

Anti-tumor Necrosis Factor Alpha (Infliximab) Attenuates Apoptosis, Oxidative Stress, and Calcium Ion Entry Through Modulation of Cation Channels in Neutrophils of Patients with Ankylosing Spondylitis

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Abstract Ankylosing Spondylitis (AS) is known to be associated with increased neutrophil activation and oxidative stress, however, the mechanism of neutrophil activation is still unclear. We have hypothesized that the antioxidant and anti-tumor necrosis factor properties of infliximab may affect intracellular Ca^{2+} concentration in the neutrophils of AS patients. The objective of this study was to investigate the effects of infliximab on calcium signaling, oxidative stress, and apoptosis in neutrophils of AS patients. Neutrophils collected from ten patients with AS and ten healthy controls were used in the study. In a cell viability test, the ideal non-toxic dose and incubation time of infliximab were found as 100 μM and 1 h, respectively. In some experiments, the neutrophils were incubated with the voltage-gated calcium channel (VGCC) blockers verapamil + diltiazem (V + D) and the TRPM2 channel blocker 2-aminoethyl diphenylborinate (2-APB).

Intracellular Ca^{2+} concentration, lipid peroxidation, apoptosis, caspase 3, and caspase 9 values were high in neutrophils of AS patients and were reduced with infliximab treatment. Reduced glutathione level and glutathione peroxidase activity were low in the patients and increased with infliximab treatment. The intracellular Ca^{2+} concentrations were low in 2-APB and V + D groups. In conclusion, the current study suggests that infliximab is useful against apoptotic cell death and oxidative stress in neutrophils of patients with AS, which seem to be dependent on increased levels of intracellular Ca^{2+} through activation of TRPM2 and VGCC.

Keywords Ankylosing spondylitis · Infliximab · Apoptosis · Calcium signaling · Oxidative stress · Neutrophil

Abbreviations

$[\text{Ca}^{2+}]_i$	Intracellular Ca^{2+}
2-APB	2-aminoethyl diphenylborinate
AS	Ankylosing spondylitis
CRP	C-reactive protein
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis[2-aminoethylether]- <i>N,N,N,N</i> -tetraacetic acid
ESR	Erythrocyte sedimentation rate
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
HBSS	Hank's buffered salt solution
ROS	Reactive oxygen species
SDU	Suleyman Demirel University
TNF- α	Tumor necrosis factor alpha
TRP	Transient receptor potential
TRPM2	Transient receptor potential melastatin 2
V + D	Verapamil + diltiazem

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Introduction

Ankylosing spondylitis (AS) is an inflammatory rheumatological disease that has a high incidence of 1 % worldwide and is seen three times more in males than in females between the ages of 20–40 years (Santiago et al. 2013). The incidence is high in patients who are HLA B27 positive. The main symptoms of AS are progressive spine involvement with pain, stiffness, fatigue, and dysfunction. Decreased quality of life and a restricted social life are very common problem in patients with AS (Santiago et al. 2013). Neutrophil activation plays key pathophysiological roles in the progression of AS symptoms such as spine ankylosis and tendon inflammation (Couto et al. 2010; Boyraz et al. 2014). Several cytokines such as interleukin (IL)-1 α and tumor necrosis factor alpha (TNF- α) are involved in each stage of AS pathogenesis (Meroni and Valesini 2014).

Treatment of the disease has progressed over the years at research with better knowledge on the pathogenesis of AS. Anti-TNF- α therapy has proven to be effective for the treatment of patients with AS (Meroni and Valesini 2014). Infliximab (INF) is a monoclonal chimeric (mouse–human) immunoglobulin G1 that binds to TNF- α (Zou et al. 2003). The effect of INF on neutrophils, one of the important cells in inflammation that also has the ability to activate caspase cascades, has not yet been established. Conflicting reports indicate that TNF molecules may show an anti-inflammatory effect by inducing apoptosis while also triggering inflammation by inhibiting apoptosis (Couto et al. 2010; Kettritz et al. 2002; González-Flores et al. 2014).

An increase in neutrophil counts in patients with AS, especially in the active period of the disease has been reported (Boyraz et al. 2014). Based on the important role played by reactive oxygen species (ROS) in some malignancies and rheumatic diseases, it is also possible that these cells play a role in the activation of AS (Couto et al. 2010). Neutrophils in inflammatory reactions form the first defense barrier. Leucocytes are stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) produced by bacteria to increase the intracellular calcium ion concentration ($[Ca^{2+}]_i$); this increase can induce free radical formation and activate apoptotic pathways (Heiner et al. 2006). In phagocytic cells, an increase in oxidative stress causes activation of calcium channels [especially transient receptor potential melastatin 2 (TRPM2) and voltage-gated calcium channels (VGCC)] by feedback mechanism; enhanced intracellular ROS can further exacerbate the stimulation of neutrophils (Heiner et al. 2006; Kaplan et al. 2013; Nazırođlu et al. 2014a; Köse and Nazırođlu 2015). Recent studies have indicated that regulation of calcium ion entry through modulation of TRPM2 and VGCC

channels may decrease apoptosis and oxidative stress (Nazırođlu et al. 2014a, b; Övey and Nazırođlu 2015; Yürüker et al. 2015). INF is known to have an antioxidant effect in the phagocytic cells of patients with AS (Túnez et al. 2007; Feijóo et al. 2009) and the effects might be induced through the modulation of TRPM2, VGCC, oxidative stress, apoptosis, and activation of caspases.

In this study, the first aim was to understand the importance of Ca^{2+} influx in neutrophils in the activation of AS. Additionally, we examined the effect of the anti-TNF drug, INF, on neutrophil $[Ca^{2+}]_i$ infiltration from extracellular sites induced by fMLP as a calcium-mobilizing agonist. The second aim of the study was to examine the effect of INF on apoptosis, caspase activity, lipid peroxidation, and antioxidant values in human neutrophils. With these data, we aimed to better understand the mechanisms of neutrophil activation in the pathogenesis of AS, which may aid in the development of new treatment methods.

Patients and Methods

Chemicals

All chemicals [cumene hydroperoxide, thiobarbituric acid, 1,1,3,3 tetraethoxypropane, 5,5-dithiobis-2-nitrobenzoic, tris-hydroxymethyl-aminomethane, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), glutathione, butylhydroxytoluol, fMLP, digitonin, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA)] were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all organic solvents (*n*-hexane, ethyl alcohol) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Fura-2-acetoxymethyl ester was purchased from Promega (Eugene, Oregon, USA). Neutrophil isolation was carried out in sterile solutions containing phosphate-buffered saline from GIBCO Invitrogen (Istanbul, Turkey), 6 % hydroxyethyl starch solution in isotonic NaCl (Plasmasteril) from Fresenius (Bad Homburg, Germany) and Ficoll-Paque Plus Solution from GE Healthcare Bio-Sciences (Uppsala, Sweden).

Patients and Controls

The study was conducted at the Biophysics Research Laboratory, Suleyman Demirel University (SDU), Turkey. The patients enrolled in the study were selected from patients attending the Rheumatology Department of SDU. The study was approved by the Local Ethics Committee of Medical Faculty, SDU. All participants gave written consent, confirming their acceptance for giving blood through

vena brachialis, and were informed about the experimental procedures. For patients included in the study, demographic characteristics, clinical information, physical examination findings, and laboratory tests were recorded.

In the current study, 10 patients with AS and 10 age-matched control subjects were included. The diagnosis of AS was provided by the Assessment of Spondyloarthritis International Society (ASAS) classification criteria for axial spondyloarthritis (Rudwaleit et al. 2009).

Inclusion Criteria

Eligible patients fulfilled ASAS classification criteria for axial spondyloarthritis, meeting the New York criteria for AS. Patients with previous exposure to biologics or DMARDs (sulfasalazine or methotrexate) were not included.

Exclusion Criteria

Patients with rheumatologic or systemic disease other than AS, active infection, malignancy, or pregnancy were excluded.

Blood with anticoagulants was used for the analysis of erythrocyte sedimentation rate (ESR). Serum and neutrophil samples were obtained from the blood samples. Serum samples were immediately used for measurement of C-reactive protein (CRP). Half of the neutrophil samples were stored at $-33\text{ }^{\circ}\text{C}$ and were used to determine lipid peroxidation, reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) within 1 month. The remaining neutrophil samples were used to determine $[\text{Ca}^{2+}]_i$ concentration and were also used for MTT, apoptosis, and caspase 3 and 9 activation assays.

Groups

Neutrophils isolated from the controls and patients with AS were divided into four subgroups as follows:

Control group ($n = 10$): Neutrophils from age-matched healthy subjects.

Control + INF ($n = 10$): Neutrophils of age-matched healthy subjects incubated with INF.

AS group ($n = 10$): Neutrophils from patients with AS not incubated with INF.

AS + INF group ($n = 10$): Neutrophils from the same patients with AS incubated with INF ($100\text{ }\mu\text{M}$) for 1 h. The dose and time of INF incubation were determined with a cell viability (MTT) test.

In Ca^{2+} signaling experiments, we investigated role of TRPM2 and VGCC on Ca^{2+} entry in the neutrophils. For this aim, the neutrophils were further divided into subgroups as follows:

A-2-APB group ($n = 10$): Neutrophils were incubated with 2-APB (0.1 mM) for 10 min before fMLP stimulation.

B-(V + D) group ($n = 10$): Neutrophils were incubated with V + D (0.01 mM) for 30 min before fMLP stimulation (Korkmaz et al. 2011; Şahin et al. 2011).

Stock solutions of 2-APB and V + D were dissolved in dimethyl sulfoxide and extracellular buffer, respectively, and were stored at $-33\text{ }^{\circ}\text{C}$ until use. 2-APB (0.1 mM) in extracellular buffer was diluted to reach the final concentration and the pH of the solution was adjusted to 7.4 with KOH.

Isolation of Human Neutrophils

Neutrophils were isolated from peripheral whole blood of healthy volunteers and patients with AS, as described previously (Korkmaz et al. 2011; Şahin et al. 2011), by centrifugation through Ficoll. Half of the cells were stored for antioxidant analyses. The remaining cells were used for the measurement of $[\text{Ca}^{2+}]_i$, MTT, apoptosis, caspases 3 and 9. 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. The measuring buffers did not contain serum but were otherwise identical to the loading buffer when a normal extracellular Ca^{2+} concentration (1.2 mM) was explored.

Cell Viability (MTT) Assay

Cell viability analyses were spectrophotometrically performed as described elsewhere (Nazıroğlu et al. 2014b; Övey and Nazıroğlu 2015). Briefly, the neutrophils were re-suspended after isolation in $150\text{ }\mu\text{l}$ PBS. The cells were then divided into 3 eppendorf tubes ($50\text{ }\mu\text{l}$ each) and $15\text{ }\mu\text{l}$ MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each tube. The tubes were then shaken and centrifuged and the supernatants were removed. DMSO ($400\text{ }\mu\text{l}$) was added to each tube and the cells were re-suspended with a pipette. Each sample was divided into 2 cuvettes with $200\text{ }\mu\text{l}$ volume. Optical density was measured in a spectrophotometer at 490 and 650 nm and presented as fold increase over the pretreatment level (experimental/control).

Measurement of $[\text{Ca}^{2+}]_i$

Neutrophils (5×10^6 cells/ml) were loaded with $4\text{ }\mu\text{M}$ Fura-2/AM in loading buffer for 45 min at $37\text{ }^{\circ}\text{C}$ in the dark, washed twice, incubated for an additional 30 min at $37\text{ }^{\circ}\text{C}$ to complete probe de-esterification and re-suspended in loading buffer at a density of 3×10^6 cells/ml according to a procedure published elsewhere (Espino et al. 2013). The four groups were exposed to fMLP to stimulate intracellular Ca^{2+} release. Fluorescence was recorded from

2 ml aliquots of a magnetically stirred cellular suspension at 37 °C using a spectrofluorometer (Carry Eclipse; Varian, Sydney, Australia) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ concentration were monitored using the Fura-2/AM 340/380 nm fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (1985). In the experiments where calcium-free medium was required, Ca^{2+} was omitted and 2 mM of the chelator EGTA was added. Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_i$ concentration for 110 s after addition of fMLP. Ca^{2+} release was expressed in nanomoles, taking a reading every second as previously described (Korkmaz et al. 2011; Şahin et al. 2011).

Lipid Peroxidation and Protein Determinations

Lipid peroxidation in the neutrophils was measured with the thiobarbituric acid reaction by the method of Placer et al. (1966). Thiobarbituric acid-reactive substances were quantified by comparing the absorption to the standard curve of malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The lipid peroxidation values in neutrophil were expressed as micromoles (μ M) per gram (g) of protein. The protein content in neutrophil samples was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Reduced (GSH) and Glutathione Peroxidase (GSH-Px) Assays

The GSH content of neutrophil samples was measured at 412 nm according to the method of Sedlak and Lindsay (1968). The samples were precipitated with 50 % trichloroacetic acid and then centrifuged at $1000\times g$ for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris-EDTA buffer (0.2 M, pH 8.9), and 0.1 ml of 0.01 M DTNB. The solution was kept at room temperature for 5 min and then read at 412 nm using a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). GSH-Px activity of neutrophil samples was measured spectrophotometrically at 37 °C and 412 nm according to the method of Lawrence and Burk (1976).

Apoptosis Assay

The apoptosis assay was performed with a commercial kit according to the manufacturer's instructions (Biocolor Ltd., UK) and elsewhere (Köse and Naziroglu 2015). In this APOpercentage dye-uptake assay, when the membrane of apoptotic cell loses its asymmetry, the APOpercentage dye is actively transported into cells, staining apoptotic

cells red, thus allowing detection of apoptosis by spectrophotometry.

Assays for Caspase 3 and 9 Activities

To determine caspase 3 and 9 activities, neutrophils were sonicated and cell lysates were incubated with 2 ml of substrate solution [20 mM HEPES (pH 7.4), 2 mM EDTA, 0.1 % 3-[(3 cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), and 8.25 μ M of caspase substrate] for 1 h at 37 °C as previously described (Uğuz et al. 2009). The activities of caspase 3 and 9 were calculated from the cleavage of the respective specific fluorogenic substrate [*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (ACDEVD-AMC) for caspase-3 and *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC) for caspase-9]. Substrate cleavage was measured with a fluorescence spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary experiments confirmed that cleavage of caspase 3 or caspase 9 substrates were not detected in the presence of the inhibitors of caspase 3 or 9, ACDEVD-AMC or AC-LEHD-AMC, respectively. The data were calculated as fluorescence units/mg protein and presented as fold increase over the pretreatment level (experimental/control).

Biochemical Analysis

ESR was measured by routine Westergren method. CRP is an acute-phase protein that increases during systemic inflammation and serum CRP values in patients with AS were measured in a nephelometer (Delta SeacRadim, Pomezia, Italy).

Statistical Analysis

All results were expressed as mean \pm SD. Significant values in four groups were assessed with an unpaired Mann-Whitney *U* test. Data were analyzed using the SPSS statistical program (version 17.0 software, SPSS Inc. Chicago, Illinois, USA). *p*-values of <0.05 were regarded as significant.

Results

Results of Demographic Values

Ten patients with AS (7 men, 3 women) and 10 age-matched control group were used in the current study. All AS patients had active disease (BASDAI score > 4). The mean age of the patients was 33 ± 6.8 years for the AS group

and 32.0 ± 5.3 years for the control group. There was no statistically significant difference in age between these two groups ($p > 0.05$). BASDAI and BASFI scores are widely applicable indicators for evaluating disease activity in AS patients. These score changes are useful to monitor treatment outcomes; BASDAI scores also help in the evaluation of treatment response to drugs (Rudwaleit et al. 2004). ESH, CRP, BASDAI, and BASFI values in control and patients with AS are shown in Table 1. ESH ($p < 0.001$), CRP ($p < 0.001$), BASDAI ($p < 0.001$), and BASFI ($p < 0.001$) values were significantly higher in the patients group than in the control group.

Lipid Peroxidation, GSH and GSH-Px Results

Lipid peroxidation, GSH levels, and GSH-Px activity investigated as indicators of oxidative stress and antioxidant values. Lipid peroxidation, GSH, and GSH-Px values are shown in Table 2. Compared to the control group, lipid peroxidation levels in patients with AS was significantly high ($p < 0.05$), although its level was low in AS + INF group. In addition, the GSH ($p < 0.001$) and GSH-Px ($p < 0.05$) values were significantly lower in AS group with a significant recovery seen in the GSH ($p < 0.01$) level and GSH-Px activity ($p < 0.05$) in the AS + INF group.

Determination of Non-Toxic Infliximab Dosage

The dosage and incubation time of INF with human neutrophils was not available in the literature. Therefore, the control neutrophils obtained in the current study were incubated with five different doses of INF (0.01 mM, 0.1, 1, 10, and 100 μ M) at four different times (1, 2, and 3 h). Neutrophil viability was analyzed with a cell viability (MTT) test. Incubation of neutrophils for 1 h with 100 μ M INF was not toxic for the cells (Fig. 1).

Effects of INF on Intracellular Ca^{2+} Concentration [Ca^{2+}]_i in Neutrophil of Patients with AS

Ca^{2+} is considered to be important for agonist-induced leukocyte activation (Br  chard and Tschirhart 2008).

Table 1 Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), bath ankylosing spondylitis disease activity index (BASDAI), and bath ankylosing spondylitis functional index (BASFI) values in control and patients with AS (Mean \pm SD and $n = 10$)

Parameters	Control	AS	<i>p</i> -value
ESR (h)	7.10 ± 4.70	23.10 ± 16.80	<0.05
CRP (mg/ml)	4.20 ± 1.50	16.20 ± 14.90	<0.001
BASDAI score	0.70 ± 0.50	5.10 ± 0.70	<0.001
BASFI score	0.30 ± 0.60	3.90 ± 1.50	<0.001

Therefore we investigated whether treatment of neutrophils of AS patients with INF could affect the mobilization of Ca^{2+} , thereby inhibiting agonist-induced ROS production. Effects of INF on [Ca^{2+}]_i concentration in neutrophil are shown in Fig. 2a, b. Intracellular [Ca^{2+}]_i concentration resulting from Ca^{2+} entry through TRPM2 and VGCC of the neutrophils was determined as significantly high in the patient (AS) group compared to the control group ($p < 0.001$). However, the pre-incubation of neutrophils with INF for 1 h, significantly ($p < 0.05$ and $p < 0.001$) prevented Ca^{2+} entry in the AS neutrophils challenged with fMLP. The [Ca^{2+}]_i concentration was significantly lower ($p < 0.05$) in the AS + INF + (V + D) group than in AS + INF + 2-APB group. The VGCC channels appear to be more important than the TRPM2 channels in determining intracellular [Ca^{2+}]_i concentration.

Effects of INF on VGCC and TRPM2 Channels in Neutrophil of Patients with AS

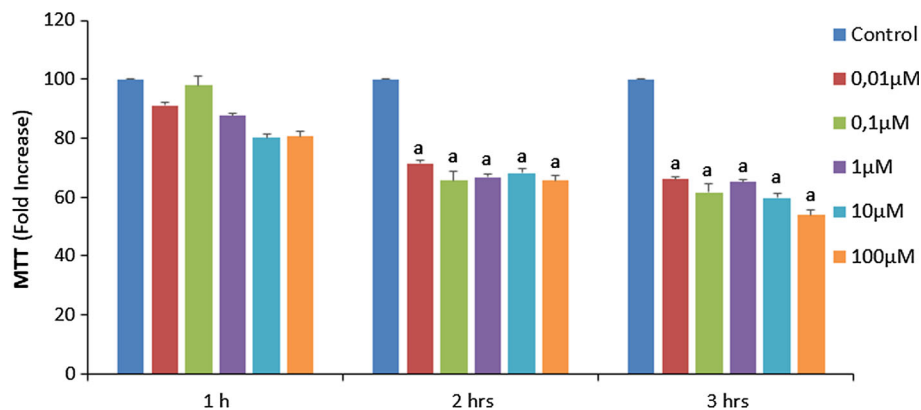
We next tested the route of Ca^{2+} entry using V + D, a VGCC blocker. In some experiments, neutrophils from the AS patient groups were also incubated with 2-APB, a drug that inhibits TRPM2 channels, on the [Ca^{2+}]_i concentration. The [Ca^{2+}]_i concentrations were determined as significantly lower in patient + V+D ($p < 0.001$) and patient + 2-APB ($p < 0.001$) compared to the patient group. The [Ca^{2+}]_i concentrations were also markedly lower ($p < 0.001$) in AS + 2-APB, AS + (V + D), AS + INF + 2-APB, and AS + INF + V+D compared to the AS group. These data highlight the importance of VGCC and TRPM2 channels on the [Ca^{2+}]_i concentrations in the neutrophils of AS patients. In addition, the [Ca^{2+}]_i concentrations were determined as significantly lower in AS + INF + (V + D) compared to the AS + INF + 2-APB group ($p < 0.01$). In the other words, Ca^{2+} entry through VGCC was more important than through the TRPM2 channels.

Effects of INF on Apoptosis

We investigated the effects of AS on programmed cell death of neutrophils through apoptosis and caspase activation assays. The results of the apoptosis assay in control, control + INF, INF, and AS + INF groups are shown in Fig. 3. The apoptosis values in AS were significantly higher ($p < 0.001$) than the control. There was a significant decrease in apoptosis of control neutrophils treated with INF ($p < 0.001$). Apoptosis of the AS groups compared to the control and control + INF groups were also markedly higher ($p < 0.001$). When INF was added to control and AS neutrophils, apoptosis was decreased and apoptosis was significantly lower in the AS + INF group compared to the AS group.

Table 2 Effects of INF on lipid peroxidation, reduced glutathione (GSH), and glutathione peroxidase values in the neutrophil of control and patients with AS (Mean \pm SD and $n = 10$)

Parameters	Control	AS	AS + INF
Lipid peroxidation ($\mu\text{M/g}$ protein)	19.60 \pm 1.52	23.30 \pm 3.02 ^a	20.30 \pm 2.82 ^c
GSH ($\mu\text{M/g}$ protein)	3.27 \pm 0.37	2.56 \pm 0.24 ^b	2.97 \pm 0.30 ^d
GSH-Px (IU/g protein)	10.65 \pm 1.44	8.17 \pm 1.39 ^a	9.79 \pm 1.20 ^c

^a $p < 0.05$ ^b $p < 0.001$ versus control group^c $p < 0.05$ ^d $p < 0.01$ versus AS group**Fig. 1** Effects of different doses and incubation times of INF on cell viability (MTT) test in the neutrophil of controls. The obtained control neutrophils were incubated at different times (30 min, 1, 2, and 3 h) and controls in five different doses (0.01 mM, 0.1, 1, 10, and100 μM) of INF. The neutrophils were analyzed by cell viability (MTT) test. We observed that 100 μM INF for 1 h incubation was not lethal dosage for the neutrophils. The dosage and incubation time were used in the current study. ^a $p < 0.05$ versus control

Effects of INF on Caspase Activity

Caspase 3 and 9 activities in control, control + INF, INF, and AS + INF groups are shown in Figs. 4 and 5, respectively. Caspase 3 and 9 activities were significantly increased ($p < 0.001$) in the neutrophils of patients with AS. In addition, the caspase 3 and 9 activities were decreased in the neutrophils of controls and AS patients after incubation with INF.

Discussion

AS is a chronic multisystem disorder characterized by rheumatoid symptoms. Neutrophil activation and extensive production of ROS have been proposed as causative reasons (Feijóo et al. 2009), although the etiology of AS has not been defined. INF has anti-inflammatory and antioxidant effects (Kageyama et al. 2008; Kurt et al. 2009). This study aimed to investigate the mechanisms of neutrophil activation, which are involved in the etiopathogenesis of

AS, as well as the protective role of INF on neutrophil Ca^{2+} entry, oxidative stress, and apoptosis. We observed here that the incubation with INF decreased the formation of ROS and apoptosis, and the entry of Ca^{2+} in neutrophils of AS patients through modulation of 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. DNA damage in the neutrophils is induced by extensive ROS production (Bréchar and Tschirhart 2008); excessive ROS production may also mediate tissue injury in patients with AS (Feijóo et al. 2009). Lipid peroxidation with increased neutrophil activity has been reported both in vivo and in vitro, suggesting increased neutrophil-induced ROS production in patients with AS (Kageyama et al. 2008; Kurt et al. 2009). Thus, by measuring the changes in the neutrophil $[\text{Ca}^{2+}]_i$ concentration of the patient groups, the importance of intracellular $[\text{Ca}^{2+}]_i$ concentration in the etiopathogenesis of the disease was

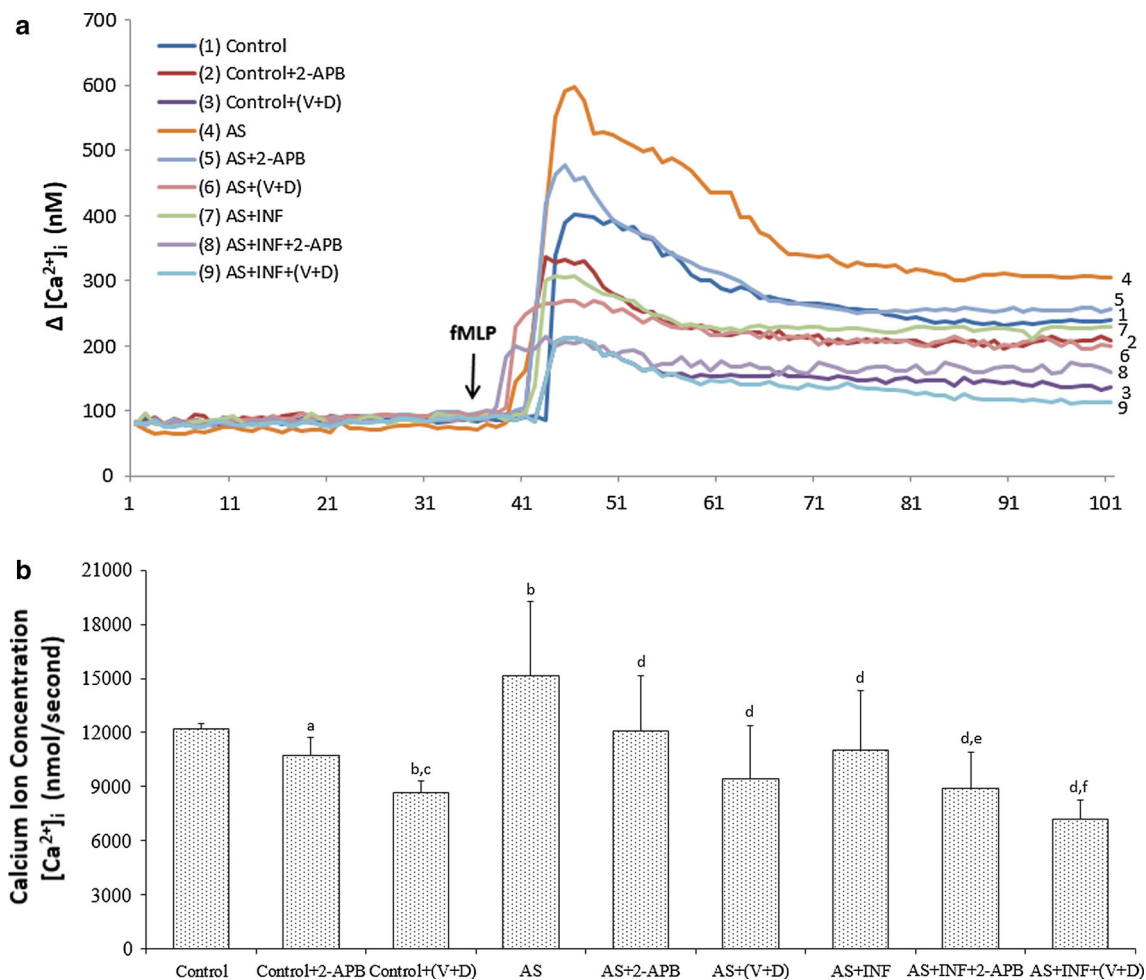


Fig. 2 Effect of infliximab treatment on intracellular calcium concentrations (a, b) of neutrophil of patients with Ankylosing Spondylitis (AS). (*n* = 10 and mean ± SD). These neutrophils were obtained from control and patients with AS. The neutrophils were incubated by infliximab (INF and 100 μM for 1 h). Fura-2-loaded neutrophils were stimulated with fMLP (0.001 mM) in the presence of normal extracellular calcium ($[Ca^{2+}]_o = 1.2$ mM) for 100 s. In

some experiments the neutrophils were incubated with TRPM2 channel blocker (2-APB, 0.1 mM for 10 min) and VGCC blockers (verapamil and diltiazem, V + D and 0.01 mM for 30 min). ^a*p* < 0.05 versus and ^b*p* < 0.001 versus control. ^c*p* < 0.001 versus control + 2-APB group. ^d*p* < 0.001 versus AS group. ^e*p* < 0.001 versus AS + 2-APB group. ^f*p* < 0.001 versus AS + (V + D) group

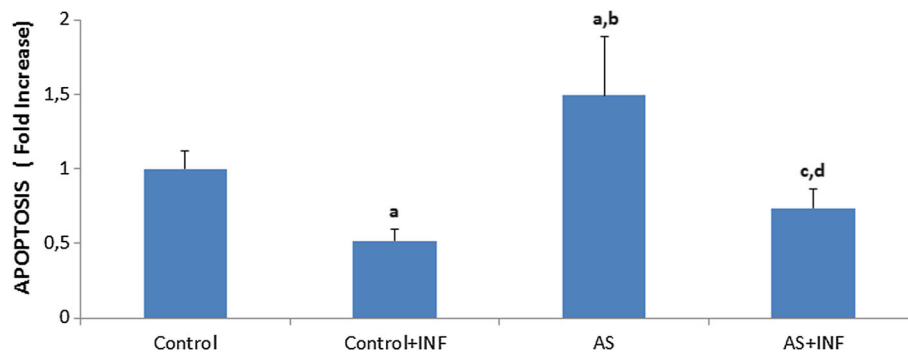


Fig. 3 Effects of infliximab (INF and 100 μM for 1 h) on apoptosis values in neutrophils of patients with AS (mean ± SD and *n* = 10). Apoptosis was estimated as described under “Patients and Methods” sections. Values expressed as fold increase over the pretreatment level

(experimental/control). ^a*p* < 0.001 versus control. ^b*p* < 0.001 versus AS and control groups. ^c*p* < 0.01 versus AS group. ^d*p* < 0.01 versus INF group

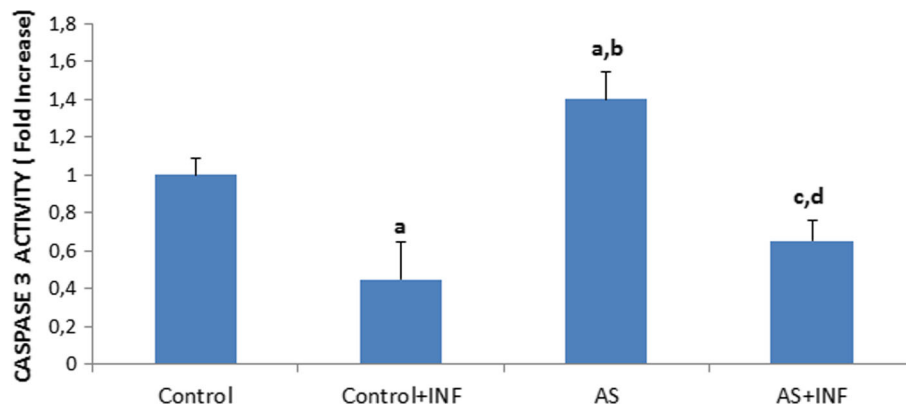


Fig. 4 Effects of infliximab (INF and 100 μ M for 1 h) on caspase 3 activity in neutrophils of patients with AS (mean \pm SD and $n = 10$). Apoptosis was estimated as described under “Patients and Methods” sections. Values expressed as fold increase over the pretreatment level

(experimental/control). ^a $p < 0.001$ versus control. ^b $p < 0.001$ versus AS and control groups. ^c $p < 0.01$ versus AS group. ^d $p < 0.001$ versus INF group

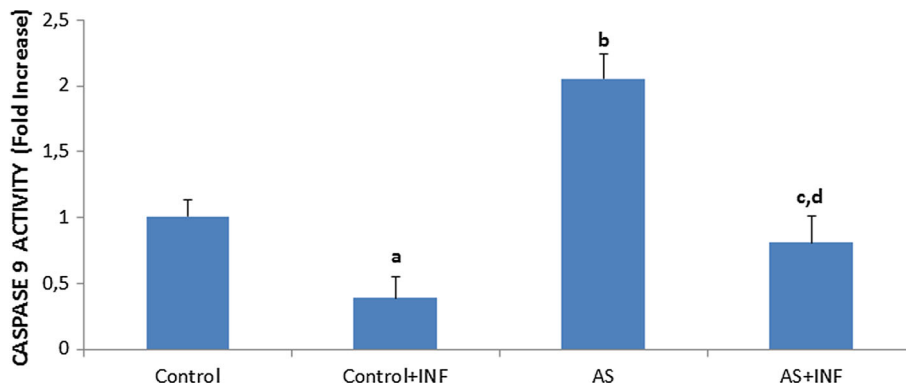


Fig. 5 Effects of infliximab (INF and 100 μ M for 1 h) on caspase 9 activity in neutrophils of patients with AS (mean \pm SD and $n = 10$). Apoptosis was estimated as described under “Patients and Methods” sections. Values expressed as fold increase over the pretreatment level

(experimental/control). ^a $p < 0.001$ versus control. ^b $p < 0.001$ versus AS and control groups. ^c $p < 0.01$ versus AS group. ^d $p < 0.001$ versus INF group

more clearly as ascertained. Basal $[Ca^{2+}]_i$ concentration measured from the patient groups were 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 disease etiopathogenesis and activation.

It was reported that excessive ROS production is stimulated by activating phagocytic NADPH oxidases in mitochondria of neutrophils (Yamashita et al. 1999; González-Flores et al. 2014; Liu et al. 2014), although NADPH oxidases-dependent cytokine, chemokines, mitogens, proteases existing products from the TNF- α network are reduced through supporting antioxidant redox system by INF treatment (Dalaklioglu et al. 2013). TRPM2 and VGCC are activated by oxidative stress and fMLP (Heiner et al. 2006; Pantaler and Lückhoff 2009; Korkmaz et al. 2011; Şahin et al. 2011) and are inhibited by antioxidants (den Broeder et al. 2003; Naziroglu et al. 2014b; Köse and Naziroğlu 2015). To our knowledge, the effect of anti-

TNF- α drugs such as INF on Ca^{2+} entry through VGCC and TRPM2 channels in neutrophils of AS patients has not been investigated. Previous studies suggest protective effects of anti-TNF agents on ROS production in healthy subjects and in patients with rheumatoid arthritis. In the current study, lipid peroxidation level was decreased in the neutrophils of AS patients incubated with INF, while GSH and GSH-Px values were increased by the treatment. Similarly, Pay et al. (2005) observed that ROS production in neutrophils of healthy subjects was decreased by INF. It was reported that the production of superoxide and ROS in neutrophils of patients with rheumatoid arthritis were decreased with human anti-TNF- α monoclonal antibody treatment (den Broeder et al. 2003). ROS production in the spinal cord tissue of rats and in urine of patients with rheumatoid arthritis was also decreased by INF treatment (Kageyama et al. 2008; Kurt et al. 2009). Karakucak et al. (2010) reported that oxidative stress levels as total

oxidative status (TOS) was decreased in the serum of AS patients while the total antioxidant status (TAS) was increased after anti-TNF- α treatment. Result of a recent study indicated that lipid peroxidation and apoptosis levels were decreased in serum and lung of rats with experimental lung fibrosis by INF treatment although GSH level and GSH-Px activity were increased by the treatment (Altintas et al. 2016). Data from the current study show that anti-TNFs can ameliorate oxidative stress-induced inflammation in neutrophils of AS patients. These data also corroborate previous results reported by den Broeder et al. (2003), Pay et al. (2005), Altintas et al. (2016), and Karakucak et al. (2010).

The intrinsic apoptosis pathway is characterized by loss of mitochondrial membrane potential, release of mitochondrial apoptotic factors into the cytosol, and activation of the caspase protease cascade (Espino et al. 2011). Activated executioner caspases cleave specific cellular substrate proteins, promoting destruction of the cell. Increased activation of TRPM2 and VGCC channels can trigger high Ca^{2+} influx, which activates a number of enzymes that damage cell structures. Ca^{2+} influx is thought to contribute to excitotoxic neuronal cell death and apoptosis in patients with AS. Mitochondrial depolarization is fueled by Ca^{2+} entry from the extracellular space via TRPM2 and VGCC channels in stimulated neutrophils (Ayub and Hallett 2004; Heiner et al. 2006; Naziroğlu et al. 2014a). It has been reported that TNF- α may have a role in the intrinsic pathway of apoptosis, especially in the activation of caspases 3 and 9. On the other hand, TNF- α inhibitors have been accepted as anti-apoptotic (Yamashita et al. 1999; Kettritz et al. 2002; González-Flores et al. 2014). In this study we examined apoptosis and caspase 3 and 9 activation in neutrophils of AS patients. We have also investigated the protective role of INF on neutrophil apoptosis. Apoptosis, caspase 3 and 9 activations were observed to be enhanced in the neutrophils of AS patients compared to healthy controls; apoptosis and caspase activities were reduced when the neutrophils were incubated with INF. Decreased apoptosis in the neutrophils of rats after intestinal transplantation has been reported (Gerlach et al. 2014). Apoptosis and caspase 3 activity were decreased in dorsal root ganglion neuron after INF treatment in TNF-TNFR1 knockout mice in an experimental model of spinal cord trauma (Genovese et al. 2008). On the other hand, apoptosis, caspase 3, and caspase 9 activities were reported to remain unchanged in intestinal mucosa of ischemia/reperfusion-induced rats after INF treatment (Yang et al. 2013).

An important factor affecting neutrophil functions is intracellular $[\text{Ca}^{2+}]_i$ concentration which plays roles in chemotaxis, adhesion, arachidonic acid metabolism, and ROS production in neutrophils (Ayub and Hallett 2004).

The current study is the first to examine the effects of Ca^{2+} signaling, oxidative stress parameters, and INF treatment on Ca^{2+} -induced alterations in neutrophils of patients with AS. One significant result obtained from this study is that INF treatment reduced cytoplasmic Ca^{2+} levels in the neutrophils through modulation of VGCC and TRPM2 channels. It is thought that INF treatment can modulate neutrophil Ca^{2+} concentration by a Ca^{2+} channel other than the VGCC.

In conclusion, we have shown here the effects of the anti-TNF- α drug INF on calcium signaling, oxidative stress, and apoptosis in neutrophils from AS patients. INF was seen to inhibit intracellular Ca^{2+} flux through modulation of TRPM2 and VGCC channels in neutrophils. We have suggested here a new concept in the mechanism of action of INF whereby the anti-TNF- α drug may improve neutrophil activity in AS patients by regulating intracellular signaling pathways and protecting them against apoptosis and oxidative stress.

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Author contributions MN and MŞ formulated the present hypothesis and MN was responsible for writing the report. YU was responsible for blood collection and patient management. MA was responsible for the analyses.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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