



Regulation of NO production by MAPK dual-specificity phosphatases (DUSP) in human neutrophils exposed to N-nitrosodimethylamine

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One of the enzymes responsible for nitric oxide (NO) production in neutrophils is the inducible nitric oxide synthase (iNOS). Changes in its expression may result from the activation of different signaling pathways, including MAPK, which lead to activation of various genes, including DUSP genes. DUSP induce the negative feedback loop leading to MAPK deactivation through their phosphorylation. Our study assessed the role of DUSP1, DUSP10 and DUSP16 with the participation of MAPK in the iNOS-dependent NO production by neutrophils exposed to xenobiotic, N-nitrosodimethylamine (NDMA). The obtained results suggest that N-nitrosodimethylamine enhances the expression of all tested proteins (except DUSP10) in the cytoplasmic and nuclear fractions of neutrophils. The JNK pathway inhibition resulted in an attenuation of iNOS, phospho-p38 and DUSP10 expression in the cytoplasmic fraction and DUSP1 expression in the nuclear fraction of neutrophils. Inhibition of the p38 pathway led to a lower expression of iNOS, DUSP16 and DUSP10 in the cytoplasmic fraction. No changes in the phospho-JNK and DUSP1 expressions were observed. With the results of this study we can conclude that DUSP are positive regulators of MAP kinases in NDMA-induced signaling pathway which lead to modulation of iNOS-dependent NO production in human neutrophils.

Keywords: iNOS expression, JNK pathway inhibition, Nitric oxide

One of the enzymes responsible to produce nitric oxide (NO) in immune system cells is an inducible nitric oxide synthase (iNOS)¹⁻³. Activation of different intracellular signaling pathways, including MAPK, cause changes in iNOS expression, in both, pathological and physiological states^{4,5}. The MAP-kinase (MAPK) signaling pathway is a family of serine-threonine kinases: JNK, p38, ERK1/2 and ERK5. All of MAP kinases contain the Thr-X-Tyr (TXY) motif, whose phosphorylation leads to their activation^{6,7}.

The activated MAP kinases, by phosphorylation of transcription factors lead to activation of several genes, including those coding for kinase phosphatases (MKPs)^{7,8}. MKPs induce the negative feedback loop leading to the inactivation of MAP kinases through their phosphorylation. MKPs phosphatases may also occur in the cells with low level expression⁹⁻¹¹. Among the MKPs proteins, three phosphatase families are distinguished based on their active sites: tyrosine phosphatases (HePTP/LC-PTP, PTP-SL/STEP), serine and threonine phosphatases (PP1, PP2A, PP2B) and

tyrosine, and serine and threonine phosphatases (DUSP)^{12,13}.

DUSP proteins exhibit varying substrate specificity MAPK, intracellular location and different action time. Presence of 10 out of 13 identified MKPs was determined in PMNs. p38 and JNK kinase inactivation in these cells primarily involves three phosphatases, DUSP1 (MKP-1; found in the nucleus), DUSP10 (MKP-5; located in the nucleus and cytoplasm) and DUSP16 (MKP-7; occurring in the cytoplasm)^{10,14-16}. Studies have demonstrated that DUSP phosphatases not only activate MAP kinases, but also can lead to elevated selective activity of a particular kinase or enhance their activity through interaction with these kinases⁹. Their significant role was determined for production of cytokines and control of iNOS expression induction in cells¹⁷.

Further, data indicate that certain mediators, including TNF and IL-1, through specific kinases may activate protein phosphatases, thus leading to further activation of MAPK cascade^{9,18}. Earlier, we demonstrated that N-nitrosodimethylamine (NDMA) increases iNOS expression and nitric oxide production in human neutrophils through parallel activation of JNK and p38 MAP kinases. But the shorter time of its

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action, activates the p38 pathway more efficiently. On the other hand, a prolonged exposure to this xenobiotic leads to stronger activation of the JNK pathway than the p38 pathway in PMNs^{19,20}. Induction of iNOS expression and NO production in these cells is not directly linked to the ERK1/2 and ERK5 kinases. Studies have also demonstrated interactions between p38 and JNK kinases and ERK1/2 and p38 kinase in regulation of iNOS-dependent NO production in polymorphonuclear leukocytes (PMN) exposed to NDMA^{21,22}.

The literature is silent on the contribution of DUSP phosphatases in regulation of iNOS-dependent NO production in leucocytes exposed to the NDMA. Because we have been studying the role of the signaling pathway induced by NDMA in human neutrophils in iNOS-dependent NO production, we have drawn a compelling conclusion that “all pathways lead to MAP kinases”. Since the MAP kinase activation leads to the activation of transcription factors, including AP-1 and NF- κ B, as well as STAT proteins⁶⁻⁸, assessment of the expression of transcription factors, including STAT1 and STAT3 induced by JNK and p38 kinases in PMN exposed to NDMA, will enable better understanding of the basics of molecular mechanism of this xenobiotic effect on the functions of the tested leukocyte population linked to the synthesis and release of NO. Here, we tried to evaluate of DUSP protein activity involved in the iNOS-dependent NO production in neutrophils exposed to the effect of NDMA. Also, we estimated the role of individual DUSP phosphatases in the activation of MAP kinases involved in NO production by neutrophils exposed to the xenobiotic (Fig. 1).

Materials and Methods

Reagents

NDMA, Griess reagent and BCIP/NBT Liquid substrate system were purchased from Sigma (Steinheim, Germany). SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA). Laemmli buffer, Tris-Buffered Saline (TBS)/Caseine buffer and TBS-T [containing: Tris-Buffered Saline and Tween 20] were purchased from BioRad Laboratories (Hercules, CA).

Antibodies

Monoclonal mouse antibodies against iNOS, phospho-p38, DUSP10 and β -actin, polyclonal goat antibodies against phospho-JNK, DUSP16, phospho-STAT1 and phospho-STAT3 protein, as well as polyclonal rabbit antibodies against DUSP-1 were

purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Goat anti-mouse IgG antibody conjugated with alkaline phosphatase (AP), donkey anti-goat IgG antibody conjugated with alkaline phosphatase and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase were obtained from Vector Laboratories (Burlingame, CA). Monoclonal mouse antibody against the Poly(ADP-ribose)polymerase (PARP-1) was purchased from Calbiochem.

Isolation and incubation of PMNs

The study involved a group of 20 healthy (age 20-50 years) volunteer blood donors from the Regional Centre for Transfusion Medicine (Bialystok, Poland). Males were chosen to avoid possible influences due to endogenous hormones on the experimental findings. The Ethics Committee of the Medical University of Bialystok (R-I-002/41/2015) approved this study. All persons gave written informed consent prior to blood donations.

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)²³. This method enables simultaneous separation of two highly purified leukocyte fractions: PMN (containing

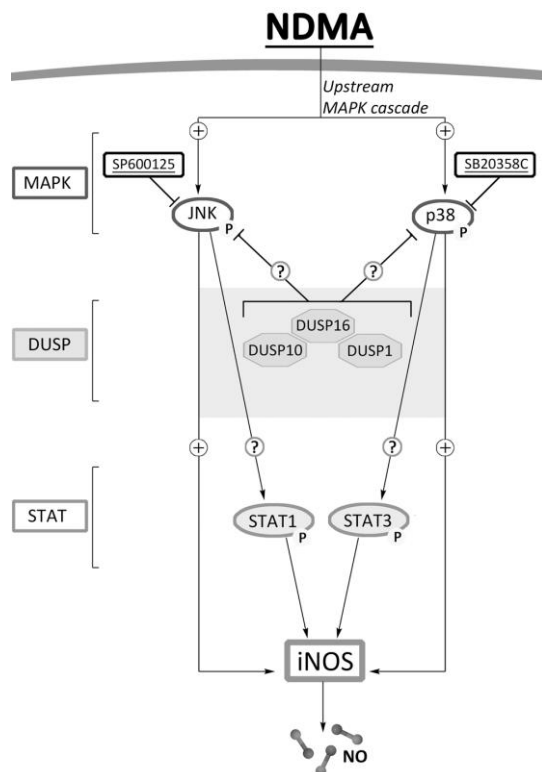


Fig. 1 — iNOS signaling

91% PMN) and PBMC (containing 94% lymphocytes). The purity of isolated PMN was determined by MayGrunewald-Giemsa. Sera were obtained from blood samples collected without anticoagulation agents.

PMNs were suspended at a concentration of 5×10^6 cells/mL in Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA) containing the subject's own serum (7.4%, 20/270 μ L), 100 U penicillin/mL, and 50 ng streptomycin/mL (Polfa Trachomin SA, Warsaw, Poland). The cells (200 μ L aliquots) were then placed into wells of microplates (Microtest III-Falcon, BD Biosciences, Bedford, MA) and incubated for 2 h at 37°C in a 5% CO₂ incubator (NuairTM US Autoflow, Plymouth, MN). PMN in the wells were then treated with 20 μ L NDMA to attain a final concentration of 0.74 μ g NDMA/ μ L in the well; control wells received vehicle only. Dose of NDMA was selected based on preliminary studies that determined the cytotoxic effects of NDMA on these cell types²⁴; metabolic activity was decreased in the leukocytes only among cells incubated with ≥ 0.74 μ g NDMA/ μ L. The PMNs were then cultured a further 2 h before culture supernatants were collected. Assessments of viability (via trypan blue exclusion) showed that PMNs were still >93% viable after the treatment.

Phospho-p38 and phospho-JNK expressions are markers of p38 and JNK pathways activation. Similarly, JAK/STAT pathway activation manifests by a presence of the phospho-STAT1 and phospho-STAT3 proteins.

In order to determine the role of MAP kinase in the DUSP family proteins and STAT engaged in regulation of iNOS expression in PMN exposed to NDMA, selective inhibitors of particular pathways were used. In these studies, cells were pre-incubated with: 40 μ M SP600125, a selective JNK pathway inhibitor²⁵ or with 40 μ M SB203580 - a selective p38 pathway inhibitor²⁶ for 1 h before the addition of NDMA. Preliminary studies showed that the presence of any of the inhibitors did not affect the cell viability.

Protein isolation and Western blot analyses

Cytoplasmic and nuclear extracts from PMNs (3×10^6 cells total/sample) were prepared using NucBusterTM Protein Extraction Kit (Calbiochem). Step-wise extraction delivered two distinct cellular protein fractions: cytoplasmic and nuclear. The concentration of protein in each was determined with a QubitTM Protein Assay Kit (Invitrogen). An antibody against PARP-1 (1:5000) and against β -actin (1:100)

were used as an internal control within the nuclear and cytoplasmic fractions, respectively.

The extracts were suspended in Laemmli buffer, loaded at 20 μ g/well, and then electrophoresed over a 4% stacking and a 10% separating SDS-PAGE gel. The resolved proteins were electrotransferred onto 0.45- μ m pore-size nitrocellulose membranes (BioRad) then blocked with Tris-Buffered Saline (TBS)/Caseine buffer, washed with TBS-T (TBS with 0.05% Tween-20), and incubated with QentixTM Western Blot Signal Enhancer (Thermo Fisher Scientific, Rockford, IL). The membranes were then incubated for 10 min at room temperature (21°C) in SNAP (Protein Detection System; Millipore, Billerica, MA) with 1:100 dilutions of primary monoclonal antibody against iNOS, phospho-p38, DUSP10 or primary polyclonal antibodies against phospho-JNK, DUSP16, phospho-STAT1 and phospho-STAT3 or DUSP1. After washing with 0.1% TBS-T, the membrane was incubated at room temperature with alkaline phosphatase anti-mouse IgG Ab, anti-rabbit IgG Ab or anti-goat IgG Ab (1:200). Immunoreactive bands were then visualized using BCIP/NBT Liquid substrate system; intensities were determined using ImageJ software (Bethesda, MD) and reported as Arbitrary Units (A.U.).

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 the nitrite and nitrate concentrations present. NO production by PMNs were determined using an indirect method based on measurement of nitrite concentration in culture supernatants according to a Griess reaction²⁷. In the analyzed samples, 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 μ M (10^6 cells in 270 μ L supernatant).

Statistical analysis

Results were analyzed using Statistica version 9.1. (StatSoft, Inc., Tulsa, OK). Data distribution normality were determined using a Kolmogorov-Smirnov test. Since data were not normally distributed, for comparison of variations between assayed groups, Mann-Whitney U non-parametric tests were applied to unrelated results. A p-value ≤ 0.05 was accepted as statistically significant. All data are presented as mean \pm SE.

Results

Total NO concentration in PMN supernatants

Exposure of neutrophils to N-nitrosodimethylamine confirmed earlier observations which demonstrated enhanced release of nitric oxide in comparison to cells incubated without this xenobiotic. In order to confirm contribution of the examined MAP kinases: JNK and p38 in the iNOS-dependent nitric oxide production by neutrophils exposed to NDMA, its concentration in the presence of selective inhibitors of individual kinases was determined. We found in the supernatants of neutrophils, which were preincubated with the JNK and p38 pathway inhibitors, and treated with NDMA, lower concentrations of total NO in comparison to the cellular supernatants without an inhibitor (Table 1).

Expression of iNOS, phospho-JNK, phospho-p38, DUSP1, DUSP10, DUSP16, phospho-STAT1 and phospho-STAT3 in PMNs

Exposure of neutrophils to N-nitrosodimethylamine led to a parallel increase of the expression of iNOS,

phospho-p38 and phospho-JNK in the cytoplasmic fraction in comparison to the cells incubated without the xenobiotic (Fig. 2). Moreover, an increased DUSP16 expression was determined in the cytoplasmic fraction and DUSP1 in the nuclear fraction of these cells. However, no changes in DUSP10 expression were observed for both neutrophil fractions (Fig. 2). Moreover, neutrophil incubation with NDMA demonstrated higher expression of phospho-STAT1 and phospho-STAT3 in both cytoplasmic and nuclear fractions. The phospho-STAT1 and phospho-STAT3 expression in the nuclear fraction of PMN exposed to NDMA was higher in comparison to expression of these proteins in the cytoplasmic fraction (Fig. 3). Experiments with the inhibitors of kinases were performed to establish the role of MAP kinases (JNK and p38) in induction of the studied DUSP proteins and STAT transcription factors. Pre-incubation with the JNK pathway inhibitor (SP600125) confirmed our

Table 1 — Concentrations of total NO ($\mu\text{M}/10^6$ cells (in 270 μL supernatant)) from PMNs

PMNs \pm SE	PMNs+NDMA \pm SE	PMNs+ SP600125+NDMA \pm SE	PMNs+ SB203580+NDMA \pm SE
10.83 \pm 3.39	21.31* \pm 4.78	12.29 ^a \pm 4.75	11.45 ^b \pm 4.02

[PMNs were treated with or without SP600125 (40 μM) or SB203580 (40 μM) for 1 h before addition of NDMA (0.74 $\mu\text{g}/\mu\text{L}$). Two hours after addition of NDMA, the nitrite concentrations were measured as a marker of NO production. * Value significantly different between cells without and with NDMA ($P < 0.05$); ^a value significantly different between cells treated with NDMA but pre-incubated without or with the inhibitor SP600125 ($P < 0.05$). ^b value significantly different between cells treated with NDMA but pre-incubated without or with the inhibitor SB203580 ($P < 0.05$). Data are expressed as $\mu\text{M}/10^6$ cells (in 270 μL supernatant) and are shown as mean (\pm SE) of 20 experiments]

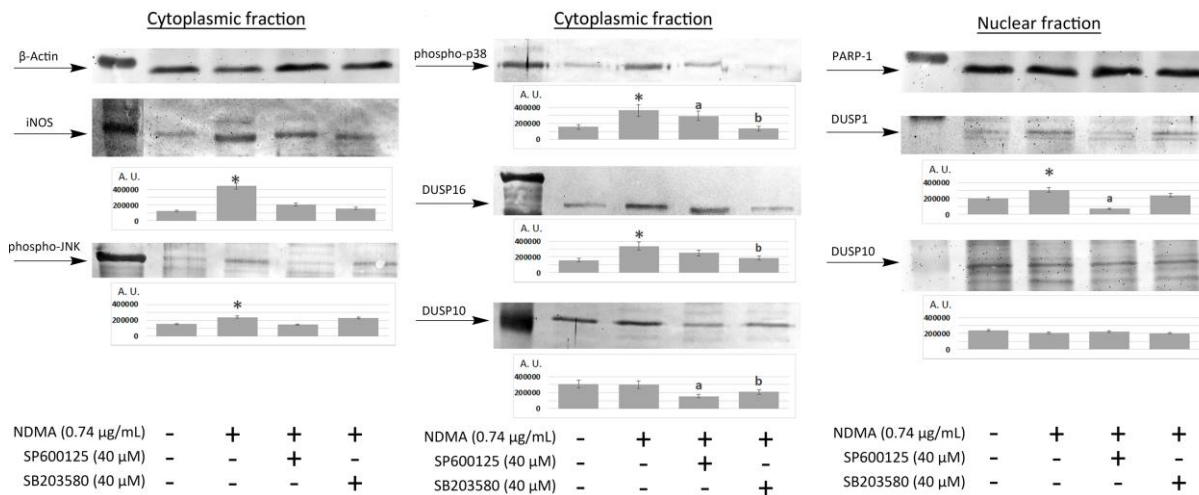


Fig. 2 — Expressions of iNOS, fosfo-JNK, fosfo-p38, DUSP1, DUSP1 and DUSP16 in PMNs. [PMNs were treated with or without SP600125 SB203580 (40 μM) or SB203580 (40 μM) for 1 h before addition of NDMA (0.74 $\mu\text{g}/\mu\text{L}$). The cytoplasmic and nuclear fractions obtained from those cells were used to detect iNOS, fosfo-JNK, fosfo-p38, DUSP1, DUSP10 and DUSP16 protein levels by western blotting. The results shown are representative of five independent experiments. Band intensity was quantified using ImageJ software and expressed in arbitrary units (A.U.). Data shown are mean (\pm SE) of five independent experiments. * Value significantly different between cells without and with NDMA ($P < 0.05$); ^a value significantly different between cells treated with NDMA but pre-incubated without or with the inhibitor SP600125 ($P < 0.05$); and ^b value significantly different between cells treated with NDMA but pre-incubated without or with the inhibitor SB203580 ($P < 0.05$)]

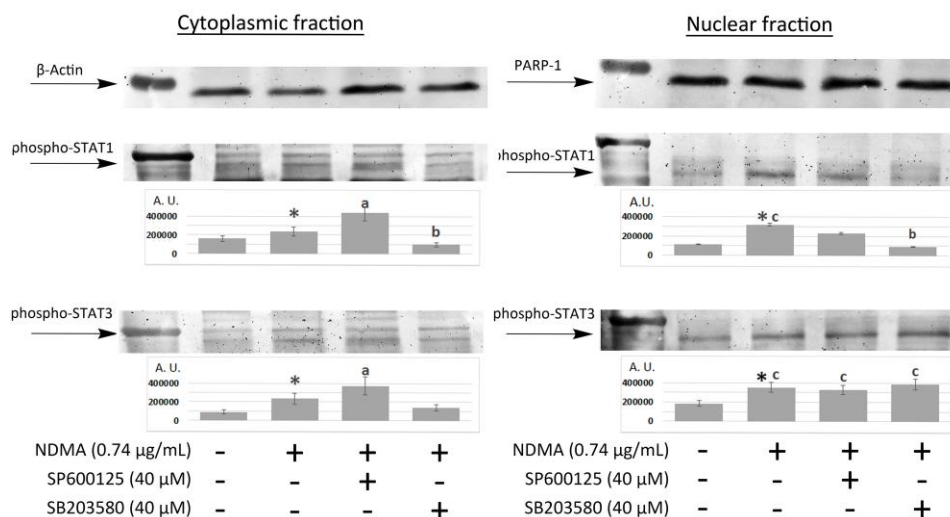


Fig 3 — Expressions of phospho-STAT1 and phospho-STAT3 in PMNs. [PMNs were treated with or without SP600125 SB203580 (40 μ M) or SB203580 (40 μ M) for 1 h before addition of NDMA (0.74 μ g/ μ L). The cytoplasmic and nuclear fractions obtained from those cells were used to detect phospho-STAT1 and phospho-STAT3 protein levels by western blotting. The results shown are representative of five independent experiments. Band intensity was quantified using ImageJ software and expressed in arbitrary units (A.U.). Data shown are mean (\pm SE) of five independent experiments. * Value significantly different between cells without and with NDMA ($P < 0.05$); ^a value significantly different between cells treated with NDMA but pre-incubated without or with the inhibitor SP600125 ($P < 0.05$); ^b value significantly different between cells treated with NDMA but pre-incubated without or with the inhibitor SB203580 ($P < 0.05$); and ^c value significantly different between cytoplasmic and nuclear fractions ($P < 0.05$)]

earlier results that decreased expression of iNOS phospho-p38 in the cytoplasmic fraction of neutrophils exposed to NDMA in comparison to cells incubated without the inhibitor²⁰. Decreased DUSP10 expression was determined in the cytoplasmic fraction and DUSP1 in the nuclear fraction. No changes in the expression of DUSP16 in the cytoplasmic fraction and DUSP10 in the nuclear fraction were observed (Fig. 2). The use of the JNK pathway inhibitor (SP600125) in the cytoplasmic fraction of PMN exposed to NDMA increased expression of phospho-STAT1 and phospho-STAT3 in comparison to the cells without the inhibitor. No changes in the expression of these proteins in the nuclear fraction were observed (Fig. 3). In the presence of the p38 pathway inhibitor (SB203580), a decreased iNOS expression in the cytoplasmic fraction of PMN exposed to NDMA was confirmed²⁰. No changes were found in phospho-JNK expression in the cytoplasmic fraction, the same as DUSP1 and DUSP10 in the nuclear fraction of neutrophils in comparison to their expression in the cells incubated without the inhibitor. However, lower expression of DUSP16 and DUSP10 in the cytoplasmic fraction was demonstrated (Fig. 2). Inhibition of the p38 pathway decreased expression of phospho-STAT1 and phospho-STAT3 in the cytoplasmic fraction of neutrophils exposed to NDMA in comparison to cells without the inhibitor. Moreover,

decreased expression of phospho-STAT1 in the nuclear fraction was observed in the tested cells. No changes in the expression of phospho-STAT3 were observed in this fraction (Fig. 3).

Discussion

The presented results and our earlier observations confirm the significant effect of N-nitrosodimethylamine (NDMA) on the iNOS expression and NO production by human neutrophils¹⁹. NDMA is a compound with high carcinogenic, mutagenic and teratogenic potentials. Therefore, the highest allowable concentration of NDMA in drinking water should not exceed 0.7 ng/L. A slight excess of this concentration with prolonged exposure time may be considered as a low-dose, chronic exposure to NDMA, which are manifested by primarily cancerous changes of different organs, particularly of the liver^{28,29}. Most information about the direct, acute effect of NDMA is mainly based on experimental studies. Data obtained from various studies demonstrate that NDMA cytotoxicity toward hepatocyte cultures are exhibited at the concentration of 0.1 mM, and for the cells of kidneys and lungs a range of concentration 3-50 μ g/mL^{30,31}. The tested cells, neutrophils, have the shortest life span among the cells of the body system. Every day, marrow releases to the blood approximately 1.5×10^9 of mature cells/kg

of body wt., where they remain for 6-12 h³². Therefore, in order to achieve the intensified effect of NDMA on these cells *in vivo*, we used higher concentration of the xenobiotic in the study²⁴. Our results for the first time demonstrated that the N-nitrosodimethylamine leads to activation of DUSP1 and DUSP16 in human neutrophils.

Following observations of different authors, expression of MKPs occurs at a lower level in non-stimulated cells³³. This is further confirmed by the results of our study, on neutrophils not exposed to the xenobiotic, which demonstrated low DUSP1 expression in the nuclear fraction and DUSP16 in the cytoplasmic fraction. In the light of earlier observations that the exposition of human PMNs to N-nitrosodimethylamine induces enhanced generation of superoxide radical in these cells, it seems an important observation that among the examined DUSP proteins, NDMA does not activate DUSP10 in neutrophils²¹. The lack of DUSP10 activation correlates with augmented cytokines and reactive oxygen species production³⁶.

There are reports that MAP kinases may stabilize, or through phosphorylation, lead to degradation of phosphatases^{33,34}. The decreased DUSP10 expression observed after the inhibition of the JNK or p38 pathway, as well as lowered DUSP16 expression after the inhibition of the p38 pathway in the cytoplasmic fraction of neutrophils exposed to NDMA indicates significant role of MAP kinases in the posttranslational regulation of these proteins in the tested cells. Available data indicate that DUSP10, due to its presence in nucleus and cytosol, may negatively regulate the activation of p38 MAPK in the cytoplasm^{35,36}. The lack of the phosphatase leads to a considerable enhanced and prolonged activity of p38 MAPK without the effect on the activity of JNK kinase in neutrophils. In lymphocytes DUSP10 regulates phosphorylation of JNK but not p38 MAPK^{35,37}. However, our study showed that the activity of DUSP10 in the cytoplasm of neutrophils exposed to NDMA depends on the activity of p38 and JNK kinases.

DUSP10 and DUSP1 control the activation of MAP kinases, but DUSP10 regulates the early phosphorylation phase and DUSP1 modulates the late phosphorylation phase. This observation is connected with the different intracellular location of these phosphatases³⁶⁻⁴⁰. Our study demonstrated that the activity of DUSP1 in the nucleus of neutrophils

exposed to NDMA depends on the JNK pathway. On the other side, the activation of DUSP16 in the cytoplasm is not regulated by p38. Therefore, the demonstrated activity of DUSP proteins as a result of the effect of p38 and JNK kinases may explain the different involvement of these kinases in the production of NO by PMNs subjected to the effect of NDMA observed in the earlier study.

The obtained results and earlier observations confirm contribution of the JNK pathway in the activation of the p38 kinase and that this activation is involved in the increase of iNOS expression in PMNs treated with NDMA. However, our study did not demonstrate the effect of the p38 kinase on activation of JNK, which may be the result of the shorter incubation time with the xenobiotic. This explains the stronger involvement of the JNK kinase in iNOS activation as a result of prolonged exposure to NDMA.

All our observations indicate that N-nitrosodimethylamine activate STAT1 and STAT3 proteins in neutrophils. Results of this study constitute the first reference to the regulation of STAT protein activation by MAP kinases in human neutrophils exposed to this xenobiotic. The enhanced expression of phospho-STAT1 and phospho-STAT3 in the cytoplasmic fraction of the tested proteins determined after inhibition of the JNK pathway indicates that this kinase has inhibitory role in the activation of these proteins. On the other hand, the decreased expression of phospho-STAT1 in both neutrophil fractions determined after inhibition of the p38 pathway suggests that p38 kinase has an activating effect on this transcription factor. Earlier studies also demonstrated that JAK2 kinase participates in activation of STAT1 and STAT3 proteins. Galdiero *et al.*⁴¹ presented different results on this line. They demonstrated that in U937 monocytes of cell line stimulated with porins that STAT1 and STAT3 activation occurs solely as a result of the effect of p38 and JNK kinases without the contribution of the JAK kinases.

Data exist on quantitative differences between the STAT1 and STAT3 proteins depending on the cellular location. The presence of STAT3 in the nucleus is not constant and it is constantly translocated between the nucleus and the cytoplasm. STAT3, contrary to STAT1, may accumulate in the nucleus independent of the phosphorylation state. Moreover, the phosphorylated STAT3 may also form a heterodimer with the phosphorylated STAT1, which may eventually lead to intensified nuclear accumulation of

the transcription factor STAT3 as a result of exposure of cells to STAT1-activating factors⁴²⁻⁴⁴. This was further corroborated by the results of our own study, in which an intensified accumulation of phospho-STAT1 and phospho-STAT3 was observed in the nucleus of neutrophils exposed to NDMA.

Conclusion

The obtained results indicate that N-nitrosodimethylamine (NDMA) activates DUSP16 and DUSP1 phosphatases in human neutrophils, whose regulation is linked with other MAP kinases. Secondly, the DUSP10 activation depends on both MAP kinases: p38 and JNK involved in iNOS regulation dependent NO production. Further, it reveals the role of MAP kinases JNK and p38 in the activation of STAT1 and STAT3 transcription factors involved in regulation of iNOS expression and NO production by human neutrophils treated by NDMA. The study confirms that MAP kinases constitute a significant role of integrator of the NDMA initiated signals. DUSP are positive regulators of MAP kinases in NDMA-induced signaling pathway which lead to modulation of iNOS-dependent NO production in human neutrophils. The observed changes in DUSP expression in human neutrophils resulting from NDMA effect may lead to changes in other functions controlled by them, not only those linked to NO production. Overall, this study provides clarification of potential causes of impaired innate responses in subjects who might be exposed to the xenobiotic NDMA in a workplace or by foods they consumed.

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Conflict of interest

Authors declare no conflict of interests.

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