Folia biologica (Kraków), vol. **56** (2008), No 1-2
doi:10.3409/fb56_1-2.83-89

Role of the p38 MAPK Pathway in Induction of iNOS Expression in Human Leukocytes

Ewa JABLONSKA, Wioletta RATAJCZAK and Jakub JABLONSKI

Accepted October 10, 2007

JABLONSKA E., RATAJCZAK W., JABLONSKI J. 2008. Role of the p38 MAPK pathway in induction of iNOS expression in human leukocytes. Folia biol. (Kraków) **56**: 83-89.

Inducible nitric oxide synthase (iNOS) is one of the enzymes responsible for NO production in neutrophils (PMN) and in peripheral blood mononuclear cells (PBMC). Several studies have demonstrated that iNOS expression is controlled by a wide group of cytokines which achieve their biological effect through, among others, the activation of the p38 MAPK pathway. The aim of the present study was to define the participation of the p38 MAPK pathway in the induction of iNOS expression and NO production by PMN and PBMC of healthy persons after stimulation of rhIL-15 and rhIL-18. We also estimated the influence of rhIL-15 and rhIL-18 on cGMP production by both population cells and the production of superoxide anion radicals by neutrophils. The results show that rhIL-15 and rhIL-18 induced an increase in the expression of iNOS and phospho-p38 MAPK in PMN and PBMC. We also found that PMN and PBMC, stimulated by these cytokines, released larger amounts of NO and cGMP in comparison with non-stimulated cells. Additionally, PMN showed a more pronounced ability to produce superoxide anions. The results suggest that iNOS activation in neutrophils and in peripheral blood mononuclear cells stimulated with rhIL-15 and rhIL-18 may be achieved through the assistance of the p38 MAPK pathway.

Key words*:* Neutrophils, peripheral blood mononuclear cells, nitric oxide, inducible synthase of nitric oxide, cGMP, rhIL-15, rhIL-18.

Ewa JABLONSKA, Wioletta RATAJCZAK, Department of Immunology Medical University of Bialystok, Waszyngtona 15A, 15-274 Białystok, Poland.
E-mail: ewaj@amb.edu.pl
Jakub JABLONSKI, Department of Toxicology Medical University of Białystok, Mickiewicza 2C, 15-089 Białystok, Poland.

Inducible nitric oxide synthase (iNOS) is one of the enzymes responsible for nitric oxide synthesis in the body. Cell types reported to express iNOS include lymphocytes, macrophages, neutrophils, fibroblasts, Kupfer cells, hepatocytes, astrocytes and chondrocytes (BECK *et al*. 1999; KRONCKE *et al.* 1995).

Nitric oxide (NO) is a biologically active molecule of an approximate molecular weight of 30 Da, which regulates the functioning of many systems including the immune system (KRONCKE *et al.* 1995; MOODLEY 2002). Its biological effect is achieved through the activation of the guanylyl cyclases (sGC) leading to cyclic guanomonophosphate synthesis (cGMP), which is considered an indirect indicator of the amount of generated NO (KOESLING *et al.* 1991; STROOP & BEAVO 1991).

The largest quantities of NO are produced in the course of the immunological reaction. Studies *in vitro* and *in vivo* have shown that NO may inhibit intracellular development and kill viruses, bacteria, fungi and protozoa. NO shows anti-inflammatory properties, including the inhibition of leukocyte adhesion to the endothelium, degranulation of mast cells, lymphocyte proliferation, and synthesis of reactive forms of oxygen by phagocytes. On the other hand, NO has also been demonstrated to have pro-inflammatory properties, such as the stimulation of TNF-α production, cyclooxygenase (COX) activation, and prostaglandin production (KRONCKE *et al.* 1995; MOODLEY *et al.* 2002).

The regulation of iNOS expression depends on the cell type, activation of appropriate transcription factors and their interaction (LIRK *et al.* 2002). A previous study has shown the inverse correlation between iNOS and phospho-IκB (a *sign* of NF-κB activation) expression and suggested that iNOS activation in rhIL-15 and rhIL-18 stimulated PMN and PBMC was not directly dependent on the NF-κB pathway and may occur with the involvement of other intracellular signaling pathways (JABLONSKA *et al.* 2006).

The p38 MAPK pathway, with the participation of the enzyme kinase MAP family, can take part in

the initiation of mRNA iNOS transcription. They have the ability to move to the cell nucleus and activate the expression of many genes, including transcription factor coding genes, such as AFT-2 or SAP-1. It is known that cytokines such as IL-15 and IL-18 are p38 MAPK pathway stimulating factors (WYMAN *et al.* 2002; PELLETIER *et al.* 2002; DE VERA *et al.* 1996).

Therefore this study was undertaken in order to determine the participation of p38 MAPK pathways in the induction of iNOS expression and NO production by PMN and PBMC after rhIL-15 and rhIL-18 stimulation. As the presence of phospho–p38 MAPK is a sign of p38 MAPK activation, its expression may confirm or exclude the involvement of p38 MAPK pathway in the activation of iNOS. Because cGMP is an indirect coefficient of the generated amount of NO, its concentration was also estimated in the examined PMN and PBMC supernatants. Moreover, an assessment was made of the ability of neutrophils to release superoxide anion radicals by PMN, which produces highly toxic pernitrate (V) with NO, responsible for the peroxidation of proteins, lipids and nucleic acids (KRONCKE *et al.* 1995).

Material and Methods

We examined 20 healthy donors aged from 21 to 40 years (mean 32 years). Cells were isolated from heparinized (10U/ml) whole blood by Gradisol G gradient 1.115g/ml (Polfa) according to ZEMAN *et al.* (1988).

Two highly purified leukocyte fractions, mononuclear cells (PBMC), and polymorphonuclear cells (PMNs), were suspended in the culture medium (HBSS) to provide $5x10^6$ cells/ml and the cells were incubated in flat-bottomed 96-well plates (Microtest III-Falcon) for 4 h at 37°C in a humidified incubator with 5% CO_2 (NUAIRE^{TM}). Recombinant human IL-15 (rhIL-15 – 50 ng/ml; R&D) and/or recombinant human IL-18 (rhIL-18 – 50 ng/ml; R&D Systems) were tested to stimulate secretion by PMN and PBMC.

Western blot analysis

The cells were incubated for 4 h and then were lysed directly by sonication. Total lysates of PMNs and PBMC were suspended in Laemmli buffer (Bio-Rad Laboratories) and electrophoresed on SDS-PAGE. The resolved protein was transferred onto 0.2 μm pore-sized nitrocellulose (Bio-Rad Laboratories, Herkules CA, USA). Membranes were then blocked with a TBS/Caseine buffer for 1 hour at room temperature. Afterwards the membranes were washed in TBS and 0.05% Tween20. Nitrocelluloses were incubated at +4°C for 18 h with the primary monoclonal antibody anti-iNOS (R&D Systems, Minneapolis, USA) and polyclonal antibody anti-phospho-p38α MAPK (ABR Affinity BioReagents, Golden, USA). After washing with 0.1% TBS-T, the membrane was incubated at room temperature for 1 h with alkaline phosphatase anti-mouse IgG Abs (Vector Laboratories, Burlingame, CA, USA). Immunoreactive protein bands were visualized by the BCIP/NBT Liquid substrate system (Sigma, Saint Louis, USA), determined using LabImage 1 Gel software and estimated by arbitrary units. The illustrations show a typical image of iNOS and phosho-p38 MAPK expression (Fig. 1).

Determination of total nitric oxide (NO_3^-/NO_2^-) concentration in the PMN and PBMC supernatants

Nitric oxide produced in cells in the presence of superoxide anion-radicals is rapidly converted to nitrate (V) and nitrate (III) (NO_3^-, NO_2^-). Nitrate (V) and nitrate (III) are stable final products of NO metabolism and may be used as indirect markers of NO presence. Total NO concentration is commonly determined as a sum of nitrate (V) and nitrate (III) concentrations. NO production by PMN and PBMC was determined using an indirect method based on measurement of NO_2^- ion concentration in culture supernatants and serum according to Griess's reaction. In the samples analyzed, nitrates (V) were reduced to nitrates (III) in the presence of cadmium, and then converted to nitric acid III that gave a colour reaction with Griess's reagent (SCHULZ *et al.* 1999). NO_2^- ion concentrations were determined by spectrophotometric analysis at $\lambda = 540$ nm with reference to a standard curve.

Analysis of generation of superoxide anion radicals by PMN using the cytochrom-c reduction test

"Oxygen burst" in neutrophils was explored by detecting the production of O_2^{-*} by these cells according to Mc Cord's method, in BHUYAN's modification, based on differences between light absorbancies of solutions containing nonreduced and reduced cytochrome-c (BHUYAN & BHUYAN 1994; BARTOSZ 2003). Cytochrome-c does not permeate through the plasmic membrane and thus its reduction by superoxide anion radicals in PMN supernatants indicates O_2^{-*} release outside of the cell.

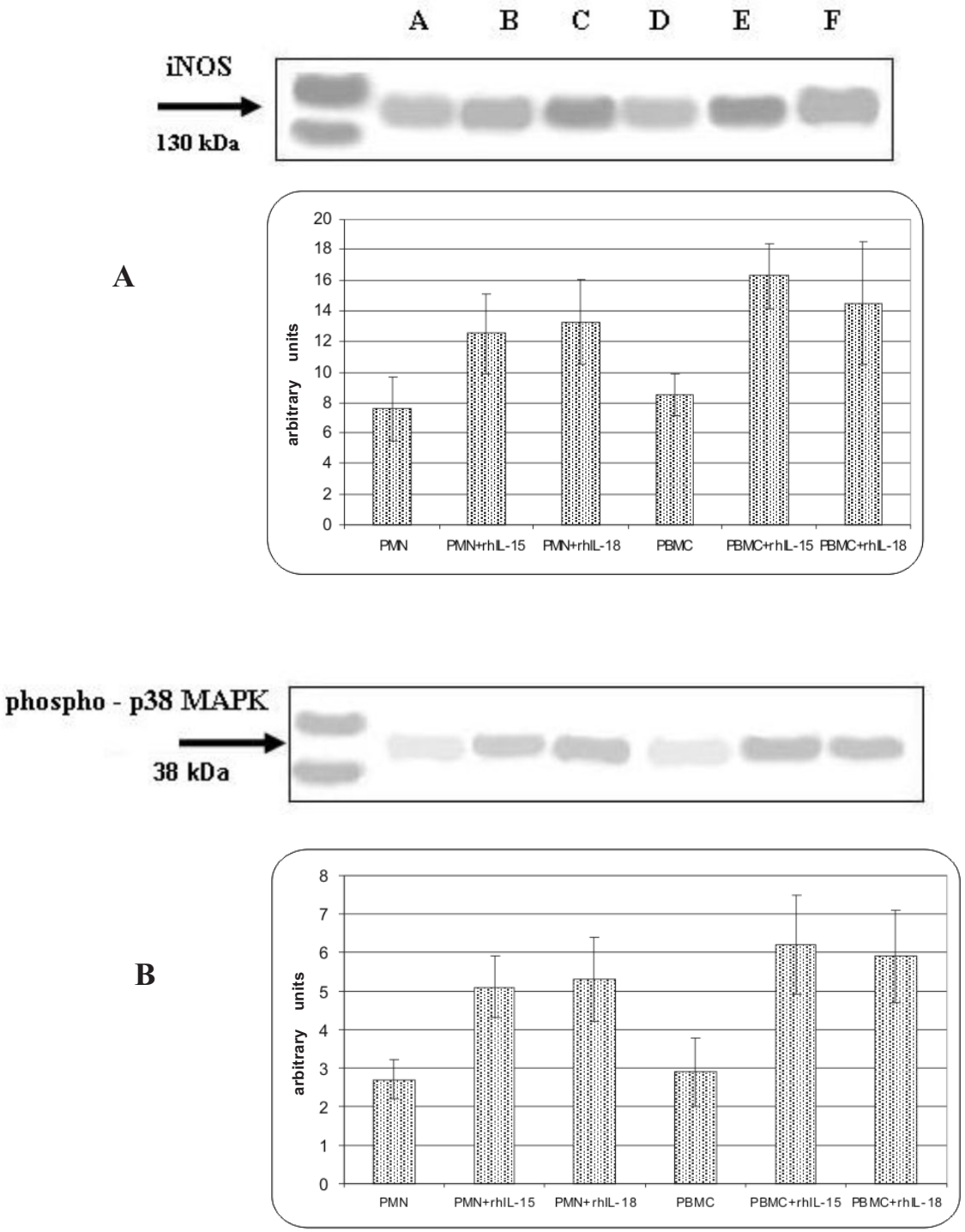

Fig. 1. Western blot analysis of iNOS and phospho-p38 MAPK protein expression in PMN and PBMC. A – PMN; B – PMN+rhIL-15; C – PMN+rhIL-18; D – PBMC; E – PBMC+rhIL-15; F – PBMC+rhIL-18.
* – statistical differences between unstimulated and stimulated cells ($P<0.05$)

Cytochrome-c solution in phosphate buffer (KH_2PO_4/K_2HPO_4), pH=7.8, containing 0.1 mM EDTA, was added to two parallel samples with isolated neutrophils. Cytochrome-c concentration was 15mg/ml. Superoxide dismutase (SOD), 5000 U/ml activity, was added to the reference sample, while buffer was added to the study sample. Next, after addition of LPS (10 μg/ml) to both test-tubes, the samples were incubated at 37°C, and then absorbance was read at λ=550 nm in the presence of

deionized water. The result was presented as non-titrated F index expressed by the absorbance of the reference sample to the reduced sample ratio.

$$F = \frac{\text{Abs Cyt-c + SOD}}{\text{Abs Cyt-c}}$$

Determination of cGMP concentration in the PMN and PBMC supernatants

cGMP levels in the cell supernatants was assessed using ELISA kit (R&D Systems).

Statistical evaluation

The results obtained were analyzed statistically using Microsoft Excel spreadsheet and Statistica 5.1 suite. Data were presented as mean ± standard deviation (SD). Data distribution normality was determined using the Kolmogorov-Smirnov. Since the data were not normally distributed, for comparison of variations between assayed groups, U-Mann-Whitney nonparametric tests were applied to unrelated results. For analysis of correlation between parameters tested, Pearson's linear correlation was used, and its significance was assessed using the Student's *t*-test for the correlation coefficient. A statistical significance level of $P<0.05$ was assumed.

Results

iNOS and phospho-p38 MAPK expression in PMN and PBMC

iNOS protein of approximate molecular mass of 130 kDa was expressed in PMN and PBMC of the control group. Equal expression was found in PMN and PBMC. rhIL-15 – and rhIL-18 – stimulated leukocytes increased iNOS expression in comparison with non-stimulated cells (Fig. 1A).

Moreover, the expression of a phospho–p38 MAPK protein of a molecular weight of 38 kDa was found in PMN and PBMC. Differences were not observed in expression of this protein between PMN and PBMC. In the presence of rhIL-15 and rhIL-18, phospho–p38 MAPK expression was elevated in both leukocyte groups in comparison to non-stimulated cells (Fig. 1B).

Table 1

Concentrations of total NO (NO_3^-/NO_2^-) in PMN and PBMC supernatants

	Concentrations of total NO (NO_3^-/NO_2^-) (μM/5x10^6 cells/ml)	
Cells	PMN±SD	PBMC±SD
Unstimulated	26.75±3.52	24.68±3.61
rhIL-15 stimulated	32.19[a]±4.02	33.22[a]±4.13
rhIL-18 stimulated	33.46[a]±4.21	31.44[a]±4.28

* – statistical differences between unstimulated and stimulated cells ($P<0.05$).

Concentrations of total NO (NO_3^-/NO_2^-) in PMN and PBMC supernatant

Incubation of PMN and PBMC in the presence of rhIL-15 and rhIL-18 caused a significant increase in the production of NO as compared to non-stimulated cells (Table 1).

Concentrations of cGMP in PMN and PBMC supernatants

There was no difference in the concentrations of cGMP between PMN and PBMC. In the supernatants of rhIL-15 – and rhIL-18 – stimulated

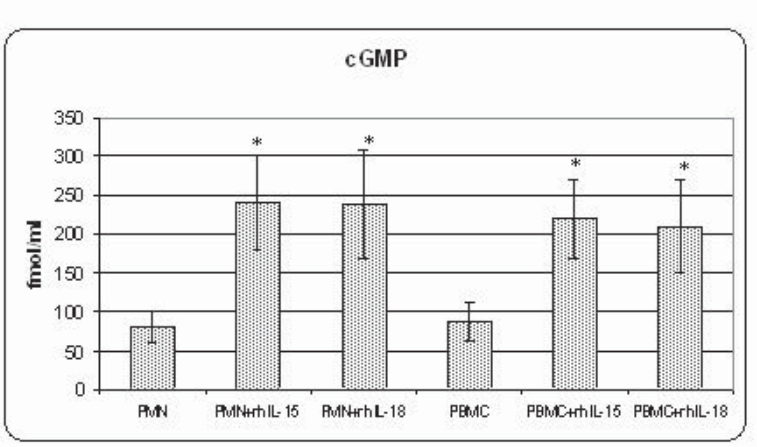

Fig. 2. Concentrations of cGMP in PMN and PBMC supernatants
* - statistical differences between unstimulated and stimulated cells ($P<0.05$).

PMN and PBMC, cGMP concentrations were higher compared to non-stimulated cells (Fig. 2).

Superoxide anion radical generation by PMN

Incubation of neutrophils in the presence of rhIL-15 and rhIL-18 caused a significant increase in the production of superoxide anion radicals compared to non-stimulated cells (Fig. 3)

Assessment of correlations between the examined parameters

A correlation was observed between total NO concentration and cGMP levels in PMN supernatants (r=0.78 P<0.05) (Fig. 4).

A correlation was also found between total NO concentration and cGMP levels in PBMC supernatants (r=0.66 P<0.05) (Fig. 5).

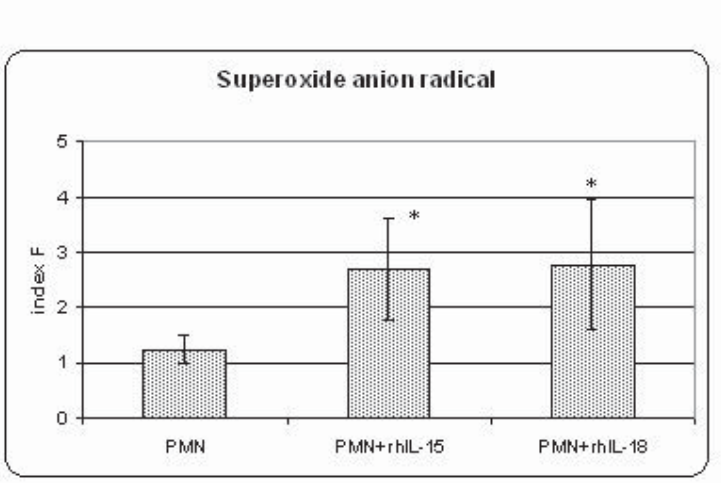

Fig. 3. Superoxide anion radical generation by PMN
* – statistical differences between unstimulated and stimulated cells (P<0.05).

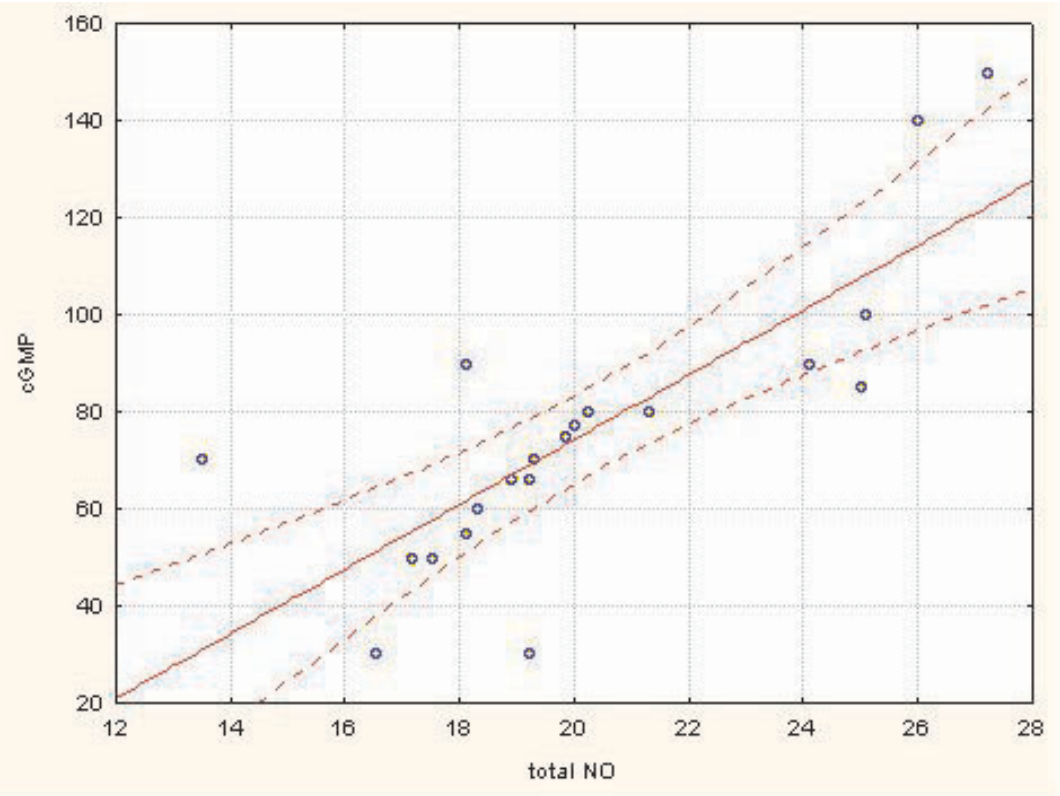

Fig. 4. Correlation between total NO concentration and cGMP level in PMN supernatants.

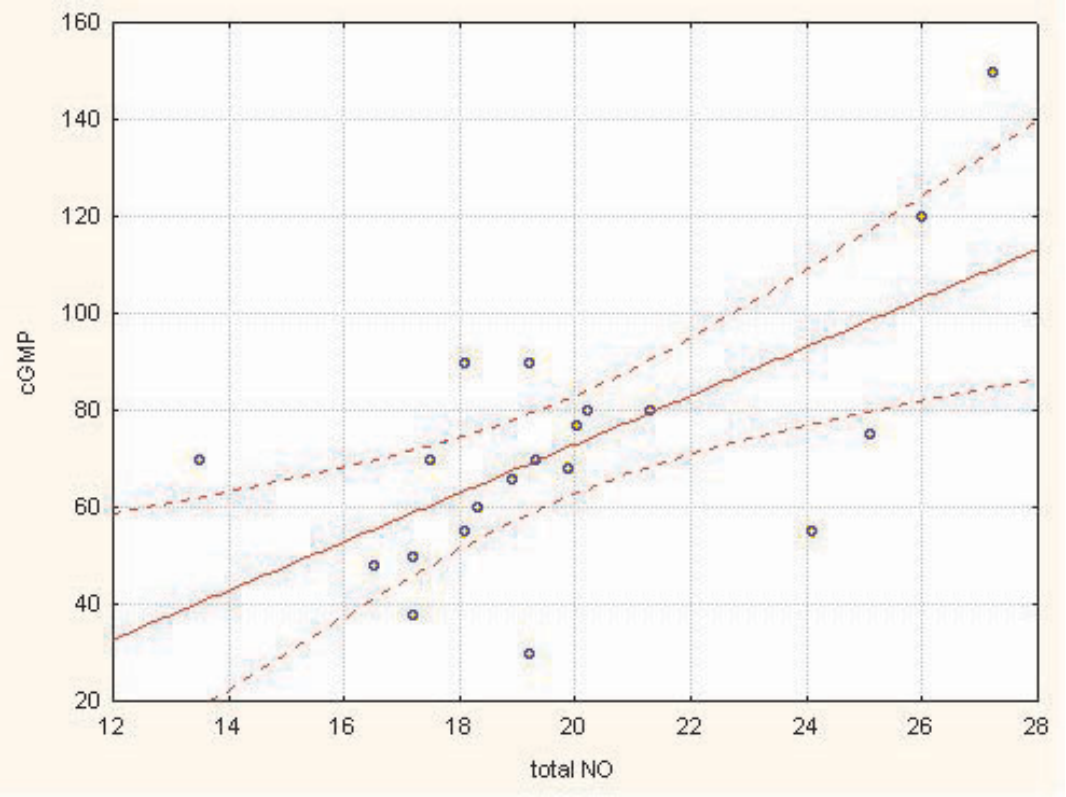

Fig. 5. Correlation between total NO concentration and cGMP level in PBMC supernatants.

Discussion

There are reports that the p38 MAPK pathway plays several different roles in the induction of iNOS expression in cells – either activation, inhibition or none (LIRK *et al.* 2002; DA SILVA *et al.* 1997). In this study, a simultaneous increase in iNOS and phospho-p38 MAPK expression and in production of nitric oxide by non-stimulated PMN and PBMC in healthy persons was observed. The same relations were observed in the presence of rhIL-15 and rhIL-18. These observations confirm the role of the p38 MAPK pathway in iNOS activation in these cells.

The results are in agreement with data presented by AJIZIAN *et al.* (1999), who assessed p38 MAPK pathway activation in macrophages and showed that its inhibition blocked iNOS expression in cells stimulated with IFN-γ + LPS. However, DA SILVA *et al.* (1997) demonstrated that the p38 MAPK pathway is necessary, but not sufficient, for iNOS induction in mouse astrocytes stimulated by TNF-α + IL-α.

In contrast, CHAN *et. al*. (2001) observed that p38 MAPK signaling pathway inhibition led to an increase in iNOS expression in mouse macrophages.

It is suggested that all signals which lead to NO synthase activation simultaneously initiate cGMP synthesis, considered an indicator of the amount of generated NO. Activation of the guanylyl cyclases (sGC) by NO leads to cGMP synthesis (KOESLING *et al.* 1991; STROOP *et al.* 1991). The results obtained in the present study show a correlation between high cGMP concentrations and elevated production of NO by PMN and PBMC.

cGMP released by the examined leucocytes may have different implications in the immune system. cGMP can lead to the inhibition of neutrophil chemotaxis and adhesion as well as aggregation of thrombocytes in auto- and paracrine way (KOESLING *et al.* 1991; STROOP & BEAVO 1991). Moreover, it was confirmed that cGMP decreases iNOS expression in mouse macrophages by blocking the activation of the transcription factor NF-κB and disturbing mRNA durability of this enzyme (KIEMER & ANGELIKA 1998). However, in smooth muscle cells, cGMP exerts an activating effect on iNOS (HAYDEN & NAKAYAMA 1999). In this study similar results were obtained, i.e. a significant increase in the production of cGMP by PMN and PBMC was accompanied by intensified iNOS expression in these cells.

It is well established that cytokines influence cGMP generation. SHINDO *et al.* (1995) reported

an increase in cGMP production by cardiac myocytes under the influence of IL-1β and LPS *in vitro*. However, other cytokines, such as TNF-α, IL-2, IL-6, IL-8, and TGF-β did not demonstrate this effect. In the current study the influence of pro – inflammatory cytokines, rhIL-15 and rhIL-18, on the increase in cGMP release by PMN and PBMC was observed.

It was also shown that in the presence of these cytokines neutrophils released increased amounts of superoxide anion radicals, with accompanied higher expression of phospho-p38 MAPK. These results confirm reports of other authors that respiratory burst in granulocytes may occur with the activation of the p38 MAPK pathway. The study of BENNA *et al.* (1997) showed that the protein $p47^{phox}$ (a component of the NADPH oxidase) is phosphorylated in activated neutrophils by p38.

In conclusion, the results suggest p38 MAPK pathway participation in iNOS activation in PMN and PBMC. Since the induction of two or more signaling pathways is necessary for total iNOS gene activation in human cells, further investigations in search of other pathways regulating iNOS expression in human PMN and PBMC are indispensable.

References

AJIZIAN S. J., ENGLISH B. K., MEALS E. A. 1999. Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. J. Infect. Dis. **179**: 939-944.

BARTOSZ G. 2003. The Oxygen Second Face. Wydawnictwo Naukowe PWN.

BECK K. F., EBERHARDT W., FRANK S., HUWILER A., MESSMER U. K., MUHL H. 1999. Inducible NO synthase: role in cellular signaling. J. Exp. Biol. **202**: 645-653.

BENNA J. E. L., PHAM MY-CHAN D., GAUDRY M., FAY M., MOREL F., HAKIM J., GOUGEROT-POCIDALO M. A. 1997. Phosphorylation of the Respiratory Burst Oxidase Subunit $p67^{phox}$ during Human Neutrophil Activation. Regulation by protein kinase c-dependent and independent pathways. J. Biol. Chem. **272**: 17204-17208.

BHUYAN D. K., BHUYAN K. C. 1994. Assessment of oxidative stress to eye in animal model for cataract. Methods Enzymol. **233**: 630-639.

CHAN E. D., DAVID W., RICHES H. 2001. IFN-γ + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and $p38^{mapk}$ in a mouse macrophage cell line. Am. J. Physiol. Cell. Physiol. **280**: C441-C450.

DA SILVA J., BENOIT P., JEAN-LUC M., WERNER L. 1997. Blockade of p38 Mitogen-activated Protein Kinase Pathway Inhibits Inducible Nitric-oxide Synthase Expression in Mouse Astrocytes. J. Biol. Chem. **272**: 28373–28380.

DE VERA M. E., SHAPIRO R. A., NUSSLER A. K., MUDGETT J. S., SIMMONS R. L., MORRIS S. M. J., BILLIAR T. R., GELLER D. A. 1996. Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. PNAS **93**: 1054-1059.

HAYDEN M. A., NAKAYAMA D. K. 1999. Cyclic nucleotides and inducible nitric oxide synthesis in pulmonary artery smooth muscle. J. Surg. Res. **82**: 222-227.

JABLONSKA E., PUZEWSKA W., CHARKIEWICZ M. 2006. Effect of IL-18 on leukocyte expression of iNOS and phospho-IkappaB in patients with squamous cell carcinoma of the oral cavity. Neoplasma **53**: 200-205.

KIEMER A. K., ANGELIKA M. 1998. Vollmar Autocrine Regulation of Inducible Nitric-oxide Synthase in Macrophages by Atrial Natriuretic Peptide. J. Biol. Chem. **273**: 13444-13451.

KOESLING D., BOHME E., SCHULTZ G. 1991. Guanylyl cyclases, a growing family of signal-transducing enzymes. FASEB J. **5**: 2785-2791.

KRONCKE K. D., FEHSEL K., KOLB-BACHOFEN V. 1995. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. Biol. Chem. Hoppe Seyler **376**: 327-43.

LIRK P., HOFFMANN G., RIEDER J. 2002. Inducible Nitric Oxide Synthase – Time for Reappraisal. Current Drug Targets – Inflammation & Allergy **1**: 89-108.

MOODLEY Y. P. 2002. The role of inducible nitric oxide in health and disease. Curr. Diagn. Pathol. **8**: 297-304.

PELLETIER M., RATTHE C., GIRARD D. 2002. Mechanisms involved in interleukin-15-induced suppression of human neutrophil apoptosis: role of the anti-apoptotic Mcl-1 protein and several kinases including Janus kinase-2, p38 mitogen-activated protein kinase and extracellular signal-regulated kinases-1/2. FEBS Lett. **532**: 164-170.

SCHULZ K., KERBER S., KELM M. 1999. Reevaluation of the Griess method for determining NO/NO2- in aqueous and protein-containing samples. Nitric Oxide **3**: 225-234.

SHINDO T., IKEDA U., OHKAWA F., KAWAHARA Y., YOKOYAMA M., SHIMADA K. 1995. Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. Cardiovasc. Res. **29**: 813-819.

STROOP S. D., BEAVO J. A. 1991. Structure and function studies of the cGMP-stimulated phosphodiesterase. J. Biol. Chem. **266**: 23802-23809.

WYMAN T. H., DINARELLO C. A., BANERJEE A., GAMBONI F., HIESTER A. A., ENGLAND K. M., KELHER M., SILLIMAN C. C. 2002. Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation. J. Leukoc. Biol. **72**: 401-409.

ZEMAN K., TCHORZEWSKI H., MAJEWSKA E. 1988. Simple and fast method of simultaneous isolation of lymphocytes and the polymorphonuclear cells from peripheral blood. Immunol. Pl. **13**: 217-225.