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Differential menopause- versus aging-induced changes in oxidative stress and circadian rhythm gene markers

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ABSTRACT

Menopause is characterized by the depletion of estrogen that has been proposed to cause oxidative stress. Circadian rhythm is an internal biological clock that controls physiological processes. It was analyzed the gene expression in peripheral blood mononuclear cells and the lipids and glucose levels in plasma of a subgroup of 17 pre-menopausal women, 19 men age-matched as control group for the pre-menopausal women, 20 post-menopausal women and 20 men age-matched as control group for the post-menopausal women; all groups were matched by body mass index. Our study showed a decrease in the expression of the oxidative stress-related gene GPX1, and an increase in the expression of SOD1 as consequence of menopause. In addition, we found that the circadian rhythm-related gene PER2 decreased as consequence of menopause. On the other hand, we observed a decrease in the expression of the oxidative stress-related gene GPX4 and an increase in the expression of CAT as a consequence of aging, independently of menopause. Our results suggest that the menopause-induced oxidative stress parallels a disruption in the circadian clock in women, and part of the differences in oxidative stress observed between pre- and post-menopausal women was due to aging, independent of menopause.

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1. Introduction

Aging is a complex process. Our current knowledge points towards oxidative stress as the main determinant in the deleterious and cumulative effects in the biology of aging (Sohal and Orr, 2012). In fact, aging increases the risk of cardiovascular diseases mainly by its association with increased oxidative stress and chronic low-grade inflammation (Herrera et al., 2009), which is also related with oxidative stress (Kabe et al., 2005). In women, menopause is a normal consequence of aging. As a major manifestation of female reproductive senescence, it is characterized by the permanent cessation of ovarian follicular activity, which produces an abrupt fall in estrogen levels, leading to the classic signs and symptoms of menopause, as well as an increased risk of cardiovascular diseases and osteoporosis (Rao et al., 2013; Greendale et al., 1999).

Oxidative stress has been implicated in various pathologies such as vasomotor disturbances, osteoporosis and cardiovascular diseases, which significantly correlate with the progressive loss of estrogen and its protective effects, combined with a deficient antioxidant defense which leads to a pronounced redox imbalance. In fact, it has been proposed that the depletion of estrogen in post-menopause could cause oxidative stress, in addition to the known symptoms (Rao et al., 2013; Greendale et al., 1999; Doshi and Agarwal, 2013). In addition, the incidence of metabolic diseases and their co-morbidities, which are sexually dimorphic, increases after menopause in women, at which time sex hormones are thought to play an important role in the development

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of cardiovascular disease (Arnlov et al., 2006; Bhupathy et al., 2010; Luczak and Leinwand, 2009). Moreover, it has been reported that post-menopausal women have higher lipoperoxide (Sanchez-Rodriguez et al., 2012), pro-inflammatory cytokines (Vural et al., 2006), and pro-oxidant biomarkers such as malonaldehyde, 4-hydroxynenal, and oxidized LDL (Signorelli et al., 2006) levels than pre-menopausal women, whereas the levels of antioxidant glutathione peroxidase (GPX) are lower in post-menopausal women (Signorelli et al., 2006). Nevertheless, hormone replacement therapies (HRT) have failed to prevent the post-menopausal increase in oxidative stress (Sekhon and Agarwal, 2013).

Most of the studies focusing on the physiological changes caused by menopause compare only pre- and post-menopausal women, with an age difference of several years (post-menopausal groups are usually on average at least 10 years older than pre-menopausal groups), without age-matched groups of men to control the potential aging-induced, menopause-independent differences between pre- and post-menopausal women. To fill this gap, the objective of this study was to performer a series of comparative expression analyses in groups of pre- and post-menopausal women, and their corresponding age-matched male control groups. All 4 groups were matched by body mass index (BMI), and in them we analyzed the expression of gene markers of cardiovascular risk-related processes, including endoplasmic reticulum stress, inflammation, oxidative stress, metabolism, and circadian rhythms, in peripheral blood mononuclear cells (PBMC). This sub-set of white blood cells modifies its gene expression profile in response to stimuli and has proved to be useful in distinguishing a disease from a healthy state (Burczynski and Dorner, 2006; Camargo et al., 2014).

2. Methods

2.1. Study subjects

The current work was conducted in a subgroup of patients as part of the CORDIOPREV study (Clinical Trials.gov.Identifier: NCT00924937), an ongoing prospective, randomized, opened, controlled trial in patients with coronary heart disease (CHD), who had their last coronary event over six months before enrolling in two different dietary models (Mediterranean and Low-fat) over a period of five years, in addition to conventional treatment for CHD (Alcala-Diaz et al., 2014). All the patients gave written informed consent to participate in the study. The trial protocol and all amendments were approved by the local ethics committees, following the Helsinki declaration and good clinical practice. We analyzed a subgroup of 76 patients from the control healthy group included in the CORDIOPREV study (without oncological or cardiovascular diseases): 17 pre-menopausal women (E2 = 109.401 ± 41.46 pg/mL, FSH = 13.47 ± 2.90 mIU/mL), 19 men matched by age as a control group for the pre-menopausal women (T = 108 ± 14 pg/mL), 20 post-menopausal women (E2 = 11.49 ± 4.47 pg/mL, FSH = 93.54 ± 7.63 mIU/mL) and 20 men (T = 92 ± 9 pg/mL) matched by age as a control group for the post-menopausal women: all 4 groups were matched by BMI. Patient's inclusion was assessed by medical history, biochemical measures and physical examination by clinicians. Exclusion criteria included cardiovascular disease, cancer, and chronic diseases and did not have severe diseases or an estimated life expectancy of less than 5 years. We also performed a diet assessment with a validated 14-item questionnaire (Schroder et al., 2011) to assess the dietary habits of the groups.

2.2. Clinical plasma parameters

The patients arrived at the clinical centre at 08:00 h. We measured anthropometric (weight, height, waist circumference, BMI and blood pressure) and took a fasting blood sample. Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) to give a final concentration of 0.1% EDTA. The plasma was separated from the red blood cells by centrifugation at 1500 × g for 15 min at 4 °C. Analytes determined in frozen samples were analyzed centrally by laboratory investigators of the Lipid and Atherosclerosis Unit at the Reina Sofia University Hospital, who were unaware of the interventions. Lipid variables were assessed with a DDPPII Hitachi modular analyzer (Roche) using specific reagents (Boehringer-Mannheim). Plasma triglycerides (TG) and cholesterol concentrations were assayed by enzymatic procedures (Allain et al., 1974; Paisley et al., 1996). High-density lipoprotein-cholesterol (HDL-c) was measured by precipitation of a plasma aliquot with dextran sulphate-Mg^{2+}, as described by Warnick et al. (Warnick et al., 1982). Low-density lipoprotein-cholesterol (LDL-c) was calculated by using the following formula: plasma cholesterol − [HDL-C + large TG-rich lipoproteins (TRL-C) + small TRL-C]. Plasma glucose concentrations were measured by using the IL Test Glucose Hexokinase Clinical Chemistry kit (Instrumentation Laboratories, Warrington, United Kingdom).

2.3. Sex hormone determination

Testosterone was determined by the commercial kit: Testosterone Assay (R & D System; Cat. No. KGE010), according to the manufacturer's instructions. Estradiol was determined by the commercial kit: Estradiol EIA kit (Cayman Chemical; Cat. No. 582251), according to the manufacturer instructions. Follicle-Stimulating Hormone was determined by the commercial kit: FSH ELISA (DRG Instruments GmbH; Cat. No. EIA-1288), according to the manufacturer's instructions.

2.4. Isolation of peripheral blood mononuclear cells and RNA extraction

Buffy coats were diluted 1:2 in phosphate saline buffer (PBS), and cells were separated in 15 mL Ficoll gradient (lymphocyte isolation solution, Axis-Shield, Oslo, Norway) by centrifugation at 2000 × g for 25 min. PBMC were collected, washed twice with cold PBS and stored in RNA Later Solution (Ambion, Thermo Fisher Scientific, MA, USA). Total RNA was extracted using Tri Reagent (Sigma, St Louis, MO, USA), according to the manufacturers' instructions. The recovered RNA was quantified using a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology®, Cambridge, UK), and its integrity was checked on agarose gel electrophoresis and stored at − 80 °C. RNA samples were digested with DNAse I (AMPD-1 Kit, Sigma) before RT-PCR.

2.5. qRT-PCR for gene expression analysis

Retrotranscription reaction was performed with 500 ng of total RNA using the Ambion WT Expression Kit (Applied Biosystems, Carlsbad, CA, USA), following the manufacturers' instructions. Real-time PCR reactions were carried out using the OpenArray™ NT Cycler system (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturers' instructions. Primer pairs for 53 target genes related to endoplasmic reticulum stress, inflammation, oxidative stress (pro-oxidants and antioxidants), metabolism and circadian rhythms were selected from the TaqMan Gene Expression assays database (Applied Biosystems, Carlsbad, CA, USA) (Appendix 1 Supplemental Table 1). We used as housekeeping genes: beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase, for which Ct values were combined by the software Bestkeeper (Pfaffl et al., 2004) in order to get a more stable reference value than each one independently. Gene expression values were obtained as a relative expression of the target gene versus the Bestkeeper value (relative expression = $2^{-(\text{Ct, Targe gene} - \text{Ct, Bestkeeper value})}$). The data set was extracted by using the OpenArray® Real-Time qPCR Analysis Software (Applied Biosystems, Carlsbad, CA, USA).

2.6. Statistical analysis

PASW statistical software, version 20.0 (IBM Inc., Chicago, IL, USA) was used for statistical analysis of the data. The normal distribution of