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Activation of the JAK/STAT pathway in human neutrophils by NDMA

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Abstract: The study objective was to assess the role of the JAK/STAT pathway in the induction of nitric oxide (NO) synthesis with the involvement of inducible nitric oxide synthase (iNOS) and in the generation of superoxide anion radical by human neutrophils (PMNs) exposed to N-nitrosodimethylamine (NDMA). Isolated PMNs were incubated in the presence of NDMA. The expression of the study proteins in the cytoplasmic and nuclear fraction was determined by western blotting. Total NO was assayed in cell cultures by Griess reaction. The ability of PMNs to generate superoxide anion radical was assessed using the cytochrome-c reduction test. The current results indicate that NDMA potentiates the expression of iNOS and phospho-JAK2 in the cytoplasmic fraction of human PMNs. Moreover, it increases the expression of phospho-STAT1 and phospho-STAT3 in both fractions of the cells studied. PMNs treated with NDMA showed greater capability to release NO and superoxide anion radical, which was reduced after administration of the JAK/STAT pathway inhibitor. The current findings indicate that NDMA in PMNs activates the JAK/STAT pathway, which plays a role in the production of NO with the involvement of iNOS and in the generation of superoxide anion radical by these cells.

Key words: Neutrophils, N-nitrosodimethylamine, inducible nitric oxide synthase, nitric oxide, superoxide anion radical, JAK/STAT

1. Introduction

Nitrosamines are compounds of very high biological activity. Among them is N-nitrosodimethylamine (NDMA), a xenobiotic with widespread occurrence in the human environment. NDMA is used in certain technological processes in the plastic, metallurgical, and rubber industries. It is present in cigarettes and tobacco smoke, processed food, heat-treated food, and pickled products. The levels of NDMA noted in food samples range from tiny fractions to several micrograms per kilogram, which, according to some authors, poses a real risk to health (Tricker and Preussmann, 1991; Dobo et al., 1997; Zeilmaker et al., 2010).

High consumption of the precursors, amines and nitrosating agents, leads to endogenous production of N-nitrosamines. The intraorganic biosynthesis of NDMA involves secondary amines (e.g., dimethylamine) and tertiary amines (e.g., N,N-dimethyl-p-hydroxyphenyl-ethylamine) (Waller and Goscinski, 1999). Reaction kinetics for nitrosation are affected by pH, amine alkalinity, and temperature. A study conducted by Jablonski et al. (2006) showed maximum nitrosylation velocity at a pH of 3–4.5. The level of hydrogen ions in this range is most common in the acidic environment of the stomach or in an inflammatory focus.

Up to now, observations have shown that NDMA exerts a toxic action, inducing morphological and functional disorders in many tissues and organs (Aiub et al., 2011). Its mutagenic and cancerogenic effects have also been proven (Glavin et al., 2008). This xenobiotic has been found to influence the activity of the immune cells, such as neutrophils (PMNs), which are a key source of reactive oxygen and nitrogen species, including superoxide anion radical and nitric oxide (NO) (Ratajczak-Wrona et al., 2011).

NO produced in PMNs can interact with superoxide anion radical released by these cells during phagocytosis to form toxic peroxynitrite (V) (Sohn et al., 2003; Chanda et al., 2013; Demir et al., 2013). This compound is converted to nitrate ion (III) and shows potential to nitrosate systemic amines, which in consequence leads to the synthesis of nitrosoamines in the body (Vermeer et al., 2001).

Inducible nitric oxide synthase (iNOS) is one of the enzymes responsible for the synthesis of NO (Demirci et al., 2001; Konuk et al., 2012). Its activity is regulated by various signaling pathways, e.g., mitogen-activated protein kinases

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(MAP kinases), which affect the expression of numerous transcription factors (Bek et al., 1999; Lirk et al., 2002). Our previous study revealed the role of MAP kinases p38 and JNK in the activation of transcription factors, such as c-Jun and NF-κB p65, engaged in the regulation of iNOS expression in human PMNs exposed to NDMA in healthy subjects (Ratajczak-Wrona et al., 2013a).

There have been some reports that NO synthesis with the involvement of iNOS is also controlled by signaling pathways other than MAP kinases. Sareila et al. (2006) demonstrated that the blockade of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway inhibits iNOS expression in the IFN-γ stimulated macrophages. Similar observations were reported by Kleinert et al. (1998) in epithelial cells stimulated with a cytokine mixture (IFN-γ, TNF-α, and IL-1β).

The JAK/STAT pathway has been shown to also be activated as a result of cell stress triggered by reactive oxygen species, apart from cytokines and growth factors (Freitas et al., 2010). The JAK kinases include JAK1, JAK2, JAK3, and Tyk2; JAK2 kinase is considered to be the most conservative isoform of this family (Yamaoka et al., 2004). The activation of JAK kinases causes phosphorylation of tyrosine or serine residues in the C-terminal domain of STAT proteins. This enables homo- and heterodimerization of STAT proteins, with the involvement of SH2 domains and their active transport to the cell nucleus, where STAT dimers bind to interferon activated site (GAS) or interferon stimulated gene (ISG) sequences in the promoter regions of genes and activate their transcription (Chatterjee-Kishore et al., 2000; Murray, 2007). Signaling processes of the JAK/STAT pathway may also undergo negative regulation, mainly via dephosphorylation of STAT factors and synthesis of specific inhibitors of the protein inhibitors of activated STAT (PIAS) or suppressors of the cytokine signaling (SOCS/SSI/JAB) family. The ultimate action of the stimulator seems to be the result of opposing and at the same time activated signal transmission pathways (Greenhalgh and Hilton, 2001; Levy and Darnell, 2002).

The STAT type proteins include STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. It is thought that the transcription factors STAT1 and STAT3 have opposite functions in the regulation of cell proliferation, differentiation, and apoptosis and in the induction of the immune response to tumor antigens (Ihle, 2001; Levy and Darnell, 2002).

The present study objective was to assess the role of the JAK/STAT pathway in the induction of NO synthesis with the involvement of iNOS and in the generation of superoxide anion radical by PMNs exposed to NDMA.

Since the activation of the JAK/STAT pathway is manifested by the presence of phospho-JAK2 kinase, phospho-STAT1, and phospho-STAT3 proteins, their expression may either confirm or exclude the involvement of this signaling pathway in iNOS activation.

The knowledge of the expression of the STAT transcription factors, e.g., STAT1 and STAT3 induced by JAK2 kinase, in both the cytoplasmic and nuclear fraction of PMNs exposed to NDMA will help fully elucidate the basic molecular mechanism of action of this xenobiotic on the leukocyte population.

2. Materials and methods

2.1. Reagents

NDMA, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), cadmium, Griess reagent, cytochrome-c, superoxide dismutase (SOD), and BCIP/NBT Liquid Substrate System were purchased from Sigma (Steinheim, Germany). (E)-N-Benzyl-2-cyano-3-(3,4-dihydroxyphenyl)acrylamide (AG490) was obtained from Calbiochem (San Diego, CA, USA). Laemmli buffer, Tris-buffered saline (TBS)/casein buffer, and TBS-T (containing Tris-buffered saline and Tween 20) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Antibodies

Monoclonal mouse antibody against the iNOS and beta-actin, and polyclonal goat antibodies against phospho-JAK2, phospho-STAT1, and phospho-STAT3 protein were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Goat antimouse IgG antibody conjugated with alkaline phosphatase (AP) and donkey antigoat IgG antibody conjugated with alkaline phosphatase were obtained from Vector Laboratories (Burlingame, CA, USA). Monoclonal mouse antibody against the poly(ADP-ribose)polymerase (PARP-1) was purchased from Calbiochem.

2.3. Isolation and incubation of PMNs

The study involved a group of 20 healthy people, aged from 25 to 40 years old, who were volunteer blood donors from the Regional Centre for Transfusion Medicine in Bialystok, Poland. Males were chosen to avoid possible influences due to endogenous hormones on the experimental findings. The study was approved by the Ethics Committee of the Medical University of Bialystok (R-I-002/11/2010) and all persons gave written informed consent.

PMNs were isolated from heparinized (10 U/mL; Heparin, Polfa, Lodz, Poland) whole blood by density centrifugation using Gradisol G gradient, 1.115 g/mL (Polfa) (Zeman et al., 1988). Sera were obtained from blood samples collected without anticoagulant agents. The PMNs were further purified by positive selection using the MidiMACS Separation System (Miltenyi Biotec, Bergisch Gladbach, Germany) that employs MicroBeads conjugated to monoclonal antihuman CD16 antibodies.

PMNs were suspended at a concentration of 5×10^6 cells/mL in Hanks' Balanced Salt Solution 1X (Invitrogen, Carlsbad, CA, USA) containing the subject's own serum (7.4%, 20/270 µL), 100 U penicillin/mL, and 50 ng streptomycin (Polfa Trachomin SA, Warsaw, Poland). The cells (200 µL aliquots) were then placed into wells of microplates (Microtest III-Falcon, BD Biosciences, Bedford, MA, USA) and incubated for 2 h at 37 °C in a 5% CO_2 incubator (NuAire, US AutoFlow, Plymouth, MN, USA). PMNs in the wells were then treated with 20 µL NDMA to attain a final concentration of 0.74 µg/µL in the well; control wells received vehicle only. The PMNs were then cultured a further 2 h before supernatants were collected. Assessments of viability (via trypan blue exclusion) showed that PMNs were >93% viable after the treatment.

In order to determine the role of JAK/STAT pathway in the regulation of iNOS expression in PMNs exposed to NDMA, selective inhibitors of this pathway were used. In these studies, cells were preincubated with 30 µM, 50 µM, or 100 µM AG490, a selective JAK/STAT pathway inhibitor, for 1 h before the addition of NDMA. Preliminary studies showed that the presence of the inhibitor did not affect cell viability.

2.4. Cellular toxicity assay

Cytotoxic effect of NDMA on neutrophils was analyzed by using the tetrazolium-based colorimetric assay (MTT reduction assay). The MTT assay is dependent upon the ability of mitochondrial dehydrogenase in living cells to reduce the yellow MTT to a blue formazan product. It has been shown that an increase in absorbance values correlates to an increase in cellular enzymatic activity and cell number. PMNs were plated in 96-well microplates at 1×10^6 cells/well in 0.2 mL of medium without NDMA or at increasing doses of the xenobiotic: 1 µg/µL, 2 µg/µL, 3 µg/µL, and 4 µg/µL. After 2 h of incubation, 20 µL of stock MTT solution (5 mg/mL) was added to each culture well and plates were incubated for 1 h at 37 °C in a 5% CO_2 incubator. Plates were then centrifuged, the medium was aspirated, and 200 µL of DMSO was added to lyse the cells and dissolve the MTT formazan crystals. Plates were mechanically agitated, and absorbance was measured at 570 nm by using a UVN-340 ASYS Hitech GmbH microplate reader (Biogenet, Eugendorf, Austria). Experiments were performed in triplicate, and data are expressed as the percentage of MTT reduction compared to control cells (unexposed PMNs). Metabolic activity decrease of studied leukocytes was observed in cells incubated with NDMA at >0.74 µg/µL concentration (Figure 1). This proved the cytotoxic effect of this compound.

2.5. Protein isolation and western blotting

Cytoplasmic and nuclear extracts from PMNs (3×10^6 cells total/sample) were prepared using the NucBuster Protein Extraction Kit (Calbiochem). Stepwise extraction delivered 2 distinct cellular protein fractions: cytoplasmic and nuclear. The concentration of protein in each was determined with a Qubit Protein Assay Kit (Invitrogen). An antibody against PARP-1 (1:5000) and against beta-actin (1:100) was used as an internal control within the nuclear and cytoplasmic fractions, respectively.

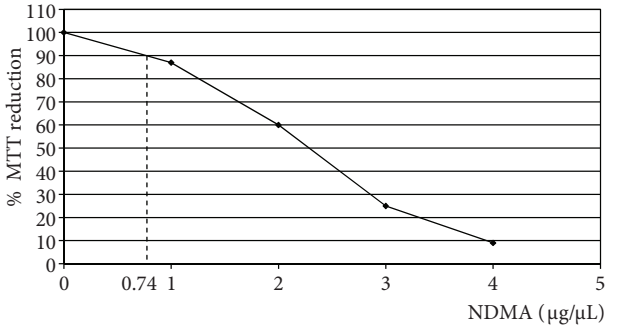

Figure 1. Cytotoxic activity of NDMA, evaluated by the MTT assay. Data shown are means of 5 independent experiments.

The extracts were suspended in Laemmli buffer, loaded at 20 µg/well, and then electrophoresed over a 4% stacking and 10% separating SDS-PAGE gel. The resolved proteins were electrotransferred onto 0.45-µm pore-size nitrocellulose membranes (Bio-Rad). These were then blocked with TBS/casein buffer, washed with TBS-T (TBS containing 0.05% Tween-20), and incubated with Qentix Western Blot Signal Enhancer (Thermo Fisher Scientific, Rockford, IL, USA). The membranes were then incubated for 10 min at room temperature in SNAP (Protein Detection System; Millipore, Billerica, MA, USA) with 1:100 dilutions of primary monoclonal antibody against iNOS and primary polyclonal antibodies against phospho-JAK2, phospho-STAT1, or phospho-STAT3. After washing with 0.1% TBS-T, the membrane was incubated at room temperature with alkaline phosphatase antimouse IgG Ab or antigoat IgG Ab (1:200). Immunoreactive protein bands were then visualized using the BCIP/NBT Liquid Substrate System, and intensities were determined using ImageJ software (NIMH, Bethesda, MD, USA) and reported as Arbitrary Units (AU).

2.6. Assay for nitrite production

Synthesis of NO was determined by an assay of the culture supernatant for nitrite, a stable reaction product of NO with molecular oxygen. Total NO concentration is commonly determined as a sum of nitrite and nitrate concentrations. NO production by PMNs was determined using an indirect method based on measurement of nitrite concentration in culture supernatants according to Griess' reaction (Schulz et al., 1999). In the samples analyzed, nitrate was reduced to nitrite in the presence of cadmium and then converted to nitric acid that yielded a

color reaction with Griess reagent. Nitrite concentrations were determined by spectrophotometric analysis at 540 nm with extrapolation from a standard curve prepared in parallel. Nitric oxide products were expressed as μM/10^6 cells in 270 μL of supernatant.

2.7. Measurement of superoxide anion radical generation

"Oxygen burst" in neutrophils was explored by detecting the production of superoxide anion radical by these cells according to McCord's (1969) method with Bhuyan's modification, based on differences between light absorbances of solutions containing nonreduced and reduced cytochrome-c. Cytochrome-c does not permeate through the plasma membrane, and thus its reduction by superoxide anion radical in PMN supernatants indicates superoxide anion radical release outside of the cell.

Cytochrome-c solution in phosphate buffer (KH_2PO_4/K_2HPO_4), pH 7.8, containing 0.1 mM EDTA, was added to 2 parallel samples with isolated neutrophils. Cytochrome-c concentration was 15 mg/mL. Superoxide dismutase, with 5000 U/mL activity, was added to the reference sample, while buffer was added to the study sample. After addition of NDMA to both test tubes, the samples were incubated at 37 °C, and then absorbance was read at 550 nm in the presence of deionized water. The generation of superoxide anion radical, expressed as nmol of cytochrome-c reduced per time, was calculated by using an absorbance coefficient of 21.1 $mmol/L^{-1}$ cm^{-1} at 550 nm.

2.8. Statistics

Results were analyzed using Statistica 9.1. (StatSoft, Inc., Tulsa, OK, USA). Data distribution normality was determined using a Kolmogorov–Smirnov test. Since data were not normally distributed, for comparison of variations between assayed groups, Mann–Whitney U nonparametric tests were applied to unrelated results. $P \leq 0.05$ was accepted as statistically significant. All data are presented as mean ± SE.

3. Results

3.1. Assessment of protein expression by western blotting

Figure 2 presents the expressions of iNOS, phospho-JAK2, phospho-STAT1, and phospho-STAT3 in PMNs. The exposure of PMNs to NDMA led to a simultaneous increase in the expression of iNOS and phospho-JAK2 in the cytoplasmic fraction as compared to the cells incubated without the xenobiotic. Higher expressions of phospho-STAT1 and phospho-STAT3 were also observed, both in the cytoplasmic and the nuclear fractions of these cells. The expressions of phospho-STAT1 and phospho-STAT3 in the cytoplasmic fraction of PMNs not exposed and exposed to NDMA were higher than in the nuclear fraction of the cells.

In order to confirm the involvement of the JAK/STAT pathway in the induction of iNOS expression in PMNs treated with NDMA, a selective inhibitor of this pathway was used in the experiment. In the presence of the JAK/STAT pathway inhibitor, only at a concentration of 50 μM, iNOS expression was lower in the cytoplasmic fraction of PMNs exposed to NDMA as compared to the cells without the inhibitor. When the inhibitor concentrations of 30 μM, 50 μM, and 100 μM were used, a reduction was noted in the expression of phospho-JAK2 in the cytoplasmic fraction of PMNs treated with NDMA as compared to the cells without the inhibitor. Since AG490 reduced the expression of both iNOS and phospho-JAK2 in PMNs exposed to NDMA only at a concentration of 50 μM, the phospho-STAT proteins were assessed in the 2 fractions using this concentration. The expressions of phospho-STAT1 and phospho-STAT3 were lower both in the cytoplasmic and the nuclear fractions of the cells studied as compared to the cells without the inhibitor.

3.2. Assessment of the level of total NO in PMNs supernatants

Figure 3 shows the levels of total concentration of NO in PMNs supernatants. The exposure of PMNs to NDMA confirmed earlier observations and showed enhanced release of nitric oxide as compared to the cells incubated without the xenobiotic (Ratajczak-Wrona et al., 2013b).

In order to determine the role of the JAK/STAT pathway in the production of nitric oxide with iNOS by PMNs exposed to NDMA, its concentration was assessed in the presence of the selective inhibitor. PMN supernatants preincubated with the JAK/STAT pathway inhibitor at a concentration of 50 μM and then incubated in the presence of NDMA showed lower levels of total NO than the supernatants without the inhibitor. However, as in the case of iNOS, no changes were found in the levels of total NO in the supernatants of PMNs preincubated with the JAK/STAT pathway inhibitor at the concentrations of 30 μM and 100 μM.

3.3. Generation of superoxide anion radical by PMNs

Figure 4 presents the generation of superoxide anion radical by PMNs. PMNs exposed to NDMA were found to show higher potential to release superoxide anion radical than the cells incubated without the xenobiotic, which is consistent with previous findings (Ratajczak-Wrona et al., 2013c).

In order to examine the involvement of the JAK/STAT pathway in the production of superoxide anion radical by PMNs exposed to NDMA, its generation was assessed in the presence of the pathway inhibitor. Contrary to iNOS expression and NO production, the presence of the AG490 inhibitor at concentrations of 50 μM and 100 μM resulted in a decreased release of superoxide anion radical by PMNs exposed to NDMA.

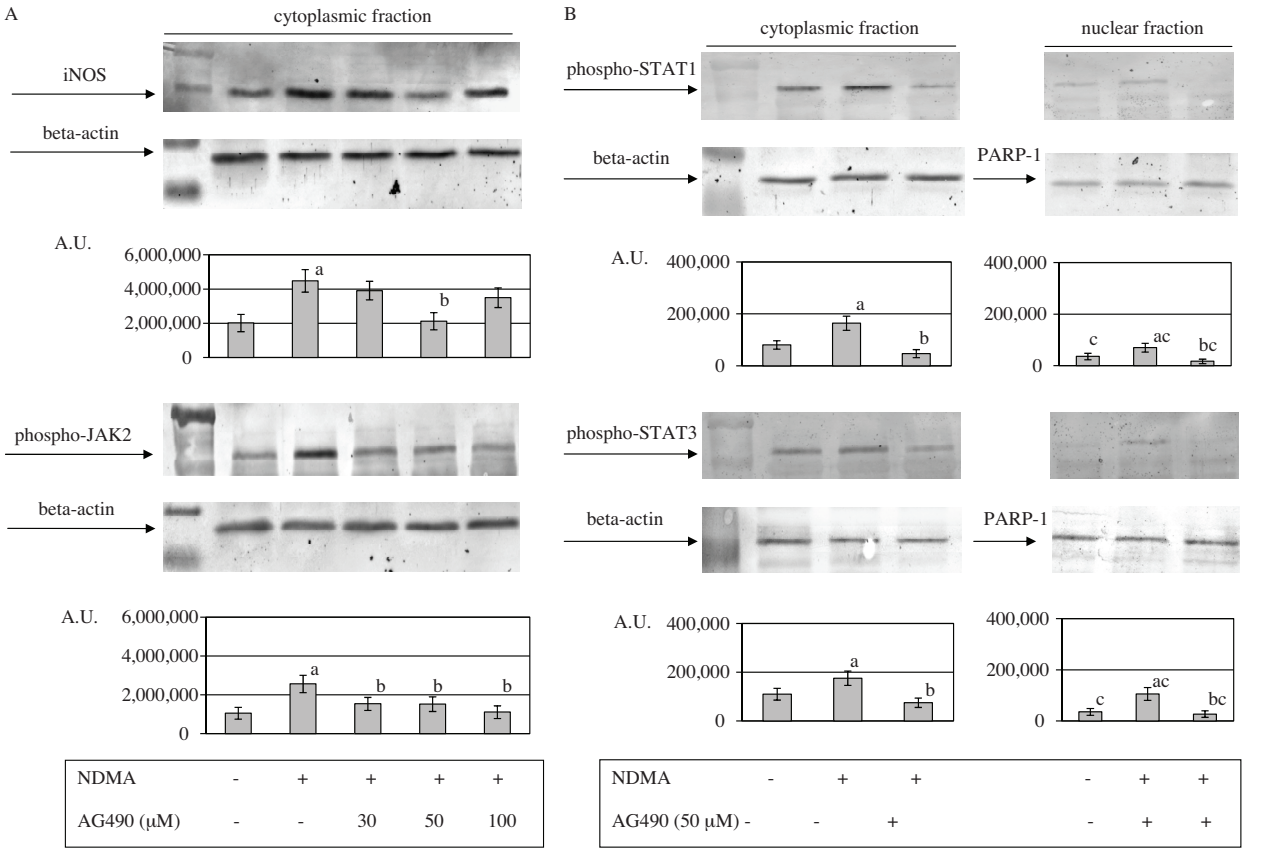

Figure 2. Expressions of iNOS, phospho-JAK2, phospho-STAT1, and phospho-STAT3 in PMNs. (A) PMNs were treated with or without AG490 (30 μM, 50 μM, or 100 μM) for 1 h before addition of NDMA (0.74 μg/μL). The cytoplasmic fractions obtained from those cells were used to detect iNOS and phospho-JAK2 protein levels by western blotting. The results shown are representative of 5 independent experiments. Band intensity was quantified using ImageJ software and expressed in AU. Data shown are mean ± SE of 5 independent experiments. a - Value significantly different between cells without and with NDMA ($P < 0.05$); b - value significantly different between cells treated with NDMA but preincubated without or with the inhibitor ($P < 0.05$). (B) PMNs were treated with or without AG490 (50 μM) for 1 h before addition of NDMA (0.74 μg/μL). The cytoplasmic and nuclear fractions obtained from those cells were used to detect phospho-STAT1 and phospho-STAT3 protein levels by western blotting. Band intensity was quantified using ImageJ software and expressed in AU. Data shown are mean ± SE of 5 independent experiments. a - Value significantly different between cells without and with NDMA ($P < 0.05$); b - value significantly different between cells treated with NDMA but preincubated without or with the inhibitor ($P < 0.05$); c - value significantly different between cytoplasmic and nuclear fractions ($P < 0.05$).

4. Discussion

The current findings and previous observations confirm a substantial effect of NDMA on iNOS expression and NO production by human PMNs (Ratajczak-Wrona et al., 2013b). The increase observed in the expression of phospho-JAK2 in the cytoplasmic fraction and in the expression of phospho-STAT1 and phospho-STAT3 in both the cytoplasmic and nuclear fractions of PMNs treated with NDMA indicates that the xenobiotic activates the JAK/STAT pathway in these cells.

The transport of STAT proteins to the nucleus from the cytoplasm depends on the binding of specific importins on their surface (STAT1 binds to α5 and β1, whereas STAT3 binds to α3, α6, and β1). As a result, STAT proteins are transported to the cell nucleus by nuclear pores (Reich and Liu, 2006).

Reports are available on the nuclear pool of STAT proteins as phosphorylated proteins. According to Haspel and Darnell (1999), STAT proteins are transported to the cell nucleus from the cytoplasm only as a result of their activation and eventual phosphorylation. Other reports provide different data on localization of the STAT protein. Sehgal et al. (2003) showed that the majority of the phosphorylated STAT1 and STAT3 proteins remain in the cytoplasm of human hepatoma Hep3B cells stimulated with cytokines (IFN-γ or IL-6) during both short- and long-term incubation. The above data can explain the higher expression of the phosphorylated STAT proteins in the cytoplasmic fraction observed in the current study in comparison with the nuclear fraction of PMNs exposed to NDMA.

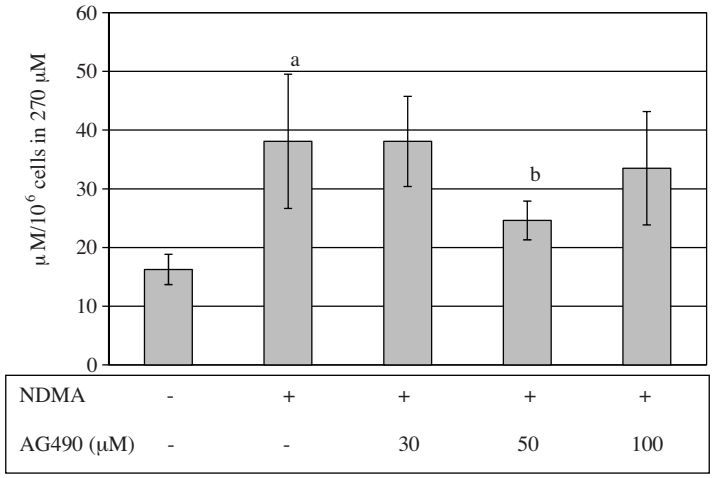

Figure 3. Concentrations of total NO from PMNs. PMNs were treated with or without AG490 (30 µM, 50 µM, or 100 µM) for 1 h before addition of NDMA (0.74 µg/µL). Two hours after addition of NDMA, the nitrite concentrations were measured as a marker of NO production. a - Value significantly different between cells without and with NDMA ($P < 0.05$); b - value significantly different between cells treated with NDMA but preincubated without or with the inhibitor ($P < 0.05$). Data are expressed as µM/10^6 cells (in 270 µL of supernatant) and are shown as mean ± SE of 20 experiments.

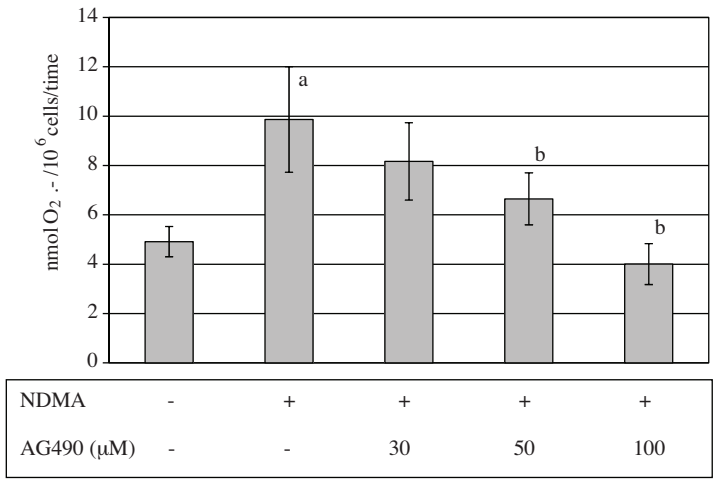

Figure 4. Superoxide anion radical generation ($O_2^{\cdot -}$) for PMNs. PMNs were treated with or without AG490 (30 µM, 50 µM, or 100 µM) for 1 h before addition of NDMA (0.74 µg/µL). Cells were incubated for 2 h and then analyzed for superoxide anion radical generation. a - Value significantly different between cells without and with NDMA ($P < 0.05$); b - value significantly different between cells treated with NDMA but preincubated without or with the inhibitor ($P < 0.05$). Data are expressed nmol $O_2^{\cdot -}$/10^6 cells/time and are shown as mean ± SE of 20 experiments.

The experiments conducted in our laboratory did not show any differences in the expression between phospho-STAT1 and phospho-STAT3 in any of the cell fractions examined. However, the available data on the human hepatoma Hep3B and HepG2 cells indicate quantitative differences between the STAT1 and STAT3 proteins depending on localization in the cell. It is suggested that STAT3 can accumulate in the nucleus not only as a phosphorylated homodimer but also irrespective of its phosphorylation status. Moreover, the phosphorylated STAT3 transcription factor can also form a heterodimer with phosphorylated STAT1, which in consequence may lead to enhanced nuclear accumulation of STAT3 as a result of the action of STAT1-activating factors on the cell (Pranada et al., 2004; Liu et al., 2005; Reich and Liu, 2006).

Based on the presented changes in NO production and iNOS expression in the presence of AG490 in PMNs exposed to NDMA, it can be assumed that the synthesis of nitric oxide with the involvement of iNOS in these cells is associated with the JAK/STAT pathway activation.

Similar results were obtained by Schmidt et al. (2010), who reported that the JAK/STAT pathway inhibition in chondrocytes stimulated with a mixture of cytokines (IFN, IL-1, and TNF) resulted in a decrease in iNOS expression.

Different findings were presented by Ravichandran et al. (2011), who, after administration of the selective inhibitor AG490, found neither lower expression of iNOS and STAT nor reduced NO production in IL-1β–stimulated human lung cancer cells. They suggested that the lack of changes in iNOS expression and NO production by these cells may result from the involvement of another signaling pathway, e.g., MAP kinases or the NF-κB pathway.

Dai et al. (2009), in a study on IFN-stimulated splenocytes from mice, demonstrated that the involvement of the NF-κB pathway synergistic to the JAK/STAT pathway is essential for the induction of iNOS expression in these cells.

However, other available data seem to indicate that the JAK/STAT pathway inhibitor exerts no effect on the activation of other pathways, including NF-κB (Nakashima et al., 1999). Our findings showed a reduction in iNOS expression and NO production by PMNs exposed to NDMA after AG490 application only at a concentration of 50 μM, and not at a higher concentration (100 μM). This can be due to the activation of other signaling pathways engaged in NO production with the involvement of iNOS by PMNs treated by NDMA (Ratajczak-Wrona et al., 2011). Moreover, a study by Nakashima et al. (1999) demonstrated complete inhibition of NO production by rat mesangial cells only after application of a mixture containing AG490 at a concentration of 300 μM and herbimycin A (HerA; 1000 ng/mL), the antibiotic inhibiting the activity of tyrosine kinases.

The differences in results concerning the role of the JAK/STAT pathway in the induction of iNOS expression and NO production by cells are probably caused by the application of another inhibitor. In their experiments on interferon-treated macrophages, Chen et al. (2002) used aurintricarboxylic acid (ATA) as a JAK/STAT pathway inhibitor to check its inhibitory action on the JAK/STAT pathway, on iNOS expression, and on NO production.

The presence of reactive oxygen species with the involvement of the JAK/STAT pathway may have an effect on the changes in iNOS expression and NO production. This has been confirmed by the findings reported by Simon et al. (1998), who showed that the activation of JAK2 kinase in vitro is regulated by reactive oxygen species. There are data indicating the involvement of JAK2 kinase in the regulation of oxygen burst in human granulocytes (Dent et al., 2000).

Our results showed changes in the production of superoxide anion radical in the presence of AG490 by PMNs exposed to NDMA, which suggests that the generation of superoxide anion radical by these cells is associated with JAK/STAT pathway activation.

In conclusion, the presented findings indicate that NDMA in human neutrophils activates the JAK/STAT pathway, which plays a role in the production of NO with the involvement of iNOS and in the generation of superoxide anion radical by these cells.

The observed changes in transcription factors' expression in human neutrophils as a result of NDMA activity may also lead to changes in the course of other functions controlled by them. These factors may have the function of an activator or repressor of the transcription of genes controlling expression of proteins functioning as factors regulating cell cycle or immunomodulating and antiapoptotic factors.

Increased production of NO by PMNs can lead to disturbances in the course of immune responses, e.g., inflammatory process, angiogenesis, or apoptosis process.

The data indicate the existence of complex mechanisms involved in the regulation of NO production with iNOS and generation of superoxide anion radical by human PMNs. Further research extended with other functions of PMNs with the involvement of kinases and transcription factors will help fully elucidate the molecular mechanism of NDMA action on these cells and determine potential threats due to exposure to this compound.

Acknowledgments

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