J Membrane Biol (2012) 245:833–840
DOI 10.1007/s00232-012-9491-x

Non-Ionic Contrast Media Induces Oxidative Stress and Apoptosis Through Ca^{2+} Influx in Human Neutrophils

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Received: 3 April 2012 / Accepted: 16 July 2012 / Published online: 18 August 2012

Abstract Non-ionic contrast media (CM) can induce tissue kidney injury via activation of phagocytosis and oxidative stress, although the mechanisms of injury via neutrophils are not clear. We investigated the effects of CM on oxidative stress and Ca^{2+} concentrations in serum and neutrophils of humans. Ten migraine patients were used in the study. Serum and neutrophil samples from patients' peripheral blood were obtained before (control) and 30 min after non-ionic (iopromide) CM injection. The neutrophils were incubated with non specific transient receptor potential 2 (TRPM2) channel blocker, 2-aminoethoxydiphenyl borate (2-APB), and voltage gated Ca^{2+} channel blockers, verapamil plus diltiazem. Serum and neutrophil lipid peroxidation, apoptosis and intracellular Ca^{2+} concentrations levels were higher in the CM group than in controls. The neutrophilic reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) levels as well as serum vitamin E and β-carotene concentrations were lower in the CM group than in controls. Neutrophil lipid peroxidation levels were lower in the CM+2-APB and CM+verapamil-diltiazem groups than in the CM group, although GSH, GSH-Px and intracellular Ca^{2+} values increased in the CM+2-APB and CM+verapamil-diltiazem groups. However, caspase-3, caspase-9, vitamin A and vitamin C values were unaltered by CM treatment. In conclusion, we observed that CM induced oxidative stress and Ca^{2+} influx by decreasing vitamin E, β-carotene and Ca^{2+} release levels in human serum and neutrophils. However, we observed protective effects of Ca^{2+} channel blockers on Ca^{2+} influx in neutrophils.

Keywords Contrast media · Inflammation · Ca^{2+} release · Oxidative stress · Neutrophil

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Introduction

The human body is equipped with a complete arsenal of defenses against external and internal aggressions. Those against the so-called reactive oxygen species (ROS) are crucial in inflammatory responses, where they participate in physiological processes such as the arachidonic acid cascade and phagocytosis (Kovacic and Somanathan 2008; Bréchard and Tschirhart 2008). ROS concentrations are kept under strict control by the activity of a complex defense system that includes enzymes and nonenzymatic species such as vitamin C, vitamin E, vitamin A and β-carotene (Traber and Stevens 2011). Vitamin E (α-tocopherol) is the most important antioxidant in the lipid phase of cells. Vitamin E acts to protect cells against the effects of free radicals, which are potentially damaging by-products of metabolism (Nazıroğlu 2007a, b). Vitamin C, as well as being a free radical scavenger, transforms vitamin E to its active form. Vitamin A (retinol) serves as a prohormone for retinoids and is involved with signal transduction at cytoplasmic and membrane sites (Traber and Stevens 2011). These enzymatic and nonenzymatic antioxidants are also essential for inhibition of phagocytic activity related to ROS production (Freitas et al. 2009).

Non-ionic contrast media (CM) have been shown to influence cellular functions such as secretion of leukotrienes, activation of phagocytic cells and oxidative stress (Blann et al. 2001; Böhm et al. 2008; Yesildağ et al. 2009). Neutrophils are primarily or secondarily involved in the pathogenesis of non-ionic CM (Fanning et al. 2002). They are key players in inflammatory processes, during which they are exposed to a variety of agonists that signal mostly through heterotrimetric G protein–coupled receptors. An increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is an important step within the multitude of serial or parallel signaling events that participate in the activation of neutrophil reactions such as chemotaxis, release of ROS and apoptosis (Ayub and Hallett 2004; Sahin et al. 2011). The release of superoxide radicals by NADPH oxidase that follows stimulation of the formyl peptide receptor requires an increase in $[Ca^{2+}]_i$ (Bréchard and Tschirhart 2008). In last decade, it was reported that iodinated CM stimulated phagocytic activity in neutrophils (Fanning et al. 2002), although its physiological mechanisms are not clear. CM may increase ROS production in kidney and neutrophils via stimulation of Ca^{2+} influx (Cruz et al. 2010; Heyman et al. 2010). However, the mechanisms of these effects of non-ionic CM are not fully understood in neutrophils.

Because of the lack of knowledge of how CM affects cellular survival and death in molecular pathways of neutrophils, we focused on the effect of CM in human neutrophil cells by checking its role in lipid peroxidation and antioxidant levels, apoptosis and Ca^{2+} release from intracellular stores evoked by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) as a Ca^{2+} mobilizing agonist.

Subjects and Methods

Chemicals

All chemicals (cumene hydroperoxide, KOH, NaOH, thiobarbituric acid, 1,1,3,3-tetraethoxy propane, 5,5-dithiobis-2 nitrobenzoic acid, tris-hydroxymethyl-aminomethane, glutathione, butylhydroxytoluol, fMLP, thapsigargin, digitonin, ethylene glycol-bis[2-aminoethyl-ether]-*N,N,N′,N′*-tetraacetic acid [EGTA]) were obtained from Sigma-Aldrich (St. Louis, MO), and all organic solvents (*n*-hexane, ethyl alcohol) were purchased from Merck (Darmstadt, Germany). Fura-2 acetoxymethyl ester was purchased from Promega (Eugene, OR). All reagents were of analytical grade. All reagents except the phosphate buffers were prepared daily and stored at +4 °C. Reagents were equilibrated at room temperature for 30 min before an analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4 °C for 1 month.

Patients

The study was approved by the Ethics Committee, of the Medical Faculty, Suleyman Demirel University (Isparta, Turkey). All participants gave written consent, confirming their acceptance for giving blood through the vena brachialis, and were informed about all experimental procedures. Patients were diagnosed and classified by clinicians of the Internal Medicine Clinic, and the control group was selected from among healthy parents or siblings.

The study was performed on 10 headache patients (five male, five female) aged 29–47 years, mean age 30.8 years (31.9 years for men, 38.4 years for women). None of them had an alcohol abuse problem. They had no proteinuria nor evidence of nephritic syndrome or kidney disease. Patients had not received any systemic therapy, which might have affected cellular immunity, during the 2 weeks prior to sample collection. The women who were included in the study had not been taking oral contraceptives for at least 6 months before sample collection.

Study Groups and Blood Sampling

Iopromide (Ultravist) was supplied as a 300 mg/ml sterile aqueous solution. A standardized volume (100 ml) was injected intravenously at a rate of 2 ml/s by means of a power injector. Blood samples were obtained before (control) and 30 min after CM (CM group) injection by sterile puncture of the cubital vein.

Blood samples of the control and CM groups were drawn from the antecubital vein into tubes with or 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 used for lipid peroxidation and antioxidant analysis within 1 month. The remaining neutrophil samples were used immediately for analysis of $[Ca^{2+}]_i$, apoptosis, caspase-3 and caspase-9.

Isolation of Human Neutrophils

Neutrophils were isolated from peripheral whole blood of healthy volunteers and patients with migraine as described previously (Sahin et al. 2011), by centrifugation through Ficoll. Half of the cells were stored for antioxidant analyses. The remaining cells were used for measurement of $[Ca^{2+}]_i$ concentration. Sterile solutions used for neutrophil isolation were phosphate-buffered saline (GIBCO Invitrogen, Istanbul, Turkey), 6 % hydroxyl ethyl starch solution in isotonic NaCl (Plasmasteril; Fresenius, Bad Homburg, Germany) and Ficoll-Paque Plus (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.

2-Aminoethoxydiphenyl Borate and Verapamil+Diltiazem Treatments

Transient receptor potential (TRP) channels can be indirectly blocked by chemicals. However, the TRP channels are of the TRP melastatin 2 (TRPM2) type, and the range of pharmacological modulators of TRPM2 is limited. Recently, 2-aminoethoxydiphenyl borate (2-APB) was described as a TRPM2 channel blocker in addition to its actions as an inositol 1,4,5-triphosphate ($InsP_3$) receptor antagonist (Togashi et al. 2008; Nazıroğlu et al. 2011). Verapamil and diltiazem are well-known blockers of high and low voltage-gated Ca^{2+} channels. Non-ionic CM-induced Ca^{2+} influx in neutrophils may be modulated by 2-APB and verapamil+diltiazem. For the investigation, neutrophils were incubated with 2-APB (0.1 mM) for 1 min and verapamil+diltiazem (10 μM) for 30 min in the CM experiments. Neutrophils were used for lipid peroxidation, reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) and cytosolic Ca^{2+} analyses.

Stock 2-APB was dissolved in dimethyl sulfoxide and stored at −33 °C. Before the experiment, 2-APB (0.05 mM) in extracellular bath solutions was diluted to reach the required final concentrations. All experiments were carried out at room temperature (approximately 20 °C). After addition of 2-APB to a standard extracellular bath solution, the pH values of these solutions were adjusted with KOH to 7.4. 2-APB was added to the cuvette of a spectrophotometer.

Measurement of Free $[Ca^{2+}]_i$ Concentrations

Neutrophils, at a density of 5×10^6 cells/ml, were loaded with 4 M fura-2/AM in loading buffer for 45 min at 37 °C in the dark, washed twice, incubated for an additional 30 min at 37 °C to complete probe deesterification and resuspended in loading buffer at a density of 3×10^6 cells/ml according to a procedure published elsewhere (Uğuz et al. 2009; Espino et al. 2010; Sahin et al. 2011). The four groups were exposed to fMLP for stimulation of $[Ca^{2+}]_i$ release. Fluorescence was recorded from 2-ml aliquots of magnetically stirred cellular suspension at 37 °C using a spectrofluorometer (Carry Eclipsys; Varian, Sydney, Australia) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ concentrations were monitored using the fura-2 340/380 nm fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (1985).

Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_i$ concentrations for 150 s after addition of fMLP (1 μM) (Espino et al. 2009; Uğuz et al. 2009). Ca^{2+} release is expressed in nanomolar concentrations, taking a sample every second as previously described (Heemskerk et al. 1997).

Lipid Peroxidation Determinations

Lipid peroxidation levels in serum and 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 a standard curve of malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. Values of lipid peroxidation in the neutrophil and serum samples were expressed as micromoles per gram of protein and nanomoles per milliliter, respectively.

GSH, GSH-Px and Protein Assay

The GSH content of neutrophil samples was measured at 412 nm using the method of Sedlak and Lindsay (1968). Samples were precipitated with 50 % trichloroacetic acid and then centrifuged at $1{,}000 \times g$ 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 5,5′-dithiobis-2-nitrobenzoic acid. 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 activities of neutrophil samples were measured spectrophotometrically at 37 °C and 412 nm according to the method of Lawrence and Burk (1976). The protein content in neutrophil samples was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Analyses for Serum Vitamins A, C and E and β-Carotene

Vitamin A (retinol) and vitamin E (α-tocopherol) were determined in serum samples by a modification of the method described by Desai (1984) and Suzuki and Katoh (1990). Serum samples (250 l) were saponified by the addition of 0.3 ml KOH (60 %, w/v in water) and 2 ml of 1 % (w/v in ethanol) ascorbic acid, followed by heating at 70 °C for 30 min. After cooling the samples on ice, 2 ml of water and 1 ml of *n*-hexane were added and mixed with the samples and then rested for 10 min to allow phase separation. An aliquot of 0.5 ml of *n*-hexane extract was taken, and vitamin A concentrations were measured at 325 nm. Then, reactants were added and the absorbance value of hexane was measured in the spectrophotometer at 535 nm. Calibration was performed using standard solutions of all-*trans* retinol and α-tocopherol in hexane.

Concentrations of β-carotene in serum samples were determined according to the method of Suzuki and Katoh (1990). Two milliliters of hexane were mixed with 250-µl serum samples. The concentration of β-carotene in hexane was measured at 453 nm in the spectrophotometer.

Ascorbic acid in serum samples was quantified using the method of Jagota and Dani (1982). The absorbance of samples was measured spectrophotometrically at 760 nm.

Assay for Caspase Activities and Apoptosis

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 % CHAPS, 5 mm DTT and 8.25 µM 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 (AC-DEVD-AMC for caspase-3 and 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 caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK or z-LEHD-FMK, respectively. The data were calculated as fluorescence units per milligram of protein.

The APOPercentage^{TM} assay (Biocolor, Belfast, Northern Ireland) was performed according to the instructions provided by the manufacturer (Uğuz and Nazıroğlu 2012).

APOPercentage is a dye-uptake assay, which stains only apoptotic cells with a red dye. When the membrane of an apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometry.

Statistical Analysis

Data are expressed as means ± SEM of the number of determinations. Statistical significance was analyzed using the SPSS program (9.05; SPSS, Inc., Chicago, IL). To compare the different treatments, statistical significance was calculated by Mann–Whitney *U* test analysis. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of CM on Intracellular Ca^{2+} Release in Neutrophils of Patients with Migraine

The effects of the CM on intracellular Ca^{2+} release in neutrophils are shown in Figs. 1 and 2. Intracellular free Ca^{2+} ($[Ca^{2+}]_c$) concentrations in the cells were significantly ($p < 0.05$ and $p < 0.01$, respectively) higher in the CM-treated than in the control group. However, intracellular $[Ca^{2+}]_c$ in the cells was significantly lower in the 2-APB ($p < 0.05$) and verapamil+diltiazem ($p < 0.01$) groups than in CM. Hence, we found that 2-APB and verapamil+diltiazem induced protective effects against fMLP-induced $[Ca^{2+}]_i$ release. Intracellular $[Ca^{2+}]_c$ in the cells was also significantly ($p < 0.05$) lower in the 2-APB ($p < 0.05$) than in the verapamil+diltiazem group.

Effects of CM on Lipid Peroxidation Levels in Serum and Neutrophil

The effects of CM on LP levels in neutrophils and serum are shown in Tables 1 and 2, respectively. Lipid peroxidation levels in neutrophils and serum were significantly ($p < 0.05$) higher in the CM group than in control. Lipid peroxidation levels were significantly lower in the 2-APB ($p < 0.05$) and verapamil+diltiazem ($p < 0.001$) groups than in the CM group. Hence, we found that 2-APB and verapamil+diltiazem induced protective effects against oxidative stress–induced lipid peroxidation levels in patients treated with CM.

Effects of CM on GSH-Px, GSH, Vitamins A, C and E and β-Carotene

The effects of CM on GSH-Px, GSH, vitamin A, vitamin C, vitamin E and -carotene values in neutrophils and serum are shown in Tables 1 and 2, respectively. Serum vitamin E ($p < 0.05$) and -carotene ($p < 0.01$) concentrations were

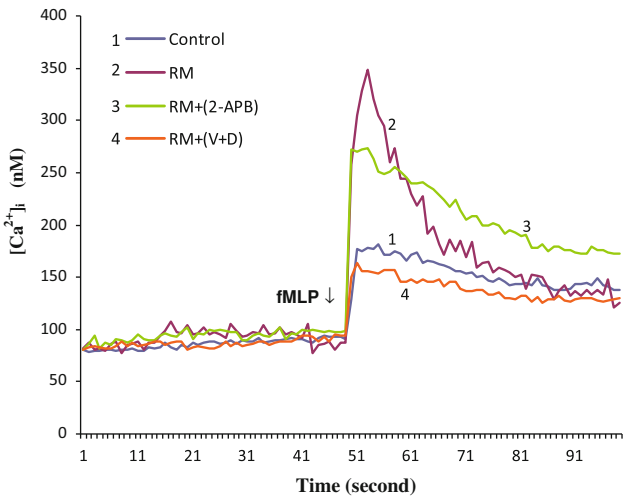

Fig. 1 Effects of non-ionic contrast media on $[Ca^{2+}]_i$ concentrations in neutrophils of control (pretreatment) and treatment patients. Stimulation was performed by fMLP (1 M). *RM* radiocontrast media, *V+D* verapamil+diltiazem, *2-APB* 2-aminoethoxydiphenyl borate, *fMLP* *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine

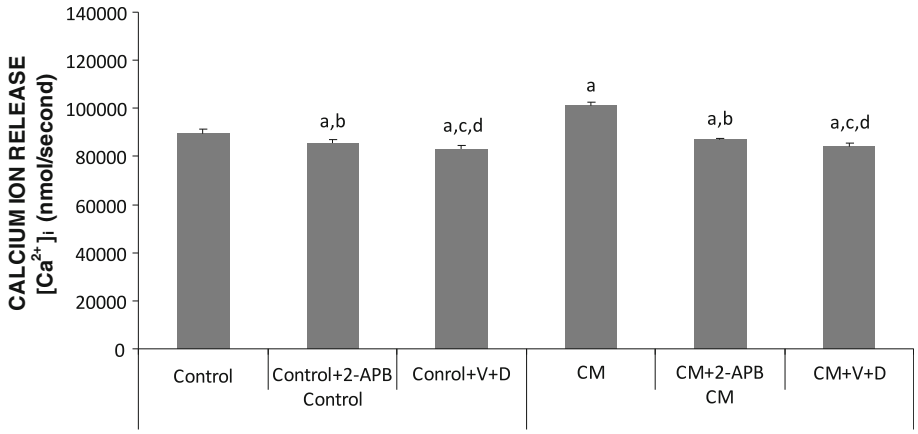

Fig. 2 Effects of non-ionic contrast media on $[Ca^{2+}]_i$ concentrations in neutrophils of control (pretreatment) and treatment patients (mean ± SD, $n = 10$). [a]$p < 0.05$ and [b]$p < 0.01$ vs. control, [c]$p < 0.01$ vs. contrast media (*CM*) group, [d]$p < 0.05$ vs. 2-aminoethoxydiphenyl borate (*2-APB*) group. *V+D* verapamil+diltiazem

Table 1 Effects of non-ionic radiocontrast media (RM), 2-APB and verapamil+diltiazem (V+D) on neutrophils lipid peroxidation (LP), reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) in control and treatment (CM) groups ($n = 10$, mean ± SD)

Parameters	Control	CM	CM+2-ABP	CM+V+D
LP (nmol/g protein)	6.02 ± 0.86	6.42 ± 1.03[a]	6.19 ± 1.70[b]	5.13 ± 0.34[c,e]
GSH (nmol/g protein)	2.29 ± 0.38	2.03 ± 0.36[a]	2.26 ± 0.40[b]	2.28 ± 0.37[b]
GSH-Px (IU/g protein)	5.20 ± 1.60	4.02 ± 1.37[a]	3.92 ± 1.43[a]	4.52 ± 1.23[b,d]

[a] $p < 0.05$ vs. control

[b] $p < 0.05$ and [c] $p < 0.001$ vs. RM

[d] $p < 0.05$ and [e] $p < 0.01$ vs. RM+2-APB

Table 2 Effects of non-ionic radiocontrast media (RM) on serum lipid peroxidation (LP) and antioxidant vitamin levels in pretreatment (control) and treatment (CM) groups ($n = 10$, mean ± SD)

Parameters	Control	CM
LP (nmol/ml)	1.34 ± 0.15	1.49 ± 0.20*
Vitamin A (μmol/l)	2.54 ± 0.21	2.70 ± 0.44
β-carotene (μmol/l)	1.54 ± 0.27	1.14 ± 0.18**
Vitamin C (μmol/l)	103.30 ± 20.35	124.90 ± 23.41
Vitamin E (μmol/l)	7.60 ± 0.94	7.12 ± 0.52*

* $p < 0.05$ and ** $p < 0.01$ vs. control

Table 3 Effects of radiocontrast media (RM) on apoptosis and caspases 3 and 9 in neutrophils of control (pretreatment) and treatment (CM) groups ($n = 10$, mean ± SD)

Parameters	Control	CM
Apoptosis (OD/mg protein)	7.96 ± 1.50	8.46 ± 1.56*
Caspase-3 (OD/mg protein)	915.09 ± 173.89	919.14 ± 178.28
Caspase-9 (OD/mg protein)	157.95 ± 28.89	162.43 ± 32.96

OD optic density

* $p < 0.05$ vs. control

significantly lower in the CM group than in the control. GSH and GSH-Px values were also significantly lower in the CM group than in the control, although neutrophil GSH and GSH-Px values were significantly ($p < 0.05$) higher in the CM+2-APB and CM+verapamil+diltiazem groups than in the CM group. However, serum vitamin A and vitamin C values in the two groups did not change significantly.

Effects of CM on Apoptosis and Caspases 3 and 9

The effects of CM on apoptosis and caspases 3 and 9 values are shown in Table 3. Apoptosis levels were significantly ($p < 0.05$) higher in the CM group than in control, although the activities of caspases 3 and 9 did not change within the two groups.

Discussion

The most important finding of this study is that serum and neutrophil lipid peroxidation levels expressed as MDA as well as neutrophil $[Ca^{2+}]_i$ values were found to be elevated in patients treated with CM compared to pretreatment, whereas serum vitamin E and -carotene concentrations in the CM-treated group were decreased by CM exposure. Lipid peroxidation and $[Ca^{2+}]_i$ values were reduced to control levels by the presence of Ca^{2+} channel blockers. To our knowledge, this is the first comparative study of the effect of CM on $[Ca^{2+}]_i$ release and oxidative/antioxidant systems in human neutrophils. There is evidence that inflammatory CM might impair antioxidant defense and increase oxidative stress.

CM is characterized by activation of the inflammatory response system with increased production of procytokines (Böhm et al. 2011). Lipid peroxidation is a major oxidative degradation product of membrane unsaturated fatty acid and has been shown to be biologically active with ROS properties (Kovacic and Somanathan 2008; Traber and Stevens 2011). In the present study, exposure to CM enhanced lipid peroxidation levels in neutrophils and serum of the human system. In diagnostic computer tomography, the upregulation of neutrophil phagocyte activity and ROS overproduction through oxidative burst were observed in cerebrospinal fluid of radiocontrast media–treated premature infants (Inder et al. 2002). The overproduction of ROS leads to an increase in lipid peroxidation and interferes with the structure and ratio of polyunsaturated fatty acids (Nazıroğlu 2007b), to cause loss of fluidity of the biological membrane. As a result of those alterations, the membranes induce cytokine production (Freitas et al. 2009).

In addition, elevation of lipid peroxidation induces phospholipase A_2, which changes receptor functions in the cell membranes, induces immune cells, leads to secretion of interleukins from T cells (Bréchard and Tschirhart 2008) and may further increase lipid peroxidation. 2-APB is an antagonist of store-operated and TRPM cation channels in neutrophils (Anderson et al. 2005; Salmon and Ahluwalia 2010; Nazıroğlu 2011), although verapamil and diltiazem are antagonists of both high and low voltage-gated Ca^{2+} channels. Treatment with the 2-APB and verapamil+diltiazem drugs effectively protected neutrophils against CM-induced damage, as shown by increased serum vitamin E and -carotene levels and decreased lipid peroxidation levels in the serum and neutrophils of patients. It has been reported that 2-APB and verapamil+diltiazem treatments may suppress immune cells including T cells and neutrophils (Anderson et al. 2005; Salmon and Ahluwalia 2010). Suppression of immune cells by treatment with 2-APB and verapamil+diltiazem may decrease oxidative stress.

ROS act as subcellular messengers in such complex processes as mitogenic signal transduction, gene expression and regulation of cell proliferation when they are generated excessively or when enzymatic and nonenzymatic defense systems are impaired (Nazıroğlu 2007b). The major intracellular antioxidant enzyme GSH-Px detoxifies H_2O_2 to water and removes organic hydroperoxides (Traber and Stevens 2011). Phagocytic cells are very vulnerable to oxidative stress because of their elevated consumption of oxygen and the consequent generation of large amounts of ROS (Freitas et al. 2009). The results of the current study provide evidence of a decrease in GSH, GSH-Px, vitamin E and β-carotene levels in response to CM treatment.

One of the most important intracellular antioxidant systems is the GSH redox cycle (Perricone et al. 2009). GSH is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in cell metabolism (Nazıroğlu 2009). The exact mechanisms of ischemia/reperfusion and phagocytic cell–induced changes in neutrophil GSH concentrations have not been completely elucidated. Thus, GSH may modulate metal reduction, and the thiol portion is very reactive with several chemical compounds, mainly with superoxide radical agents (Arthur et al. 2003). In other words, this may be due to the fact that ischemia/reperfusion injury causes a significant decrease in cysteine and cystine in phagocytic cells because cysteine is the rate-limiting precursor for glutathione synthesis (Perricone et al. 2009). In this study, GSH levels were significantly lower in the CM group than in controls. The decrease in GSH concentration in the liver as a result of ischemia/reperfusion injury may account for the increased lipid peroxidation and oxidative stress.

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 2007b). Ca^{2+} mobilizing agonists can effectively produce a rapid, simultaneous and reversible cessation of the movements of both endoplasmic reticulum and mitochondria, which is strictly dependent on a rise in $[Ca^{2+}]_i$. This inhibition in mitochondrial motility reflects increased mitochondrial Ca^{2+} uptake and, thus, enhances the local Ca^{2+} buffering capacities of mitochondria, with important consequences for signal transduction (Rathore et al. 2008). Ca^{2+} overloading in mitochondria can induce an apoptotic program by stimulating the release of apoptosis-promoting factors like cytochrome *c* and by generating ROS due to respiratory chain damage (Espino et al. 2011; Uğuz et al. 2012). 2-APB and verapamil+diltiazem have a direct regulatory effect on SOC and L-type voltage-gated Ca^{2+} channels in phagocytic cells, respectively (Anderson et al. 2005; Salmon and Ahluwalia 2010). Our results indicated that blockade of both Ca^{2+} uptake into mitochondria with 2-APB and verapamil+diltiazem rises in $[Ca^{2+}]_i$ concentrations in the CM group was able to decrease apoptosis and lipid peroxidation mediated by oxidative stress, which were able to release Ca^{2+} from intracellular stores. On the contrary, Schick et al. (2002) reported that the ionic CM agents diatrizoate and ioxaglate, but not the nonionic compounds iohexol and iodixanol, decreased $[Ca^{2+}]_i$ concentrations when incubated with Madin-Darby canine kidney cells.

In conclusion, we are the first to indicate that iodinated CM induced antioxidant reduction and affected Ca^{2+} release in neutrophils and serum of patients with migraine. We suggest that the persistent activation of neutrophils may be the cause of oxidative damage. However, treatment with the Ca^{2+} channel antagonists 2-APB and verapamil+diltiazem protected against oxidative stress and

preserved the antioxidant redox system and Ca^{2+} release in neutrophils of patients. Antioxidant systems may be improved in patients given non-ionic CM by vitamin E and β-carotene supplementation, but this suggestion needs to be investigated.

Acknowledgements The authors declare that there are no conflicts of interest. M.K. formulated the present hypothesis, and M.N. was responsible for writing the report. İ.S.Ö., M.A. and A.C.U. were responsible for data analysis. A.S. and V.A.Y. made critical revisions to the manuscript. The authors thank Dr. Peter J. Butterworth (King's College, London, UK) for polishing the English of the manuscript.

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