postprandial levels in Med+CoQ diet with respect to the other diets. Presumably, CoQ supplementation contributed to a reduction in the production of H_2O_2 and in this way suppressed the GPx1 gene expression for which H_2O_2 serves as a substrate (Stachowska et al. 2005) since CoQ deficiency interferes with assembly or stability of the respiratory chain enzymes leading to unbalanced oxidative phosphorylation and enhanced ROS production (Quinzii et al. 2008). This phenomenon, together with the fact that there were observed an increase in TrxR mRNA levels after intake of the SFA diet compared with the Med and Med+CoQ diets could be due to the fact that the damage caused by oxidative stress is higher in the SFA diet than in the other diets.

NADPH oxidases are likely to be the predominant source of ROS in the vasculature (Brandes and Kreuzer 2005; Forstermann 2008; Grienling 2004). Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac) with the membrane-bound cytochrome b558 ($gp91^{phox}$ and $p22^{phox}$; Brandes and Kreuzer 2005). High-fat diet had a significant increase in NADPH oxidase expression compared to low-fat diet in mice (Coate and Huggins 2010). Thus, we have observed that during the postprandial period, the intake of the SFA diet induced an increase in fasting and postprandial $p22^{phox}$ and postprandial $p47^{phox}$ mRNA levels, which could be due to an increased ROS production after the ingestion of this diet compared with the Med and Med+CoQ diets.

The present study has the advantage of a randomized crossover design in which all the participants have experienced the three diet periods, each individual acting as his/her own control and strengthening the fact that the effects observed are due to the influence of the type of diet. We acknowledge that our study has certain limitations, since ensuring adherence to dietary instructions is difficult in a feeding trial. However, adherence to the recommended dietary patterns was satisfactory, as

can be judged by the measurements of compliance. Another limitation of this study is that we did not study if supplementation with CoQ of the saturated fatty acid-rich diet may partly reduce the negative effects of this dietary model on postprandial oxidative stress.

A thorough nutrition status may be basic to understand the elderly total health. Therefore, our results support that consumption of a Med diet rich in olive oil (combined with vegetables, fruits, cereals, and a healthy lifestyle) contributes to redox homeostasis and is involved in prevention caused by H₂O₂-based oxidative stress. This protection is enhanced by exogenous CoQ supplementation, lowering of postprandial oxidative stress, modifying the postprandial antioxidant protein levels, and reducing the postprandial expression of antioxidant genes in PBMCs in elderly men and women. We can conclude that specific dietary intervention might be a new, interesting, and promising challenge in the treatment (and mainly prevention) of processes that lead to a rise in oxidative stress, such as cardiovascular, neurodegenerative diseases, and aging.

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