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Non-steroidal anti-inflammatory drug modulates oxidative stress and calcium ion levels in the neutrophils of patients with primary dysmenorrhea

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ABSTRACT

Primary dysmenorrhea is a common inflammatory disease with an uncertain pathogenesis, although one consistent finding is increased neutrophil activity. We aimed to investigate the effects of a non-steroidal anti-inflammatory drug (NSAID) on oxidative stress and Ca^{2+} levels in neutrophils from patients with primary dysmenorrhea. Blood samples were obtained for neutrophil isolation from six female patients with primary dysmenorrhea (patients) and six healthy female subjects. The NSAID (diclofenac) was taken daily by the patient group for 6 weeks before a second blood sample was taken. Neutrophils isolated after diclofenac treatment were investigated in three settings: (1) after incubation with verapamil and diltiazem (V + D), (2) after incubation with 2-aminoethoxydiphenyl borate (2-APB), and (3) with neither exposure. Neutrophil lipid peroxidation and stimulated intracellular Ca^{2+} levels were higher in the patients than in the controls, although their levels were reduced after six weeks of treatment with diclofenac. Ca^{2+} levels from neutrophils obtained after diclofenac treatment were further decreased after incubation with V + D or 2-APB, compared with those exposed to neither agent. Neutrophil glutathione peroxidase and total antioxidant status were lower in the patients than in the controls and higher post-treatment with diclofenac. Reduced glutathione levels were similar in the control, patient, and treatment groups. In conclusion, we observed the importance of Ca^{2+} influx into the neutrophils and oxidative stress in the pathogenesis of the patients with primary dysmenorrhea. The NSAID diclofenac appeared to provide a protective effect against oxidative stress and Ca^{2+} entry through modulation of neutrophil VGCC and TRP calcium channels.

1. Introduction

Dysmenorrhea is characterized by abdominal or lower back pain that lasts for at least two days during the menstrual cycle and is very common in young women (20–24 years of age), with a prevalence of 45–95% (Moore, 2007). Dysmenorrhea is categorized as primary dysmenorrhea, which is menstrual pain in the absence of any apparent organic disorder, and secondary dysmenorrhea, which occurs in association with an identifiable illness (Harel, 2012). Although primary dysmenorrhea is very common, its causes are unclear.

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drug; ROS, reactive oxygen species; SOD, superoxide dismutase; V + D, verapamil + diltiazem; VGCC, voltage-gated calcium channels.

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Increased levels of proinflammatory cytokines, interleukins (Yeh et al., 2004), and tumor necrosis factor-α (TNF-α), neutrophil hyperfunction (Marchini et al., 1995), and excessive reactive oxygen species (ROS) production (Marchini et al., 1995; Dikensoy et al., 2008) have been reported in patients with primary dysmenorrhea. Neutrophils are cells that play an important role in immune responses (Ayub and Hallett, 2004). In primary dysmenorrhea, there is an increase in neutrophil function-dependent inflammatory metabolites, such as interleukins and prostaglandins, in the peripheral blood (Harel, 2012). Hence, understanding the physiological mechanisms in neutrophils from patients with primary dysmenorrhea may help to clarify the disease etiology.

Ischemia is induced during uterine contraction because of decreased blood flow to the myometrium (Buhimschi et al., 1995). This can trigger the accumulation of free radicals, such as ROS (Sirmali et al., 2007). Free radicals are the products of biological reduction reactions (Nazıroğlu, 2007), and overproduction of ROS has been implicated in the pathogenesis of dysmenorrhea (Dikensoy et al., 2008). ROS can cause disease and cell damage by damaging other molecules, including proteins, lipids, and DNA. Antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase protect cells from ROS damage (Nazıroğlu, 2007, 2012; Kovacic and Somanathan, 2008). ROS-induced changes to proteins and DNA can lead to altered cellular function or activation of proteolytic cascades that ultimately result in endometrial damage and inflammation (Güney et al., 2008; Güney, 2012).

Ca^{2+} is an important ion that controls several intracellular processes, such as exocytosis, secretion, and apoptosis (Nazıroğlu, 2007; Ayub and Hallett, 2004). In neutrophils, intracellular free Ca^{2+} ions control chemotaxis and adhesion (Korkmaz et al., 2011). Ca^{2+} entry also plays an important role in the regulation of superoxide radical production by neutrophils (Şahin et al., 2011). Therefore, a change in intracellular Ca^{2+} levels in neutrophils directly affects the neutrophil response (Yamazaki et al., 2006).

Since there is no specific treatment for primary dysmenorrhea, symptomatic and empirical treatment methods are used. Non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac are frequently used for the treatment of inflammation and pain in a wide variety of disorders, including primary dysmenorrhea (Yamazaki et al., 2006; Harel, 2012). Diclofenac has been shown to be effective in the treatment of primary dysmenorrhea, and its use is widespread. Although the mechanisms of action of NSAIDs in patients with primary dysmenorrhea are not yet fully understood, they have been shown to have anti-inflammatory, antioxidant, and inhibitory effects on cardiac and neuronal cells (Yamazaki et al., 2006; Yarishkin et al., 2009), and diclofenac has been reported to inhibit voltage-gated calcium channels (VGCC) in neonatal rat ventricular cardiomyocytes (Yarishkin et al., 2009).

In the present study, we investigated the mechanisms involved in neutrophil activation and inflammation in patients with primary dysmenorrhea. Our first aim was to research the importance of Ca^{2+} in the neutrophil cytosol in patients with primary dysmenorrhea and the effect of NSAID (diclofenac) treatment on neutrophil cytosolic Ca^{2+} release from intracellular stores evoked by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). Our second aim was to investigate the effects of NSAID treatment on neutrophil lipid peroxidation and antioxidant status.

2. Subjects and methods

2.1. Patients and controls

The study was conducted at the Biophysics Research Laboratory, Suleyman Demirel University, Turkey. The patients enrolled in the study were selected from the Gynecology Department of Suleyman Demirel University, and they fulfilled the diagnostic criteria for primary dysmenorrhea (Proctor and Farquhar, 2006). Their main complaint was dysmenorrhea, and each patient underwent a detailed gynecological examination, pelvic ultrasound, and laboratory tests. The medical history of each patient was recorded, and their pain score was monitored for three ovulation cycles. The exclusion criteria for the patients were the presence of inflammatory disease, fibromyalgia, premature coronary artery disease, diabetes mellitus, or hypertension. Six healthy controls were also included, and informed consent was obtained from all the study participants. The patients and controls were women who were not undergoing hormone replacement therapy and had not taken vitamin or mineral supplements for 6 months. They were non-smokers and did not drink alcohol. Demographic characteristics, clinical information, physical examination findings, and laboratory tests were recorded for all the subjects included in the study.

2.2. Study groups

Baseline blood samples were obtained from the patient and control groups ($n = 6$ in each). The patient group was then administered a 50-mg diclofenac potassium tablet daily for 6 weeks (Dolerex; Abdi Ibrahim Medicine Inc., Vefa, Istanbul, Turkey), and blood samples were obtained.

2.3. Isolation of neutrophils

After fasting overnight, 35-mL blood samples from the antecubital vein were drawn into tubes with an anticoagulant. Peripheral whole blood was obtained, and neutrophils were isolated by centrifugation using Ficoll, as described previously (Şahin et al., 2011).

2.4. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Neutrophils were loaded with fura-2 acetoxymethyl ester (fura-2/AM) by using a previously described method (Uğuz et al., 2009). The neutrophils (5×10^6 cells/mL) were incubated with 4 μM fura-2/AM in a loading extracellular buffer for 45 min at 37 °C in the dark. They were then washed twice, incubated for an additional 30 min at 37 °C to complete probe de-esterification, and resuspended in loading buffer at a density of 3×10^6 cells/mL.