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Oxidative stress, mitochondrial dysfunction and, inflammation common events in skin of patients with Fibromyalgia

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ABSTRACT

Fibromyalgia is a chronic pain syndrome with unknown etiology. Recent studies have shown some evidence demonstrating that oxidative stress, mitochondrial dysfunction and inflammation may have a role in the pathophysiology of fibromyalgia. Despite several skin-related symptoms accompanied by small fiber neuropathy have been studied in FM, these mitochondrial changes have not been yet studied in this tissue. Skin biopsies from patients showed a significant mitochondrial dysfunction with reduced mitochondrial chain activities and bioenergetics levels and increased levels of oxidative stress. These data were related to increased levels of inflammation and correlated with pain, the principal symptom of FM. All these parameters have shown a role in peripheral nerve damage which has been observed in FM as a possible responsible to allodynia. Our findings may support the role of oxidative stress, mitochondrial dysfunction and inflammation as interdependent events in the pathophysiology of FM with a special role in the peripheral alterations.

1. Introduction

Allodynia, a perception of pain from what would usually be an innocuous stimulation of the skin, and peripheral and central nociception alterations have been involved in fibromyalgia (FM) (Staud, 2010a).

FM is a common chronic pain syndrome accompanied by other symptoms such as fatigue, headache, sleep disturbances, and depression, which pathophysiological mechanisms are difficult to identify. Although it is diagnosed according to the classification criteria established by the American College of Rheumatology (ACR) (Lawrence et al., 2008) the diagnosis is not easy and may frequently be overlooked; so new diagnostic markers of FM are needed. Despite being a common disorder that affects at least 5 million individuals in the United States (Lawrence et al., 2008), its pathogenic mechanism remains elusive. In FM, some abnormalities can be observed in the periphery. Because the main symptoms in FM (pain, stiffness and fatigue) are located in the muscles, muscle biopsies, mostly from the trapezious, have been studied. Biopsies of muscle have demonstrated inflammatory markers, subsarcolemmal mitochondrial accumulation, abnormal mitochondria, higher incidence of ragged red fibers, and defects of cytochrome-c-oxidase (complex IV of oxidative phosphorylation) (Lucas et al., 2006; Kalyan-Raman et al., 1984; Yunus et al., 1986). It is interesting to mention that ragged red fibers, subsarcolemmal mitochondrial accumulation and alteration in ultrastructure, number and size of mitochondrial are typical defects and markers found in genuine mitochondrial diseases (MELAS, MERRF, Kearns–Sayre syndrome, Pearson syndrome, Leigh syndrome, etc) (Haas et al., 2008). However, skin biopsies have also been studied. Unusual patterns of unmyelinated nerve fibers, as well as associated Schwann cells, and inflammatory foci (neurogenic inflammation) have been observed (Kim et al., 2008a; Salemi et al., 2003a), and recently, we have demonstrated oxidative stress, mitochondrial dysfunction, and CoQ_{10} deficiency in skin biopsies from two cases of FM patients (Naik and Dixit, 2011). According to this, mitochondrial dysfunction, oxidative stress, and an inflammatory component could play an important role in pathophysiology of FM. The relation between mitochondria and up-regulation of inflammatory cytokine has been recently suggested (Cordero et al., 2009). Recently, we demonstrated CoQ_{10} deficiency, mitochondrial dysfunction, oxidative stress and mitophagy in blood mononuclear cells (BMCs) from FM patients (Cordero et al., 2010), and we have proposed the hypothesis that inflammation could be a mitochondrial dysfunction-dependent event implicated in the pathophysiology of FM in several patients (Cordero et al., 2013a). Furthermore, an inflammatory component in

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the induction of pain in FM has been suggested. However, all our experiments are assayed in BMCs.

Because skin has been involved in nociception alterations and allodynia (Staud, 2010b), in the present work we have studied the presence and relation between oxidative stress, mitochondrial dysfunction and inflammatory cytokine TNF-α in skin biopsies from FM patients.

2. Patients and methods

2.1. Ethical statements

Written informed consent and the approval of the ethical committee of the University of Seville were obtained, according to the principles of the Declaration of Helsinki.

2.2. Patients

The study consisted of 25 women diagnosed with FM and 20 healthy women. Inclusion criteria were FM that had been diagnosed in the previous 2 to 3 years, based on the current ACR diagnostic criteria (Lawrence et al., 2008). Exclusion criteria were acute infectious diseases in the previous 3 weeks; past or present neurological disorder (i.e. Alzheimer, Parkinson or Amyotrophic lateral sclerosis) conditions that cause pain (i.e. arthrosis, discal hernia), history of severe psychiatric illness (i.e. current major depression, bipolar disorder, schizophrenia), metabolic diseases (i.e. type I and type II diabetes), autoimmune, allergy-related, dermal or chronic inflammatory diseases; undesired habits (e.g., smoking, alcohol, etc.); oral diseases (e.g., periodontitis); medical conditions that required glucocorticoid treatment, use of analgesics, statin or antidepressant drugs; past or current substance abuse or dependence as well as pregnancy or current breastfeeding. Twenty healthy women volunteers were included in the study and matched with the recruited female FM patients for age range, gender, ethnicity and demographic features (completion of at least 9 years of education and belonging to the middle socioeconomic class). Healthy controls had no signs or symptoms of FM and were free of any medication for at least 3 weeks before the study began. All patients and controls had taken no drugs or vitamin/nutritional supplements during the 3 weeks prior to the collection of blood samples and had a sedentary lifestyle. However, before the study, patients reported that they used paracetamol on demand. Clinical data was obtained from physical examination, and evaluated using the Fibromyalgia Impact Questionnaire (FIQ) including visual analogue scales regarding general and diffuse pain typical of FM (VAS).

2.3. Sample collection

After informed consents were signed, coagulated blood, saliva and skin biopsies from non-tender left deltoid region of patients and healthy age- and sex-matched control subjects were obtained. Coagulated bloods were collected after 12-hour fasting, between 8:00 and 10:00 AM from patients and healthy age- and sex-matched control subjects, centrifuged at 3800 *g* for 5 min, and serum was stored at −80 °C until testing. Unstimulated whole saliva was obtained by passive drooling into 1.5 ml tubes (Eppendorf, Hamburg, DE) and was stored at −80 °C until analysis. 3 ml of whole saliva from different subjects was separately centrifuged at 1500 ×*g* at 4 °C for 10 min and supernatant was isolated.

2.4. CoQ10 level determination

Samples were lysed with 1% SDS and vortexed for 1 min. A mixture of ethanol:isopropanol (95:5) was added and the samples were vortexed for 1 min. To recover CoQ, 5 ml of hexane was added and the samples were centrifuged at 1000 ×*g* for 5 min at 4 °C. The upper phases from three extractions were recovered and dried using a rotary evaporator. Lipid extracts were suspended in 1 ml of ethanol, dried in a speed-vac and stored at −20 °C. Samples were suspended in the suitable volume of ethanol prior to HPLC injection. Lipid components were separated by a Beckmann 166-126 HPLC system equipped with a 15-cm Kromasil C-18 column in a column oven set to 40 °C, with a flow rate of 1 ml/min and a mobile phase containing 65:35 methanol/npropanol and 1.42 mM lithium perchlorate. CoQ levels were analyzed with ultraviolet (System Gold 168), electrochemical (Coulochem III ESA) or radioactivity (Radioflow Detector LB 509, Berthod Technologies) based detectors as necessary. Coenzyme Q9 (CoQ9).

2.5. Mitochondrial enzyme activities

Activities of NADH:coenzyme Q1 oxidoreductase (complex I), succinate dehydrogenase (complex II), cytochrome c oxidase (complex IV), ubiquinol:cytochrome c oxidoreductase (complex III), succinate:cytochrome c reductase (complex II + complex III) and citrate synthase (CS) were determined in homogenate extracts using previously described spectrophotometric methods (Quinzii et al., 2006; Rustin et al., 1994). Results are expressed as units/CS (mean ± SD).

2.6. Western blotting for mitochondrial protein

Whole cellular lysate from homogenate was prepared by gentle shaking with a buffer containing 0.9% NaCl, 20 mM Tris–ClH, pH 7.6, 0.1% triton X-100, 1 mM phenylmethylsulfonylfluoride and 0.01% leupeptine. Electrophoresis was carried out in a 10–15% acrylamide SDS/PAGE. Proteins were transferred to Immobilon membranes (Amersham Pharmacia, Piscataway, NJ). Mouse anti-Complex I (39 kDa subunit), complex II (30-kDa subunit I), mouse anti-Complex III (Core 1 subunit), Complex IV (cytochrome c oxidase; COX II), and DNA repair enzyme 8-oxoguanine DNA glycolase-1 (OGG-1) antibodies were used to detect proteins by Western blotting. Proteins were electrophoresed, transferred to nitrocellulose membranes and, after blocking overnight at 4 °C, incubated with the respective antibody solution, diluted at 1:1000. Membranes were then probed with their respective secondary antibody (1:2500). Immunolabeled proteins were detected by using a chemiluminescence method (Immun Star HRP substrate kit, Bio-Rad Laboratories Inc., Hercules, CA).

2.7. ATP levels

ATP levels were determined by a bioluminescence assay using an ATP determination kit from Invitrogen-Molecular Probes (Eugene, OR, USA) according to the instructions of the manufacturer.

2.8. Quantification of mtDNA

Nucleic acids were extracted from BMCs by standard cellular lysis. The primers used were: for mitochondrial DNA, mtF3212 (5′-CACCCAAGAA CAGGGTTTGT-3′) and mtR3319 (5′-TGGCCATGGGTATGTTGTTAA-3′) and those for nuclear DNA for loading normalization, 18S rRNA gene 18S1546F (5′-TAGAGGGACAAGTGGCGTTC-3′) and 18S1650R (5′-CGCT GAGCCAGTCAGTGT3′). Arbitrary units were computed as the ratio between the optical density band corresponding to the mitochondrial DNA studied in the 20–30th cycle and that of nuclear DNA in the 15th amplification cycle. One unit was considered to be the ratio corresponding to the control patient.

2.9. Lipid peroxidation

Lipid peroxidation (LP) in tissue homogenate was determined by analyzing the accumulation of lipoperoxides using a commercial kit from Cayman Chemical (Ann Arbor, Michigan).