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Hypericum perforatum Modulates Apoptosis and Calcium Mobilization Through Voltage-Gated and TRPM2 Calcium Channels in Neutrophil of Patients with Behcet's Disease

Mustafa Nazıroğlu · Mehmet Şahin · Bilal Çiğ · Mehmet Aykur · İjlal Erturan · Yunus Ugan

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Abstract Behcet's disease (BD) is a chronic, inflammatory, and multisystemic condition although its pathogenesis is uncertain. Main component of St. John's wort (*Hypericum perforatum*, HP) is hyperforin and induces antiinflammatory and antioxidant properties. We aimed to investigate effects of HP on oxidative stress, apoptosis, and cytosolic-free Ca^{2+} $[Ca^{2+}]_i$ concentration in neutrophil of BD patients. Nine new-diagnosed active patients with BD and nine control subjects were included in the study. Disease activity was considered by clinical findings. Neutrophil samples were obtained from the patients and controls. The neutrophils from patients were divided into three subgroups and were incubated with HP, voltage-gated calcium channel (VGCC) blockers, (verapamil+dilitiazem) and non-specific TRPM2 channel blocker (2-aminoethyl diphenylborinate, 2-APB), respectively. The neutrophils were stimulated by fMLP as a Ca^{2+}-concentration agonist and oxidative stress former. Caspase-3, caspase-9, apoptosis, lipid peroxidation, and $[Ca^{2+}]_i$ values were high in the patient groups, although cell viability, glutathione (GSH), and glutathione peroxidase (GSH-Px) values were low in patient group. However, the $[Ca^{2+}]_i$, caspase-3, and caspase-9 values decreased markedly in patient+HP group although GSH and GSH-Px values increased in the group. The $[Ca^{2+}]_i$ concentration was also decreased in the patient group by V+D, 2-APB, and HP incubations. In conclusion, we observed the importance of neutrophil Ca^{2+} entry, apoptosis, and oxidative stress through gating VGCC and TRPM2 channels in the neutrophils in the pathogenesis and activation of the patients with BD. HP induced protective effects on oxidative stress by modulating Ca^{2+} influx in BD patients.

Keywords Antioxidant · Behcet's disease · *Hypericum perforatum* · Calcium ion · Inflammation · Oxidative stress

Abbreviations

2-APB	2-Aminoethyl diphenylborinate
BD	Behcet's disease
CRP	C-reactive protein
ESR	Erythrocyte sedimentation rate
fMLP	*N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
MDA	Malondialdehyde
MPO	Myeloperoxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TRPM2	Transient receptor potential melastatin 2
V+D	Verapamil+diltiazem
VGCC	Voltage-gated calcium channels

M. Nazıroğlu (✉)
Neuroscience Research Center, Süleyman Demirel University, Isparta, Turkey
e-mail: mustafanaziroglu@sdu.edu.tr

M. Nazıroğlu · B. Çiğ · M. Aykur
Department of Biophysics, Faculty of Medicine, Süleyman Demirel University, Isparta, Turkey

Present Address:
M. Nazıroğlu
Department of Physiology and Biophysics, Weill Cornell Medical College in Qatar, Qatar Foundation, Doha, Qatar

M. Şahin · Y. Ugan
Division of Rheumatology, Department of Internal Medicine, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey

İ. Erturan
Department of Dermatology, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey

Introduction

Behcet's Disease (BD) was first defined in 1937 by the dermatologist, Dr Hulusi Behcet, as a three-symptom complex of recurring oral and genital ulcers and uveitis (Behcet 1937). After the first report, BD is better understood as a multisystem disease involving skin, joints, vascular, and neuronal systems (Evereklioglu 2005). It can occur between the second and fourth decades of life (range 0–72). Most of body tissues including lung, kidney, inner ear, and cornea with psychosomatic manifestations are affected by the disease. BD is seen over a wide geographical area, but a higher prevalence has been found among Asian and European populations along the Silk Road, stretching to the countries of Mediterranean region including Turkey (Öztas et al. 2007).

Neutrophils are cells which play an important role in immune response, and neutrophil hyperfunction induces excessive production of reactive oxygen species (ROS) in blood of patients with inflammatory disease (Direskeneli 2001; Kökçam and Nazıroğlu 2002). The etiology and pathogenesis of BD haven't been certainly clarified, but considerable data indicate that inflammation and oxidative stress as the most significant cause of inflammation are important (Direskeneli 2001; Korkmaz et al. 2011; Tamer et al. 2012). Free radicals are the products of biological reduction reactions (Nazıroğlu 2007). Over production of ROS such as hydrogen peroxide, superoxide radical, and singlet oxygen induces oxidative stress in the induction of the inflammatory responses such as phagocyte activity and leukocyte proliferation (Bréchard and Tschirhart 2008). Uncontrolled free radicals in cells cause diseases and cell damage by affecting many molecules such as proteins, lipids, nucleic acids, and DNA. Enzymatic antioxidant systems include catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) (Nazıroğlu 2009). SOD enzyme modulates dismutation of superoxide radical to hydrogen peroxide and molecular oxygen. Then, the hydrogen peroxide is independently detoxified to water by catalase and GSH-Px enzymes. Results of some studies indicated that lipid peroxidation levels increased in blood and neutrophils of patients with BD although the antioxidant enzyme activities decreased (Kökçam and Nazıroglu 2002; Erkılıç et al. 2003; Korkmaz et al. 2011). However, there is no report on the values in the patients with BD.

All cellular functions are triggered by cellular ion changes. The basic ion Ca^{2+} is the most important trigger of these functions. Ca^{2+} is an ion which controls several physiological events such as exocytosis, secretion, and apoptosis (Ayub and Hallett 2004; Nazıroğlu 2007). Cytosolic-free Ca^{2+} $[Ca^{2+}]_i$ concentration is one of the most important ions affecting neutrophil functions. Ca^{2+} ion affects chemotaxis, adhesion, and the expression and production of free oxygen radicals and arachidonic acid products in the neutrophils. Therefore, a change in the intracellular Ca^{2+} levels in the neutrophils directly affects the neutrophil response (Bréchard and Tschirhart 2008). Transient receptor potential (TRP) channels were first described in *Drosophila*, where photoreceptor carrying *trp* gene mutations exhibits a transient voltage response to continuous light (Nazıroğlu 2011; Nazıroğlu et al. 2012). One subgroup of the TRP family is TRPM2 channels, and the channels have been activated by oxidative stress products (Nazıroğlu et al. 2012). Although TRPM2 is predominantly expressed in brain, it is also detected in many other phagocyte cells, including neutrophils (Heiner et al. 2003).

Among different plant extracts, St. John's wort (*Hypericum perforatum*, HP) has been used in the treatment of different diseases such as depression, inflammatory, and skin diseases (Meinke et al. 2012). Main component of HP is hyperforin, and it induces anti-inflammatory and antioxidant properties, by inhibiting the proliferation and induction of apoptosis phagocyte cells (Meinke et al. 2012). Research on antioxidant effects of extracts within different *Hypericum* species indicated that HP has the highest free oxygen radical scavenging effects (Heilmann et al. 2003). Result of a recent study indicated that hyperforin administration after middle cerebral artery occlusion ischemia also decreased apoptotic cell death in brain of rats through modulation of TRPC6 cation channels (Lin et al. 2013) and it may have also modulator role on TRPM2 channels in neutrophil of patients with BD. Although the mechanism of the effect of HP on oxidative stress and inflammation is not yet fully understood, it has been shown to have an anti-inflammatory, antioxidant, and inhibitor effect on neutrophils of different inflammatory diseases (Feisst and Werz 2004).

In this study, the first aim was to research the importance of the Ca^{2+} flow to the neutrophil cytosol in BD activation and the effect of HP treatment on the neutrophil cytosol Ca^{2+} release from intracellular stores evoked by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) as a calcium-mobilizing agonist. Second aim of the study was the dual effect of HP in human neutrophil cells by checking its role on lipid peroxidation and antioxidant concentrations. Thus, the mechanisms held responsible for neutrophil activation in BD pathogenesis will be able to be understood and new treatment methods will be able to be developed.

Subjects and Methods

Chemicals

All chemicals (cumene hydroperoxide, KOH, NaOH, thiobarbutiric acid, 1,1,3,3-tetraethoxy propane, 5.5-dithiobis-2-nitrobenzoik, tris-hydroxymethil-aminomethan,

5.5-dithiobis-2-nitrobenzoik asit, cumene hydroperoxide, glutathione, butylhydroxytoluol, fMLP, 2-APB, V+D, digitonin, and ethylene glycol-bis(2-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid were obtained from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA), and all organic solvents (*n*-hexane and ethyl alcohol) were purchased from Merck Chemicals (Darmstadt, Germany). Fura-2-acetoxy-methyl ester was purchased from Promega Inc (Eugene, Oregon, USA). All reagents were analytical grade. All reagents except the phosphate buffers were prepared daily and stored at +4 °C. The reagents were equilibrated at room temperature for half an hour before an analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4 °C for 1 month. HP was collected around of Isparta,Turkey, and its ethanolic extract as gift was prepared by Forestry Faculty of Suleyman Demirel University (SDU). The HP extract contained 3.6 % hyperforin. The dry HP extracts were dissolved in DMSO and diluted in DMEM medium before use (Nazıroğlu et al. 2014a).

Patient and Control Subjects

The study was conducted at the Neuroscience Research Center (NÖROBAM) Laboratory, SDU, Turkey. The patients enrolled in the study were selected from patients attending the Dermatology and Rheumatology department of SDU between January 2012 and December 2012. Local ethical committee approval was obtained, and written consent, confirming their acceptance for giving blood through vena brachialis, was taken from both the patients and controls. New-diagnosed active BD patients were included in the current study, whereas inactive BD patients were not included in the current study. A diagnosis of BD was given by calculating the BD current activity index according to the criteria defined by the BD International Working Group. Patients were accepted as active with a total index score of 4 (oral aphthae, genital ulcers, anterior/posterior uveitis, and skin lesions) or more. According to the current activity index, the study comprised seven active patients with BD and seven healthy individuals. The active BD patient group comprised six males and one female of mean age 36.3 ± 5.46 years. The control group comprised six males and one female of mean age 38.1 ± 7.19 years. There was no statistically significant difference between the ages of the groups in the study.

Neutrophils were isolated by taking a venous blood sample from each individual. For all those included in the study, demographic characteristics, clinical information, physical examination findings, and laboratory tests were recorded. Informed consent was obtained from all study participants.

Systemic exclusion criteria in control and patient groups were hepatic and kidney disease, diabetes, hypertension, pregnancy, malignancy or another chronic inflammatory disease, and the history of recent medical treatments. Patients' details were obtained from case notes, and ocular examinations were performed by an ophthalmologist using a standard procedure.

Study Groups

Seven control and seven patients with BD were used in the study as it was mentioned above. Neutrophil and serum samples were taken from the control and patients. The neutrophil cells were divided into four groups as fellows:

Control group ($n = 7$): Age-matched healthy people were used in the group.

Patient group ($n = 7$): The neutrophils were obtained from patients with BD, and they were not incubated with HP.

Patient+HP group ($n = 7$): The neutrophils were obtained from the same patients with BD, and they were incubated with HP (20 μg/ml) for 2 h. The dose and time of HP were determined in the neutrophil by cell viability (MTT) test.

The patients and patients + HP groups were divided into two subgroups as follows:

A-2-APB group ($n = 7$): The neutrophils were incubated with 2-APB (0.1 mM) for 5 min before fMLP stimulation.

B–V+D group ($n = 7$): The neutrophils were incubated with V+D incubation (0.01 mM) for 30 min before fMLP stimulation (Şahin et al. 2011; Korkmaz et al. 2011).

After an overnight fast, blood samples of the control and patients were drawn from the antecubital vein into tubes with and without anticoagulant. Serum and neutrophil samples were obtained from the blood samples. Serum and half of the neutrophil samples were stored at −33 °C, and they were used for lipid peroxidation and antioxidant analysis within 1 month. Remaining neutrophil samples were used for daily $[Ca^{2+}]_i$ concentration analysis.

Stock solutions of 2-APB and V+D were dissolved in dimethyl sulfoxide and extracellular buffer, respectively, and they were stored at −33 °C before the experiment. The 2-APB (0.1 mM) in extracellular buffers was diluted to reach the final concentrations. After addition of 2-APB and ACA to standard extracellular bath solution, the pH values of these solutions were adjusted with KOH to 7.4.

Method

After an overnight fast, blood samples of the control and patients were drawn from the antecubital vein into tubes with and without anticoagulant. Blood with anticoagulant was used for analyses of erythrocyte sedimentation rate (ESR) and leukocytes count. Serum and neutrophil samples were obtained from the blood samples. Serum and half of

the neutrophil samples were stored at −33 °C, and they were used for lipid peroxidation and antioxidant analysis within 1 month. The remaining neutrophil samples were used for daily $[Ca^{2+}]_i$ analysis.

Isolation of Human Neutrophils

Neutrophils were isolated from peripheral whole blood of healthy volunteers and patients with BD, as described previously (Şahin et al. 2011), by centrifugation through Ficoll. Half of the cells were stored for antioxidant analyses. The remaining cells were used for measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$). Sterile solutions used for neutrophil isolation were phosphate buffer saline from Gibco Invitrogen (Istanbul, Turkey) and 6 % hydroxyl ethyl starch solution in isotonic NaCl from plasmasteril Fresenius AG (Bad Homburg, Germany); and Ficoll-Paque PLUS from GE Healthcare Bio-Sciences (Uppsala, Sweden). The loading buffer contained HEPES (20 mM), NaCl (138 mM), KCl (6 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.6 mM), and glucose (5.5 mM), pH 7.4, and was supplemented with 2 % (*v*/*v*) of autologous serum. The measuring buffers did not contain serum but were otherwise identical in composition to the loading buffer when a normal extracellular Ca^{2+} concentration was explored.

Measurement of Cytosolic-Free Calcium ($[Ca^{2+}]_i$) Concentration

Neutrophil cells were loaded with 4 μM fura-2/AM in loading buffer with 5×10^6 cells per milliliter for 45 min at 37 °C in the dark, washed twice, incubated for additional 30 min at 37 °C to complete probe de-esterification, and re-suspended in loading buffer at a density of 3×10^6 cells per milliliter according to a procedure published elsewhere (Uğuz et al. 2009, 2012). The three groups were exposed to fMLP for stimulating the $[Ca^{2+}]_i$ concentration. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspension at 37 °C using a spectrofluorometer (Carry Eclipsys, Varian Inc, Sydney, Australia). The fluorescence at 505 nm was measured after excitation at 340 and 380 nm. The $[Ca^{2+}]_i$ concentrations were calculated according to the method of Grynkiewicz et al. (1985). Maximal fluorescence (F_{max}) was obtained by lysing the cells with 1 % Triton-X 100 and F_{min} by chelating with 10 mM EDTA.

Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 150 s after addition of fMLP (Espino et al. 2009; Uğuz et al. 2009). Ca^{2+} release is expressed nanomolar taking a sample every second as previously described (Heemskerk et al. 1997).

Cell Viability (MTT) Assay

Cell viability in the neutrophils was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product (Uğuz and Nazıroğlu 2012). The enzymatic reduction of the tetrazolium salt happens only in living, metabolically active cells but not in dead cells. The medium was removed, and MTT was added into each well and then incubated for 90 min at 37 °C in a shaking water bath. The supernatant was discarded, and dimethyl sulfoxide was added to dissolve the formazan crystals. Treatments were carried out by duplicate. Optical density (OD) was measured in an automatic plate reader at 490 and 650 nm and presented as fold increase over the pretreatment level (experimental/control).

Apoptosis Assay

The APOPercentage assay (Biocolor Ltd., Belfast, Northern Ireland) was performed according to the instructions provided by Biocolor Ltd. and described elsewhere (Uğuz and Nazıroğlu 2012).

Assay for Caspase-3 and -9 Activities

The determination of caspase-3 and caspase-9 activities was based on a method previously reported (Espino et al. 2010; Espino et al. 2011; Nazıroğlu et al. 2013) with minor modifications. Stimulated or resting cells were washed once with PBS. After centrifugation, cells were re-suspended in PBS at a concentration of 10^3 cells/ml. Fifteen microliters of the cell suspension were added to a microplate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer that was composed of 100 mm HEPES, pH 7.25, 10 % sucrose, 0.1 % CHAPS, 5 mm DTT, 0.001 % NP40, and 40 ml of caspase-3 substrate (AC-DEVD-AMC) or 0.1 m MES hydrate, pH 6.5, 10 % PEG, 0.1 % CHAPS, 5 mm DTT, 0.001 % NP40, and 0.1 mM of caspase-9 substrate (AC-LEHD-AMC). Substrate cleavage was measured with the microplate reader (Infinite pro200) with excitation wavelength of 360 nm and emission at 460 nm. The data were calculated as fluorescence units/mg protein and presented as fold increase over the pretreatment level (experimental/control).

Lipid Peroxidation Determinations

Lipid peroxidation is frequently investigated in biomedical research, and the assays for thiobarbituric acid reaction are much more widely used than any other index of lipid

peroxidation (Erkiliç et al. 2003). The thiobarbituric acid reacts with lipid peroxidation aldehydes, such as malondialdehyde (MDA). Therefore, assessment of thiobarbituric acid reactions is a useful indicator of oxidative reactions and lipid peroxidation assay in body fluids (Placer et al. 1966). Lipid peroxidation levels in the neutrophil samples were measured with the thiobarbituric acid reaction by the method of Placer et al. (1966). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The values of lipid peroxidation in the neutrophil samples were expressed as μmol/g protein.

Reduced Glutathione (GSH), Glutathione Peroxidase (GSH-Px), and Protein Assay

The GSH content of the neutrophil samples was measured at 412 nm using Ellman's reagent (Sedlak and Lindsay 1968) as described elsewhere (Nazıroğlu et al. 2004; Nazıroğlu et al. 2014a). GSH-Px activities of neutrophil samples were measured spectrophotometrically at 37 °C and 412 nm according to the Lawrence and Burk method (1976). The protein content in neutrophil samples was measured by method of Lowry et al. (1951) with bovine serum albumin as the standard.

Biochemical Analysis

Leukocytes counts were measured in a cell counter (Beckman Coulter LH 70 hematology analyzer, Beckman Coulter Inc. Brea, CA 92,822-8000 USA). ESR was measured by routine Westerngreen methods. C-reactive protein (CRP) is one of the acute phase proteins that increases during systemic inflammation, and serum CRP values in patients with BD were measured in a Nephelometry (Delta Seac Radim, Pomezia RM—Italy).

Results

Blood Biochemical Values

Levels of biochemical values are shown in Table 1. CRP ($p < 0.001$), ESR ($p < 0.001$), and leukocyte ($p < 0.05$) values were significantly higher in active group than in control, whereas their values were significantly ($p < 0.05$ and $p < 0.001$) lower in inactive groups than in active group.

Lipid Peroxidation and Antioxidant Results

Values of lipid peroxidation and GSH and GSH-Px values in neutrophil of the patients with BD are shown in Table 2.

Table 1 Levels of biochemical values in control and patients with BD (mean ± SD, $n = 7$)

Parameters	Control	Patients
CRP (mg/ml)	3.80 ± 1.90	4.45 ± 1.59[a]
ESR (h)	2.72 ± 0.86	14.83 ± 4.83[b]
Leukocytes ($\times 10^3$/mm^3)	7.01 ± 1.66	9.15 ± 1.87[a]

CRP serum C-reactive protein, *ESR* erythrocyte sedimentation rate

[a] $p < 0.05$ and [b] $p < 0.001$ versus control

Table 2 Effects of *Hypericum perforatum* (HP) on lipid peroxidation (LP), reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) values in neutrophil of control and patients with BD (mean ± SD, $n = 7$)

Parameters	Control	Patients	Patients+HP
LP (μmol/g prot)	2.41 ± 0.27	2.85 ± 0.10[a]	2.43 ± 0.26[b]
GSH (μmol/g prot)	5.51 ± 0.71	3.89 ± 0.25[a]	3.91 ± 0.14[a]
GSH-Px (IU/g prot)	10.52 ± 1.53	7.65 ± 1.79[a]	7.58 ± 1.13[a]

[a] $p < 0.001$ versus control

[b] $p < 0.05$ versus patient group

In both patient and control groups, the lipid peroxidation, GSH, and GSH-Px values, as indicators of oxidative stress, were examined from the isolated neutrophil samples. In the active patient group the serum lipid peroxidation level was higher ($p < 0.001$) in the patient group than in control, although the GSH levels and GSH-Px activities were lower in active group than in control. However, HP induced modulator role on the lipid peroxidation, GSH, and GSH-Px values. The neutrophil lipid peroxidation levels were significantly ($p < 0.05$) lower in patient+HP group than in patient group. The GSH levels and GSH-Px activity of the patient+HP groups were determined as significantly ($p < 0.05$) higher compared to the patient group.

Effects of HP on İntracellular Ca^{2+} $[Ca^{2+}]_i$ Mobilization in Neutrophil of Patients with BD

Ca^{2+} is considered important for antagonist for agonist-induced leukocyte activation (Bréchard and Tschirhart 2008), and we investigated if HP in the neutrophil of patients with BD could affect the mobilization of Ca2 + , thereby inhibiting the against-induced ROS production. Effects of HP on cytosolic Ca^{2+} $[Ca^{2+}]_i$ mobilization in neutrophil are shown in Figs. 1, 2. The mobilization values of Ca^{2+} from the neutrophils into the cytosol were determined as significantly high in the patient group compared to the control group ($p < 0.001$). However, the pre-incubation of neutrophils with HP for 2 h significantly ($p < 0.05$) prevented the Ca^{2+} mobilization in the neutrophil of patients with BD challenged by fMLP.

Stimulation of the leukocytes with fMLP caused also Ca^{2+} release from internal stores such as endoplasmic reticulum, followed by an entry of Ca^{2+} from the external medium. Accordingly, chelation of external Ca^{2+} by 1 mM EDTA impaired the fMLP-induced Ca^{2+} mobilization in the neutrophil (Fig. 1). When HP was added to the EDTA-treated neutrophils for 2 h min, elevation against-induced intracellular Ca^{2+} mobilization decreased (Figs. 1, 2), indicating that HP affects the Ca^{2+} release from endoplasmic reticulum.

Effects of HP on VGCC and TRPM2 Channels in Neutrophil of Patients with BD

In the neutrophil, we tested Ca^{2+} entry way through voltage-gated calcium channel (VGCC) blocker, V+D. In some experiments, the neutrophils from the BD patient groups were also incubated with 2-APB for testing the inhibitor role of the TRPM2 channels on the Ca^{2+} concentrations. The $[Ca^{2+}]_i$ concentrations were determined as significantly low in patient+V+D ($p < 0.05$) and patient+2-APB ($p < 0.05$) compared to group patient group. The $[Ca^{2+}]_i$ concentrations were also lower in patient+HP+V+D ($p < 0.05$) and patient+HP+2-APB ($p < 0.05$) compared to patient+V+D ($p < 0.05$) and patient+2-APB ($p < 0.05$) patient+HP. The $[Ca^{2+}]_i$ concentrations were determined as significantly lower in patient+HP+V+D compared to the patient+HP+2-APB group ($p < 0.05$).

Effects of HP on MTT (Cell Viability) Values

The cell viability (MTT) results in control, patient, and patient+HP groups are shown in Fig. 3. The apoptosis values in patient and patient+HP groups were significantly ($p < 0.001$) lower than in the control group. There is no significant effect of HP on the MTT values in the neutrophils of patients with BD.

Effects of HP on Apoptosis Values

We investigated the effects of BD on the rate of programed cell death as apoptosis and caspase values in the neutrophils of patient with BD. The results of apoptosis in control, patient, and patient+HP groups are shown in Fig. 4.

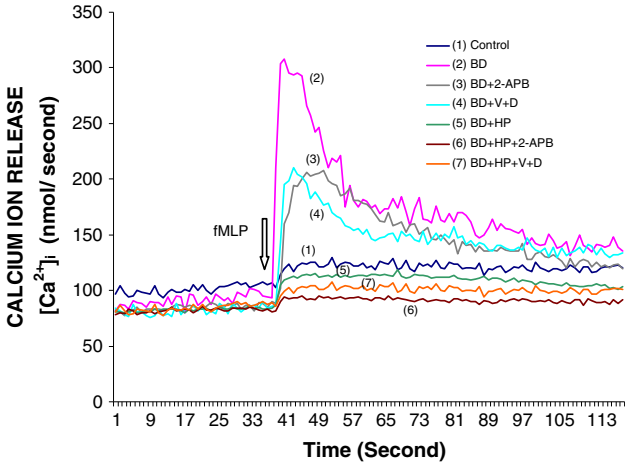

Fig. 1 Effects of *Hypericum perforatum* (HP) on cytosolic Ca^{2+} release in neutrophils of control and patients with BD (mean ± SD and $n = 7$)

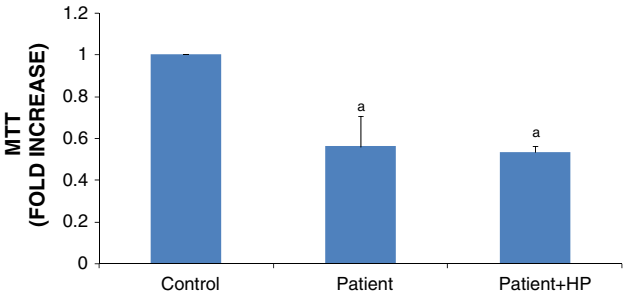

Fig. 3 Effects of *Hypericum perforatum* (HP) on cell viability (MTT) values in neutrophils of patients with BD (mean ± SD and $n = 7$). [a] $p < 0.001$ versus control

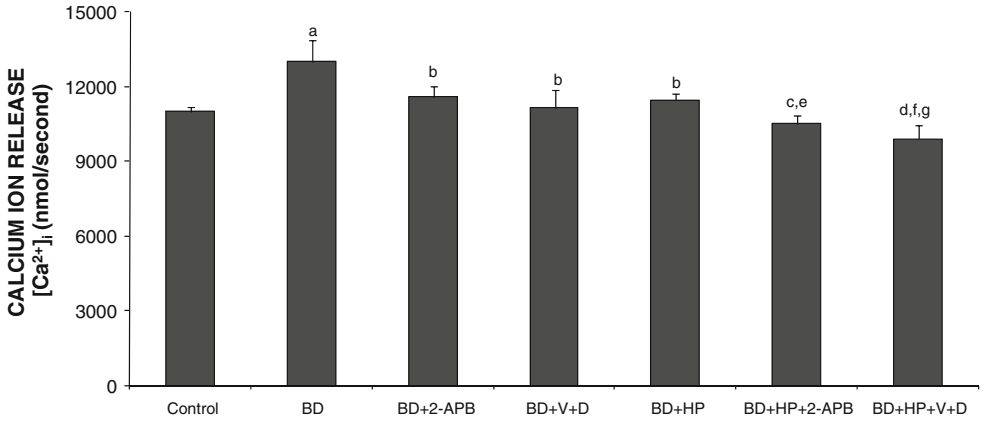

Fig. 2 Effects of *Hypericum perforatum* (HP) on cytosolic Ca^{2+} release in neutrophils of control and patients with BD (mean ± SD and $n = 7$). [a] $p < 0.05$ versus control. [b] $p < 0.05$, [c] $p < 0.01$, and [d] $p < 0.001$ versus BD group. [e] $p < 0.05$ and [f] $p < 0.01$ and [c] $p < 0.001$ versus BD+HP group. [g] $p < 0.05$ versus BD+HP+2-APB group

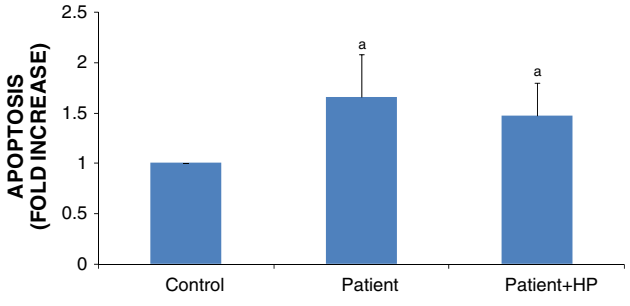

Fig. 4 Effects of *Hypericum perforatum* (HP) on apoptosis values in neutrophils of patients with BD (mean ± SD and $n = 7$). Apoptosis was estimated as described under "Subjects and Methods" sections. Values are presented as mean ± SD of 7 separate experiments and expressed as fold increase over the pretreatment level (experimental/control). $^{a}p < 0.001$ versus control

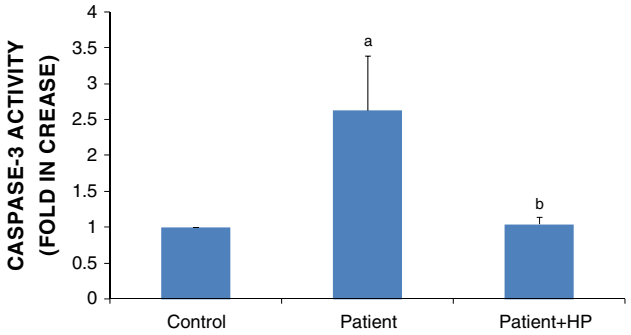

Fig. 5 Effects of *Hypericum perforatum* (HP) on caspase-3 activities in neutrophils of patients with BD (mean ± SD and $n = 7$). $^{a}p < 0.001$ versus control. $^{b}p < 0.001$ versus patient group. The caspase activity was estimated as described under "Subjects and Methods" sections. Values are presented as mean ± SD of 7 separate experiments and expressed as fold increase over the pretreatment level (experimental/control)

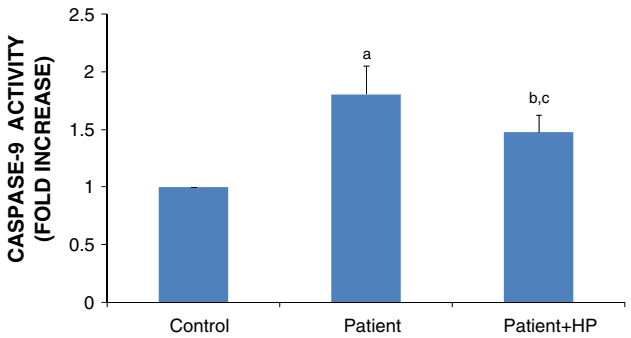

Fig. 6 Effects of *Hypericum perforatum* (HP) on caspase-9 activities in neutrophils of patients with BD (mean ± SD and $n = 7$). $^{a}p < 0.001$ and $^{b}p < 0.05$ versus control group. $^{c}p < 0.001$ versus patient group. The caspase activity was estimated as described under "Subjects and Methods" sections. Values are presented as mean ± SD of 7 separate experiments and expressed as fold increase over the pretreatment level (experimental/control)

The apoptosis values in patient and patient+HP groups were significantly ($p < 0.001$) higher than in the control group. There is no significant effect of HP on the apoptosis values in the neutrophils of patients with BD.

Effects of HP on Caspase Activities

The caspase-3 and -9 activities values in control, patient, and patient+HP groups are shown in Figs. 5, 6, respectively. The caspase-3 and -9 activities were increased ($p < 0.001$) in neutrophil of patients with BD. In addition, caspase-3 ($p < 0.001$) and caspase-9 ($p < 0.05$) activities were decreased in neutrophil of patients with BD by HP incubations.

Discussion

HP has also antioxidant, antidepressant, antibacterial, and antioxidant effects (Meinke et al. 2012). Although it is known that there is increased neutrophil activation and oxidative stress in BD, by what mechanism this neutrophil activation occurs is still unclear (Evereklioglu 2005). BD is a chronic multisystem disorder characterized by oro-genital ulceration and associated with inflammatory lesions. The etiology of BD has not been defined, but neutrophil activation and over production of oxidative stress have been proposed as causative reasons (Direskeneli 2001). This study aimed to explain the mechanisms of neutrophil activation which are involved in the etiopathogenesis of BD as well as protective role of HP on the neutrophil Ca^{2+} entry and apoptosis. We observed here that HP decreased the formation of ROS, apoptosis, and entry of Ca^{2+} in the neutrophil of BD patients through VGCC and TRPM2 channels.

In inflammatory diseases, neutrophils are stimulated by fMLP to form the first barrier of defense against the attacking bacteria. By increasing the amount of intracellular Ca^{2+}, this stimulation increases free radical production. Increased ROS in the neutrophils causes DNA damage (Karaman et al. 2009). Over production of ROS by neutrophil has been suggested to mediate tissue injury in patients with BD (Erkiliç et al. 2003). Lipid peroxidation with increased neutrophil activity has been reported both in vivo and in vitro, suggesting increased neutrophil-induced ROS production in patients with BD (Erkiliç et al. 2003; Korkmaz et al. 2011). Thus, by measuring the changes in the neutrophil $[Ca^{2+}]_i$ concentration of the patient groups, the role of changes in the $[Ca^{2+}]_i$ concentration in the neutrophil in the etiopathogenesis of the disease was more clearly evaluated. The measurements of basal $[Ca^{2+}]_i$ concentration obtained from the patient groups were determined as significantly higher compared to the healthy subjects. Therefore, it is thought that an increase in neutrophil $[Ca^{2+}]_i$ concentration may have an effect on the disease etiopathogenesis and activation.

Although oxidative stress of neutrophils has a role in patients with BD, precisely how oxidative stress plays a role in the pathogenesis of patients with BD is not known (Evereklioğlu 2005). Unlike other studies, it was thought that more robust results would be obtained from the current study by examining, as indicators of oxidative stress, the lipid peroxidation and antioxidant enzyme levels in neutrophils which have an important role in the disease pathogenesis, rather than in serum and erythrocytes which can be affected by many factors. As neutrophils are nucleated cells, when there is oxidative stress in the nucleus and an increase in intracellular calcium leading to DNA damage, they may thus play a role in neutrophil activation (Ayub and Hallett 2004; Karaman et al. 2009). In the current study, the lipid peroxidation levels of the active patient group were found to be significantly high compared to the inactive patient group and the control group; it is thought that the increase in oxidative stress may be responsible for BD activation.

GSH is the most abundant thiol antioxidant in mammalian cells. Reduced form of GSH is an endogenous tripeptide that acts both as a nucleophilic scavenger of numerous compounds and as a substrate in the selenium-dependent GSH-Px-mediated destruction of hydroperoxides (Nazıroğlu et al. 2011). In the current study, GSH and GSH-Px values were markedly decreased in neutrophil of patients with BD although lipid peroxidation levels increased in the neutrophil. Therefore, the results of the current study supported our previous reports and suggested an increased need for antioxidants in such active patients. Similarly, it was reported that GSH-Px activity with active and inactive ocular involvement and non-ocular involvement was shown to be increased to a significant degree in patients with BD compared to the control group (Taysi et al. 2002). In studies by Sandıkci et al. (2003) and Kökçam and Nazıroğlu (2002), the plasma and erythrocyte lipid peroxidation levels of active patients with BD compared to the control groups were observed to be significantly high whereas the GSH-Px level was significantly low. Korkmaz et al. (2011) reported also that GSH levels in neutrophils of patients with active BD were lower in patient group than in the controls. The current study GSH and GSH-Px results were confirmed by results of Kökçam and Nazıroğlu (2002), Sandikci et al. (2003), Taysi et al. (2002), and Korkmaz et al. (2011).

As there is no specific treatment for BD, treatment is administered according to the system involved. Although the mechanism of the effect of HP on BD is not clearly understood, it has been shown to have an anti-inflammatory effect as an antioxidant and by inhibiting leukocyte chemotaxis and it is known that even at very low doses adhesion of neutrophils to the endothelium and neutrophil migration decrease (Evereklioğlu 2005; Bréchard and Tschirhart 2008). In the study by Feisst and Werz (2004), the intracellular Ca^{2+} release of the subject who had incubated HP was significantly low compared to the neutrophils of control group and it was suggested that HP had an effect on Ca^{2+} release. In the current study, thinking that HP may have an effect on neutrophil $[Ca^{2+}]_i$ concentration variations in BD pathogenesis and activation, neutrophils were incubated with HP in vitro.

Caspases are cysteine proteases, using the sulfur atom in cysteine to cleave the polypeptide chain, and they are the executioners of apoptosis. Caspase-3 and -9 are members of the initiator caspase group and are generally thought to be responsible for initiating the caspase activation cascade during apoptosis. Recent reports have focused on the insufficient apoptosis of lymphocytes, which are the effectors cells in the BD (Öztaş et al. 2007). HP has been used as an antidepressant with few side effects (Jang et al. 2002). It induced anti-apoptotic effects in human neuroblastoma cells (Jang et al. 2002). In this study, we examined the apoptosis, MTT, caspase-3, and -9 values in neutrophil of patients with BD and we have also investigated protective role of HP on the values in the neutrophils. The apoptosis, MTT, caspase-3, and -9 values increased in neutrophil of patients with BD as compared to the results of health controls although caspase activities were modulated by HP incubations. Similarly, it was reported that caspase-9 activity and apoptosis values increased in oral endothelial of active BD (Öztaş et al. 2007). Todaro et al. (2005) reported that caspase-3 activity increased in leukocytes of patients with BD.

This study has importance in respect of it being the first study to examine the effects of Ca^{2+} signaling, oxidative stress parameters, and the level of HP molecules on the Ca^{2+} mechanism in the neutrophils of patients with BD. As increased oxidative stress and neutrophil Ca^{2+} entering into the cytosol has been observed particularly in the active phase of BD and oxidative stress and neutrophil Ca^{2+} concentration has been determined to have increased especially in the activation phase, it is thought that the neutrophils playing a role in BD etiopathogenesis are activated by oxidative stress caused by Ca^{2+} release into the cytosol. One of the significant results obtained from this study is that HP used in BD treatment is seen to lead to a reduction in cytoplasmic Ca^{2+} release in the neutrophils. It is thought that HP implements the neutrophil Ca^{2+} concentration by a Ca^{2+} channel other than the VGCC. Thus, future studies may be able to develop new treatment approaches by targeting VGCC with the substantial reduction from V+D seen in intrastoplasmic Ca^{2+} levels in the neutrophils of patients with BD.

Intracellular Ca^{2+} has been presented as a key regulator of cell survival, but this ion can also induce apoptosis in response to a number of pathological conditions (Nazıroğlu

2007). One subgroup of the TRP family is TRPM2 channels, and the channels have been activated by oxidative stress products. The formation of ROS in the neutrophil can be induced by naturally occurring agonist, fMLP, and fMLP was indicated as an activator of TRPM2 channels in neutrophils (Heiner et al. 2003). In the current study, fMLP-induced Ca^{2+} entry was blocked by V+D, indicating that L-type voltage-gated Ca^{2+} channels mediated the Ca^{2+} entry. Moreover, 2-APB blocked the Ca^{2+} entry suggesting an involvement of TRP channels, especially TRPM2. Similarly, Krishtal et al. (2001) reported that hyperforin acts via interaction with voltage-gated P-type Ca^{2+} channels or through calmodulin-activated pathways involving at least one second messenger. The modulator role of HP on voltage- and ligand-gated ionic conductance in hippocampal pyramidal neurons and cerebellar Purkinje neurons of rat was also reported by patch-clamp experiments (Chatterjee et al. 1999). Modulator role of HP on the Ca^{2+} entry in healthy human and multiple sclerosis neutrophils was also recently reported (Shima et al. 2008; Nazıroğlu et al. 2014b). Hyperforin administration after middle cerebral artery occlusion ischemia also decreased apoptotic cell death in brain of rats through modulation of TRPC6 cation channels (Lin et al. 2013). Similarly, we recently observed a protective role for HP on Ca^{2+} entry through a TRPM2 channel in the dorsall root ganglion neurons of rats (Nazıroğlu et al. 2014a).

In conclusion, results of the study confirmed our previous studies and demonstrated increased role of oxidative stress in neutrophil of patients with BD (Korkmaz et al. 2011). However, we firstly indicated that the HP was capable of breaking lipid peroxidation chain reaction in neutrophil membranes of neutrophil and thus reduced oxidative stress and apoptosis. There is now thought to be the possibility that the HP Ca^{2+} signal in the neutrophils is made through the VGCC and TRPM2 channels. Future studies targeting voltage-dependent calcium channels will have implications for new treatment approaches.

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