

H. TAHA<sup>1</sup>, A. GROCHOT-PRZECZEK<sup>1</sup>, H. WAS<sup>1</sup>, J. KOTLINOWSKI<sup>1</sup>, M. KOZAKOWSKA<sup>1</sup>, A. MAREK<sup>1</sup>,  
K. SKRZYPEK<sup>1</sup>, B. LACKOWSKA<sup>2</sup>, A. BALCERCZYK<sup>3</sup>, S. MUSTAFA<sup>4</sup>, J. DULAK<sup>1</sup>, A. JOZKOWICZ<sup>1</sup>

## MODULATION OF INFLAMMATORY RESPONSE BY PENTOXIFYLLINE IS INDEPENDENT OF HEME OXYGENASE-1 PATHWAY

<sup>1</sup>Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; <sup>2</sup>Department of Pathology, Oncology Center, Krakow, Poland; <sup>3</sup>Department of Molecular Biophysics, University of Lodz, Lodz, Poland; <sup>4</sup>Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Vienna, Austria.

**Objective:** It was reported that some effects of pentoxifylline (PTX) are mediated by heme oxygenase-1 (HO-1) induction. We investigated the role of HO-1 in anti-inflammatory activity of PTX. **Methods:** Experiments were performed in human and murine monocytes and endothelial cells and in HO-1 deficient mice. **Results:** PTX dose-dependently decreased expression of HO-1 in cell lines studied. As expected, PTX reduced also production of TNF. This effect was independent of HO-1 activity, as demonstrated in cells treated with HO-1 activators and inhibitors or in cells overexpressing HO-1. Moreover, inhibition of TNF was the same in human endothelial cells of different HO-1 genotypes, showing that PTX is similarly efficient in carriers of more and less active HO-1 promoter variants. In mice, PTX did not influence HO-1 expression, as measured in liver, kidney, spleen, heart, and skin. Accordingly, the response of PTX treated animals to LPS was the same in wild type and HO-1 deficient mice. PTX to a similar extent increased influx of leukocyte into peritoneal cavity, decreased production of TNF and reduced expression of VCAM-1 in vascular intima. **Conclusion:** PTX inhibits production of TNF and may decrease inflammatory reaction both *in vitro* and *in vivo*, but these effects are independent of HO-1.

**Key words:** *endothelial cells, monocytes/macrophages, cytokines, inflammation, HO-1 knockout mice*

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### INTRODUCTION

Pentoxifylline (PTX), 3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydropurine-2,6-dione, is commonly used as a vasodilator and anti-coagulant, which decreases the resistance of vessels and augments blood flow in ischemic tissues. It also improves erythrocyte flexibility, while reduces blood viscosity, plasma fibrinogen concentration, platelet aggregation, and thrombus formation (1, 2). Since the 1980s PTX has been prescribed for the treatment of symptomatic vascular dysfunctions, such as occlusive disease, intermittent claudication, venous ulcers, brain ischemia and vascular dementia (3-5). Additionally, it is applied to induce motility of spermatozoa during *in vitro* fertilization (6).

A very important effect of PTX is inhibition of inflammatory response, especially the downregulation of TNF. PTX suppresses TNF gene transcription, expression of TNF mRNA (7, 8), and secretion of TNF protein in macrophages and monocytes (9, 7, 10). Accordingly, the attenuation of morbidity and mortality in septic shock has been attributed to capability of PTX of suppressing the induction of TNF (11, 12).

PTX is a non-specific inhibitor of phosphodiesterases (PDEs), which decreases hydrolysis of both cAMP and cGMP, and thereby augments cyclic nucleotide-dependent signal transduction (13, 14). The exact mechanisms underlying the therapeutic effects of PTX are not fully recognized. It was postulated that decreased synthesis of TNF results from cAMP

elevation, protein kinase-A (PKA) activation and cytosolic calcium decline (15). On the other hand, PTX may attenuate oxidative stress by induction of manganese superoxide dismutase (MnSOD) (16), and direct scavenging of free-radicals (17). In consequence, it inhibits activity of NF $\kappa$ B and AP-1 transcription factors (18, 19), the effect regarded to be crucial in inhibiting the proinflammatory cytokines.

Interestingly, some studies suggested one more mechanism contributing to the activity of PTX. Experiments performed in fibrosarcoma L292 cells showed that PTX strongly induces the expression of heme oxygenase-1 (HO-1), a cytoprotective and anti-inflammatory enzyme, degrading heme to biliverdin, iron ions and carbon monoxide (CO) (20). Cytoprotective effects of PTX were imitated by incubation of cells with hemin (an HO-1 activator) or with CORM (a compound releasing CO), while reduced in the presence of ZnPPIX (zinc protoporphyrin-IX, an HO-1 inhibitor) (20). These observations strongly suggested that effects of PTX may at least partly depend on the induction of HO-1.

The similarities between cellular effects of HO-1 upregulation and PTX treatment are striking. Thus, both PTX and HO-1 have anti-inflammatory properties, as exemplified by inhibition of IL-1 $\beta$ , IL-6, IL-8, TNF or GM-CSF and induction of IL-10 (21-23). Furthermore, they both were shown to reduce proliferation, migration and adhesion of leukocytes to endothelial cells through inhibition of CD25 and ICAM-1 (24, 25). Potential involvement of HO-1 in regulation of PTX

signaling appears to be particularly important, because of the human HO-1 promoter polymorphism. The presence of less active alleles, resulting in lower HO-1 activation, was demonstrated as a factor increasing risk of cardiovascular complications, at least in some populations of patients (26-29). Thus, one can expect that also efficacy of PTX may be influenced by this polymorphism and may be low in patients with less active HO-1 variants.

The aim of our paper was to verify this supposition. Therefore we investigated the involvement of HO-1 pathway in anti-inflammatory activities of PTX *in vitro* (in murine and human macrophages and endothelial cells), and *in vivo*, in murine model of endotoxemia. The obtained results clearly show that PTX acts independently of HO-1.

## MATERIALS AND METHODS

### Reagents

PTX, triton X-100, phenylmethyl sulfonyl fluoride, leupeptin, aprotinin, bicinchonic acid protein assay kit, BCIP/NBT alkaline phosphate substrate solution, HEPES, MTT, LPS, hemin and G418 antibiotic were purchased from Sigma (Poznan, Poland). Tin protoporphyrin-IX (SnPPIX) was from Porphyrin Products (Logan, UT, USA). The total RNA extraction kit, reverse transcription system, PCR system, agarose, and Cytotox-96 NonRadioactive Cytotoxicity Assay were obtained from Promega (Madison, WI, USA). SuperFect transfection reagents were purchased from Qiagen (Hilden, Germany). ELISA kits for human and mouse TNF were obtained from R&D Systems (Minneapolis, MN, USA). BrdU incorporation ELISA was obtained from Roche Diagnostics (Mannheim, Germany). ELISA kit for human HO-1 and Rabbit anti-HO-1 polyclonal IgG were obtained from Stressgen Biotechnologies (Victoria, BC, Canada). DyNAmo™ HS SYBR® Green qPCR kit was purchased from FinnZymes (Espoo, Finland). Nitrocellulose membrane HybondECL was procured from Amersham Pharmacia Biotech (Boston, MA). Mouse anti-tubulin polyclonal IgG was purchased from Calbiochem. Anti-mouse monoclonal HRP-linked IgG and anti-rabbit monoclonal HRP-linked IgG were obtained from Cell Signaling Technology (Danvers, MA, USA). Expression plasmid pcDNA3.1 was obtained from Invitrogen (San Diego, CA, USA), while heparin and endothelial cell growth supplement from Upstate Biotechnology (Charlottesville, VA, USA). Adeno-X adenoviral expression system, adeno-X rapid titer ELISA kit and anti-VCAM-1 antibodies were purchased from BD Bioscience (Erembodegem, Belgium), while TransAM ELISA for measurements of NFκB and AP-1 binding activity were from Active Motif (Rixensart, Belgium). All other reagents were procured from PAA Laboratories, GmbH, (Pasching, Austria).

### Cell culture

The human monocyte U937 cell line was kindly provided by Dr. Guenter Weigel, Vienna, Austria. The cells were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (10 µg/ml) and L-glutamine (2 mM). Both murine macrophage J774 cell line (ATCC, Manassas, VA, USA) and murine brain microvascular endothelial cell-1 line MBEC-1 (kindly provided by Dr. Joanna Bereta, Krakow, Poland) were cultured in DMEM-HG medium supplemented with 10% FCS, L-glutamate (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). HUVEC were freshly isolated from

human umbilical veins of newborn babies by collagenase digestion. HUVEC were grown in M199 medium supplemented with 20% FCS, HEPES (20 mM), L-glutamine (2 mM), heparin/endothelial cell growth supplement (30 ng/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells of the second to fourth passages were used in experiments. Human embryonic kidney cells (HEK 293) obtained by courtesy of Dr. Stefan Kochanek (Ulm, Germany), and used for production of adenoviral vectors, were maintained in  $\alpha$ -MEM medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (10 µg/ml).

### Stable transfection

Cells were transfected with pcDNA3.1+ plasmid vector harboring human HO-1 (pcDNA-HO-1) or with empty plasmid (pcDNA) using SuperFect® Transfection Reagent. Procedure was carried out according to vendor's instruction, with 0.5 µg of DNA and 2.5 µl of SuperFect per one well in a 24-well plate. Transfected cells were selected in the presence of G418 antibiotic (1 mg/ml) for 3 weeks. Presence of stably introduced plasmids and overexpression of transgene were confirmed with PCR, RT-PCR and western-blotting, respectively. Cells were then cultured with G418 at the dose of 200 µg/ml.

### Transduction with adenoviral vectors

Endothelial cells were infected with Ad-HO-1 (harboring human HO-1 cDNA) or Ad-GFP (containing control GFP gene) at the dose of 10 multiplicity of infection (MOI). Vectors were poured into the cells in 30% volume of normal amount of media. The remaining medium was added after 2 h. After 24 hours, medium was changed and cells were stimulated with PTX (100 µM and 1 mM) for 24 h.

### Cell viability assay

Cell viability was assessed by colorimetric measurement of lactate dehydrogenase (LDH) release, and MTT reduction assays according to the manufacturers' protocols.

### Real-time RT-PCR

Total RNA was isolated from cells by acid guanidinium thiocyanate-phenol-chloroform extraction as described earlier (30). Reverse transcription with oligo-dT primers was performed on 2 µg of total RNA, for 1 hr at 42°C, using MMLV reverse transcriptase, according to vendor's instruction. Real-time polymerase chain reactions using qRT-PCR DyNAmo HS SYBR Green qPCR Kit, were performed in samples containing 50 ng cDNA, 0.5 µl of primers, 7.5 µl of DyNAmo SYBR Green master-MIX, and nuclease-free water added to 15 µl of total volume. Reaction was carried out using the following protocol: 95°C for 10 min, followed by 40 three-step cycles 95°C for 30 s, 60°C for 60 s, 72°C for 45 s, and by 72°C for 5 minutes as a final elongation. The primers specific for HO-1 (5' GTG GAA MCG CTT YAC RTA GYG C 3' and 5' CTT TCA GAA GGG YCA GGT GWC C 3'), and for housekeeping EF2 (5' GCG GTC AGC ACA ATG GCA TA 3' and 5' GAC ATC ACC AAG GGT GTG CAG 3'). Relative quantification of gene expression was calculated based on  $\Delta\Delta C_T$  method.

### ELISA assays

ELISAs for human and murine TNF (measured in conditioned media or in plasma) and for human HO-1 (measured in cell lysates) were performed according to the vendors' protocols.

### Western blotting

Expression of HO-1 protein was checked in cells cultured in six-well plates or in murine organs. Cells or tissues were lysed in ice-cold lysis buffer (1% Triton X-100, 1 µg/ml phenylmethyl sulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) and centrifuged for 20 minutes, 8000g, at 4°C. Clear supernatants were collected, and protein samples (15-20 µg/well) were subjected to electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel followed by transfer to nitrocellulose membrane HybondECL. Then, membranes were probed with polyclonal antibodies against HO-1, treated with secondary antibodies linked with horseradish peroxidase (diluted 1:10,000 in Tris-buffered saline with 3% albumin), and visualized using BCIP/NBT blue liquid substrate.

### Animal experiments

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Jagiellonian University. All mice were maintained on a standard diet and water *ad libitum*.

Eight week old C57BL/6 were used to assess the expression of HO-1 after treatment with PTX. To this aim, animals were injected intraperitoneally (i.p) with PTX (10 mg/kg body weight, total volume of 100 µl) for 7 consecutive days. Control mice received the same volume of vehicle (PBS). Then, mice were sacrificed and HO-1 protein in the liver, kidney, spleen, heart and skin was analyzed using western-blot.

Second experiment was designed to determine the role of HO-1 in PTX activities. Here, eight week old HO-1<sup>-/-</sup> and HO-1<sup>+/-</sup> mice of C57BL/6×FVB background (obtained thanks to courtesy of Dr. Anupam Agarwal, Birmingham, USA) were treated with PTX as described above, and then subjected to endotoxemia induced by i.p. injection of LPS (5 µg/kg body mass). After 1.5 hours, animals were sacrificed, and the peritoneal cavities were washed with 1 ml of Ca/Mg-free PBS. Aliquots of the lavage fluids were stained in Turk's solution (0.01% crystal violet in 3% acetic acid), and total infiltrating leukocytes were counted using a Burkner chamber under a light microscope and then seeded in a 24 well plate (100,000/well) in 1 ml of RPMI medium supplemented with 10% FCS. Concentrations of cytokines released to the culture media were measured 24 h later, using ELISA. Proportion of different leukocyte populations was assessed using flow cytometry and FSC/SSC analysis. Additionally, mice were bled for measurement of plasma TNF, and their aortas were isolated for immunohistochemical staining.

### Immunohistochemistry

Frozen aorta sections (8 µm) were air-dried and fixed for 10 minutes in acetone at 4°C. After blocking of nonspecific binding sites with 10% goat serum (in 0.1% Triton X-100 and 0.05% tween in PBS solution for 90 minutes), slides were incubated with primary antibodies (rat anti-mouse CD106 (VCAM-1) diluted 1:50) overnight at 4°C. After a PBS rinse, the slides were incubated with anti-rat IgG-PE conjugated antibodies diluted 1:250 in PBS for 45 min at room temperature. After a PBS rinse, the slides were air-dried and mounted.

### Statistical analysis

*In vitro* experiments were performed in duplicates or triplicates and repeated at least three times. *In vivo* experiments were carried out in 5 animals per group. Results are presented as mean ± SD. Statistical significance was determined using

student t-test (comparison of two groups) or ANOVA followed by Tukey test (comparison of more than two groups).

## RESULTS

### *Expression of HO-1 in cells incubated with PTX*

Murine monocytes (J774), human monocytes (U937), murine endothelial cells (MBEC-1), and human primary endothelial cells (HUVEC) were incubated with PTX at the wide range of concentrations from 0.1 µM to 1 mM for 24 h. Even the highest doses applied did not influence cell viability, as checked using LDH activity and MTT reduction assays (data not shown).

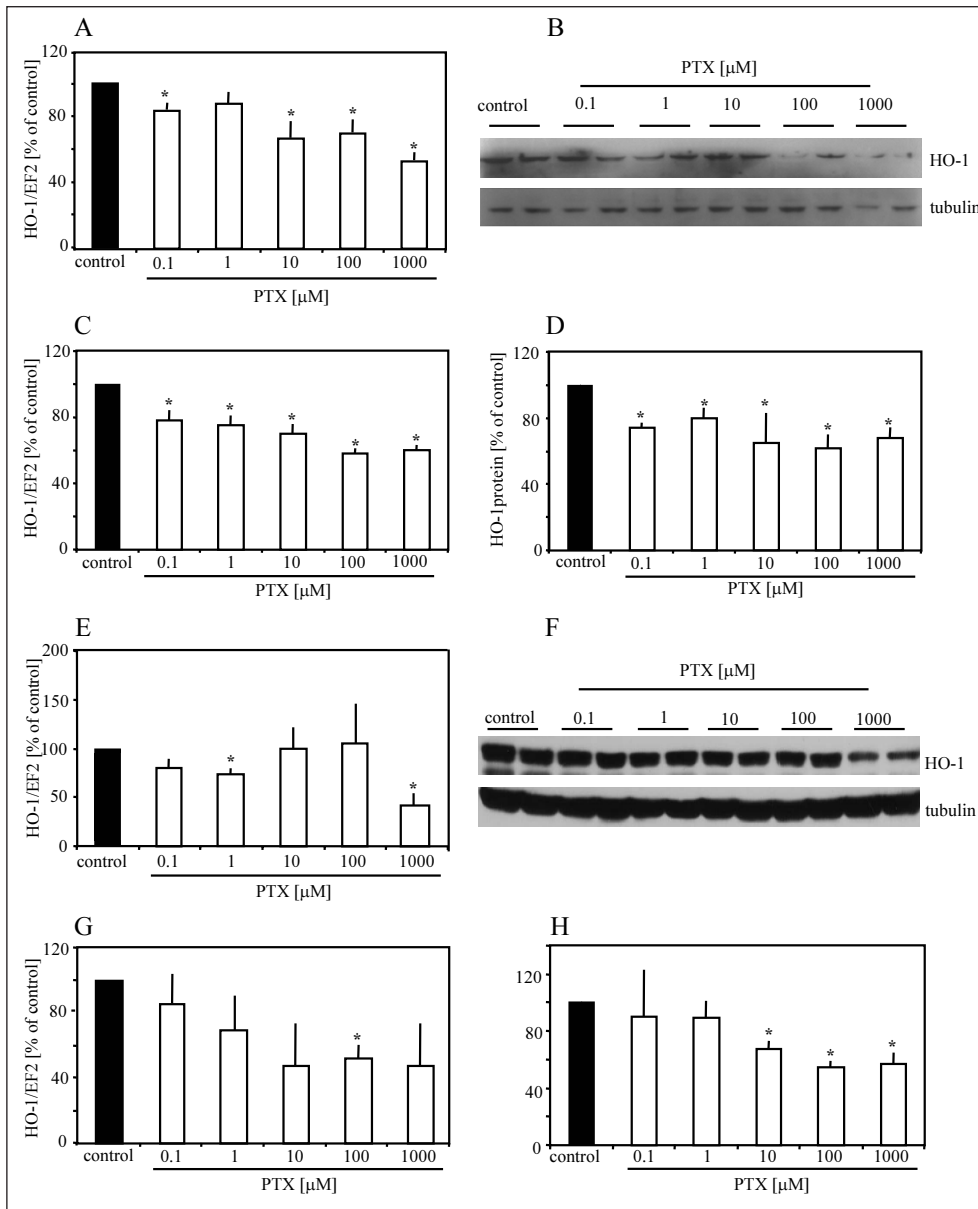
Effect of PTX on expression of HO-1 was measured at mRNA level by qRT-PCR and at protein level using ELISA (for human cells) or western-blotting (for murine cells). Surprisingly, we did not observe an induction of HO-1 in any cell line studied, except some experiments where the very high and potentially toxic doses of PTX (3 mM or 10 mM) were used. In such extreme conditions HO-1 mRNA expression could be induced up to 3-fold in endothelial cells and up to 40-fold in monocytes, but the results were very variable. Instead, within the range of non toxic concentrations (0.1 µM - 1 mM), PTX dose-dependently decreased expression of HO-1, to about 40-50% of control values in all cell lines, as demonstrated both at mRNA and protein levels (*Fig. 1*). The same modest downregulation of HO-1 expression by PTX was observed after 6 h incubation period, as well as in cells treated concomitantly with typical HO-1 inducers, namely with 10 µM hemin, 10 µM cobalt protoporphyrin (CoPPiX), or 10 µM 15-deoxy-Δ<sup>12,14</sup>-prostaglandin-J<sub>2</sub> (15d-PGJ<sub>2</sub>; data not shown).

### *Anti-inflammatory effects of PTX in cells of different HO-1 activity*

To determine whether modulation of HO-1 activity may influence the anti-inflammatory action of PTX, we checked the effect of PTX on synthesis of TNF in cells preincubated with 10 µM hemin (HO-1 activator) or 10 µM tin protoporphyrin-IX (SnPPiX, HO-1 inhibitor).

MBEC-1 did not produce measurable amounts of TNF protein, therefore the experiments were performed in U937, J774, and HUVEC cells. Mean concentrations of TNF released to the culture media by control cells after a 24 h incubation were 19.4 ± 2.3 pg/ml, 26.2 ± 3.4 pg/ml, 20.1 ± 3.7 pg/ml, respectively. In all cell lines studied, PTX at the concentrations of 100 µM and 1 mM significantly decreased synthesis of TNF, as measured by ELISA. The extent of reduction was similar, regardless of stimulation or inhibition of HO-1 activity, indicating that HO-1 does not play a role in PTX signaling (*Fig. 2*). Instead, PTX at the concentrations of 100 µM and 1 mM downregulated DNA binding activity of NFκB and AP-1 (data not shown). This suggests that the reduced expression of TNF may result from the well-known inhibitory effect PTX on those transcription factors, as described in the other experimental models (18, 19). Interestingly, cells treated with hemin produced similar amounts of TNF as control ones. This may be associated with relatively weak induction of HO-1 (up to three fold at mRNA level) in response to hemin. Inhibition of HO-1 activity by SnPPiX resulted in weak but statistically significant increase in synthesis of TNF (*Fig. 2*), supporting the involvement of HO-1 pathway in regulation of TNF expression.

Next, to avoid possible non-specific effects of hemin or SnPP, we checked the influence of PTX on TNF synthesis in cells genetically modified to overexpress HO-1. To this aim we established U937 and J774 cell lines overexpressing HO-1 after a stable transfection with pcDNA-HO-1 plasmid. Control cell



**Fig. 1.** Effect of PTX (0.1  $\mu$ M – 1 mM, 24 h) on expression of HO-1 mRNA (qRT-PCR) and protein (ELISA or western-blotting) in cultured cells. A – J774 cells, HO-1 mRNA; B – J774 cells, HO-1 protein; C – U937 cells, HO-1 mRNA; D – U937 cells, HO-1 protein; E – MBEC-1 cells, HO-1 mRNA; F – MBEC-1, HO-1 protein; G – HUVEC, HO-1 mRNA; H – HUVEC, HO-1 protein. Each bar represents mean  $\pm$  SD of at least three experiments. \* -  $P < 0.05$  in comparison with control. Pictures show representative western-blot.

lines were stably transfected with the empty pcDNA construct. To overexpress HO-1 in HUVEC cells we transduced them using adenoviral vectors harboring HO-1 cDNA or control GFP cDNA (transfection efficacy was estimated as ~80%). In all cases strong overexpression of HO-1 has been confirmed by western blot or ELISA (data not shown). Likewise pharmacological modulation of HO-1 activity, also genetic overexpression of HO-1 did not influence inhibitory effect of PTX on synthesis of TNF (Fig. 3). Noteworthy, cell lines stably overexpressing HO-1 released less TNF than their control counterparts. Similar tendency was observed in HUVEC transduced with AdHO-1 vectors, but here this effect did not reach statistical significance.

We also decided to investigate the effect of HO-1 on the postulated cytoprotective potential of PTX (20). To this aim we treated control (GFP expressing) or HO-1 overexpressing endothelial cells with PTX (100  $\mu$ M) and then with high dose of  $H_2O_2$  (100  $\mu$ M) for 24 h. Both in the presence or absence of PTX, HO-1 showed similar tendency to protect HUVEC cells, whereas in MBEC-1 this effect was negligible. PTX (100  $\mu$ M) did not show any cytoprotection regardless of the HO-1 expression level (Fig. 4).

Finally, we checked whether HO-1 promoter polymorphism, characteristic for human populations, may modulate the efficacy of PTX-mediated inhibition of TNF production. Here we employed HUVEC cells of different HO-1 promoter genotype: i) cells carrying at least one allele with short sequence of GT repeats ( $N \leq 25$ , allele S) or ii) cells without allele S. As demonstrated using ELISA, mean basal concentrations of HO-1 protein in HUVEC carrying S allele was significantly higher ( $792 \pm 108$  pg/mg of total protein) than in the cells without S allele ( $549 \pm 74$  pg/mg of total protein). In both groups expression of HO-1 was similarly increased in response to LPS. Inhibitory effects of PTX on TNF production, observed both in resting and LPS-stimulated cells were comparable, regardless of the genotype of HO-1 promoter (Fig. 5).

#### *Expression of HO-1 in mice treated with PTX*

To determine the effect of PTX on HO-1 expression not only in cell cultures but also *in vivo*, we decided to inject C57Bl mice with PTX (10 mg/kg, i.p., 100  $\mu$ l/mouse), for seven consecutive days. Control animals were injected with the same volume of

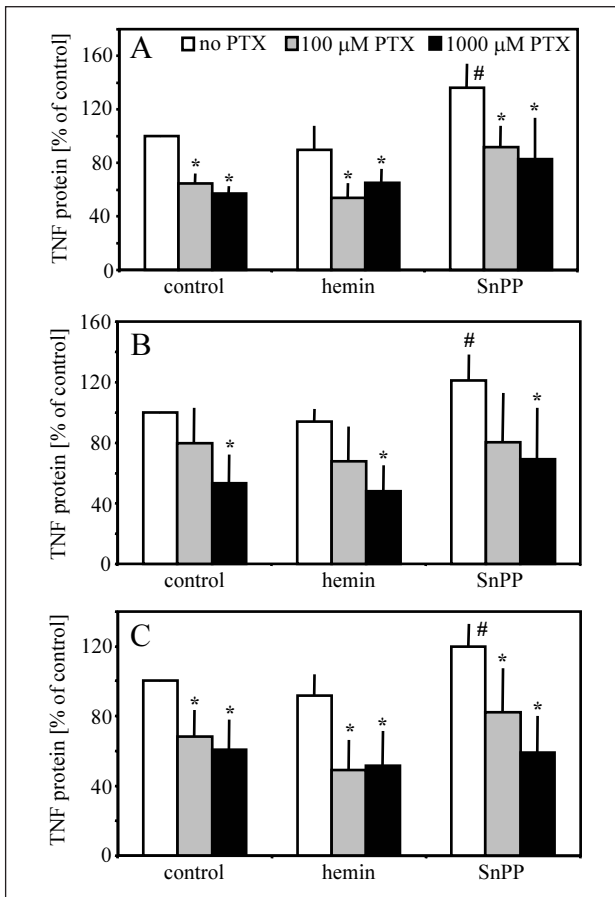


Fig. 2. Effect of PTX (100 μM and 1 mM, 24 h) on synthesis of TNF in cells preincubated with hemin (10 μM, HO-1 activator) or SnPP (10 μM, HO-1 inhibitor), measured by ELISA. A – U937 cells; B – J774 cells; C – HUVEC cells. Each bar represents mean ± SD of at least three experiments, presented as a percentage of control value, obtained for intact cells. \* - P < 0.05 in comparison with cells incubated without PTX; # - P < 0.05 in comparison to intact cells.

PBS. Then, mice were sacrificed and expression of HO-1 protein in their livers, spleens, kidneys, hearts, and skins was analyzed using western-blotting. As shown in Fig. 6, injections of mice with PTX did not influence the expression of HO-1 in any organ studied.

#### Modulation of inflammatory response by PTX in mice of different HO-1 genotype

To conclusively establish whether HO-1 plays any role in anti-inflammatory activities of PTX *in vivo*, we performed experiments in C57Bl/FVB mice devoid of HO-1 gene (HO-1 KO) and in mice of the same genetic background, but with normal level of HO-1 (HO-1 WT). KO and WT mice were injected i.p. with PTX (10 mg/kg, 100 μl/mouse) or with PBS (100 μl) for seven consecutive days. Then they were injected i.p. with LPS (5 μg/kg) for 1.5 h. As an additional control served the intact, untreated WT and KO animals.

After sacrificing the mice, we first checked number of leukocytes in their peritoneal cavities. Calculating the cells in the Burker chamber showed that intact mice had relatively small amounts of peritoneal leukocytes, regardless of the HO-1 status (672,000 ± 312,000 cells and 599,000 ± 369,000 cells in WT and

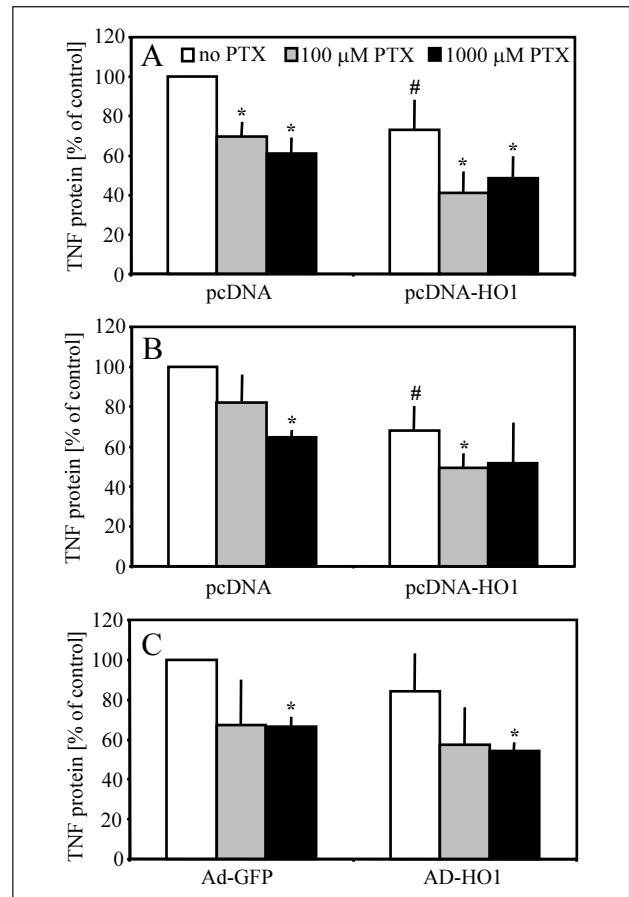
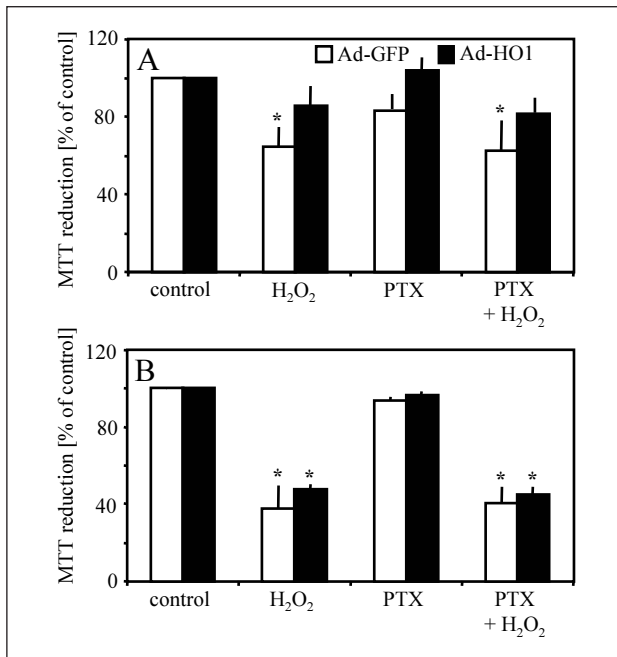


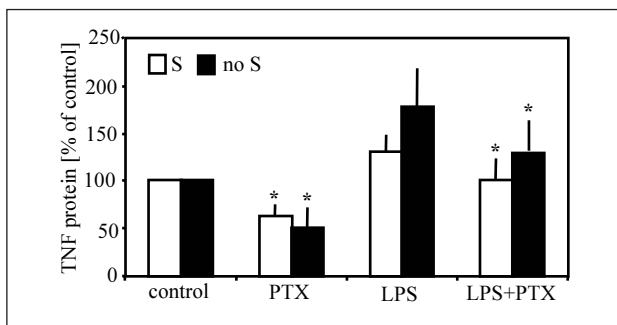
Fig. 3. Effect of PTX (100 μM and 1 mM, 24 h) on synthesis of TNF in cells overexpressing HO-1, measured by ELISA. A – U937 cells stably transfected with pcDNA-HO-1 or with control, empty pcDNA plasmid; B – J774 cells stably transfected with pcDNA-HO-1 or with control, empty pcDNA plasmid; C – HUVEC cells transduced with adenoviral vectors harboring HO-1 (Ad-HO-1) or control GFP cDNA (Ad-GFP). Each bar represents mean ± SD of at least three experiments, presented as a percentage of control values. \* - P < 0.05 in comparison with cells incubated without PTX; # - P < 0.05 in comparison to control cell (transfected with empty plasmid or transduced with Ad-GFP vectors), incubated without PTX.

KO, respectively). Intraperitoneal injection with LPS led to the rapid increase in leukocyte number already in 1.5 h. This effect was significantly less pronounced in the HO-1 deficient individuals (3,140,000 ± 1,177,709 cells and 1,256,000 ± 95,433 cells in WT and KO, respectively). As shown in Fig. 7A, also PTX considerably influenced leukocyte motility – infiltration of peritoneal cavity in response to LPS was much stronger in animals treated with PTX than in those injected with PBS. Flow cytometric analysis showed that these differences occurred in lymphocytes and granulocytes but not in a monocyte population (Fig. 7B). Expression of HO-1 did not influence effects of PTX, which were very similar in the WT and KO animals (Fig. 7).

We measured also concentration of TNF in plasma of mice and production of TNF by peritoneal leukocytes, cultured *ex vivo* for 24 h. In intact animals of both HO-1 genotypes, TNF in the blood was undetectable using ELISA method. However, at 1.5 h after i.p. injection with LPS, mice had significantly increased level of TNF. Concentration of TNF was higher in HO-1 deficient mice (1,819.45 ± 642.59 pg/ml), than in their wild-type



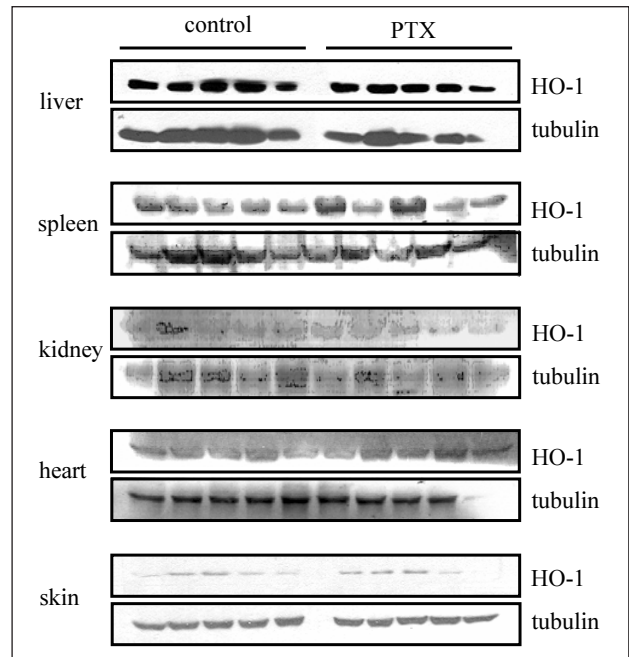
**Fig. 4.** Effect of PTX (100  $\mu$ M) on viability of endothelial cells transduced with adenoviral vectors harboring HO-1 (Ad-HO-1) or control GFP cDNA (Ad-GFP), treated with H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M, 24 h). A – HUVEC; B – MBEC-1. MTT reduction assay. Each bar represents mean  $\pm$  SD of at least three experiments, presented as a percentage of control values. \* - P < 0.05 in comparison with control.



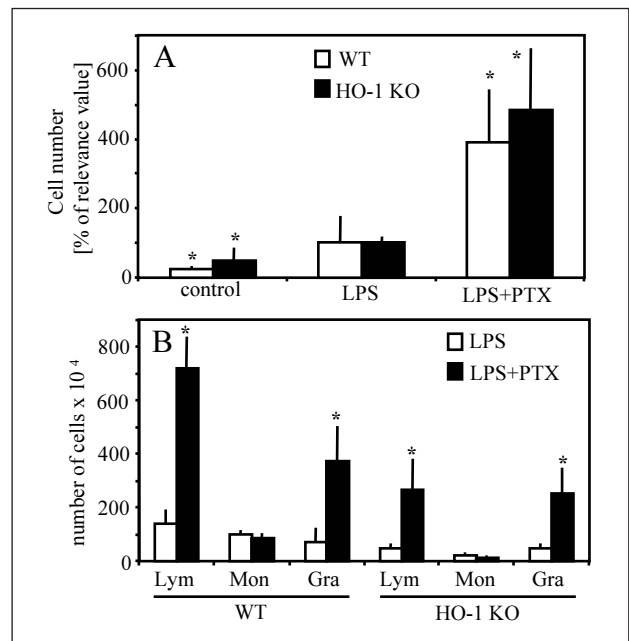
**Fig. 5.** Effect of PTX (100  $\mu$ M) on synthesis of TNF in control or LPS-stimulated (100 ng/ml) HUVEC cells of different genotype of HO-1 promoter: carrying at least one allele with short sequence of GT repeats (S) or without such alleles (no S). Each bar represents mean  $\pm$  SD of at least 30 cell batches, presented as a percentage of control values. \* - P < 0.05 in comparison with control.

counterparts (636.08  $\pm$  444.09 pg/ml). One-week exposure to PTX resulted in a tendency to decrease the production of TNF in animals responding to LPS, as demonstrated by lower concentration of the cytokine in the blood (Fig. 8A). The level of this inhibition did not depend on expression of HO-1 and was comparable in WT and KO animals.

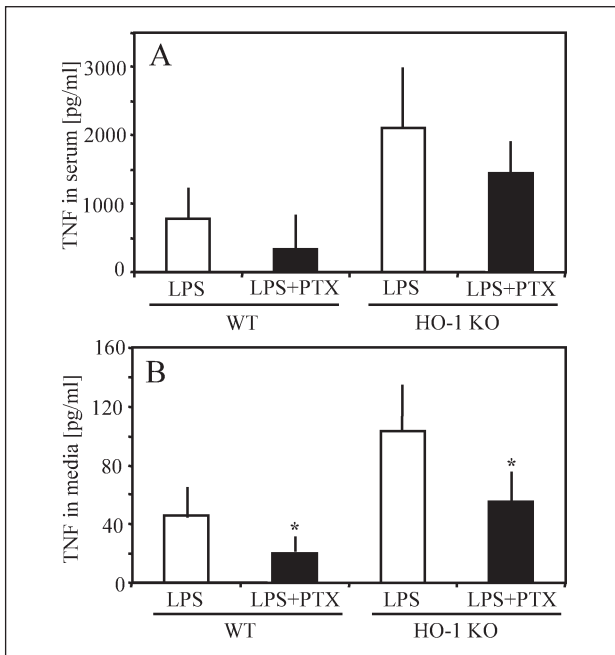
Accordingly, the leukocytes harvested from the peritoneal cavity of LPS-injected animals produced measurable amounts of TNF, and production was stronger in the cells obtained from HO-1 deficient mice. Importantly, generation of TNF was significantly decreased in leukocytes collected from PTX-treated animals. Again, inhibitory effect of PTX was independent of HO-1 genotype and was similar in HO-1 KO and



**Fig. 6.** Expression of HO-1 protein in the liver, spleen, kidney, heart and skin of mice injected i.p. with PTX (10 mg/kg) for seven consecutive days. Control – mice injected with PBS. Western blot; 5 animals per group.



**Fig. 7.** Effect of PTX on leukocytes infiltration in peritoneal cavity of the wild type and HO-1 deficient mice injected with LPS (5  $\mu$ g/kg, 1.5 h), pretreated or not with PTX (10 mg/kg, i.p. for seven consecutive days). A – Total leukocyte number, calculated using Burker chamber and presented as a percentage of reference value (number of cells after treatment with LPS only); control – intact animals, non-injected with LPS. B – Number of lymphocytes, monocytes and granulocytes in peritoneal cavity. Cells were calculated using Burker chamber, while proportion of leukocyte populations was determined using flow cytometer and FSC/SSC analysis. Each bar represents mean  $\pm$  SD of measurements for 5 animals.\* - P<0.05 in comparison with mice injected with LPS alone.



**Fig. 8.** Effect of PTX on production of TNF by the wild type and HO-1 deficient mice injected with LPS (5  $\mu\text{g}/\text{kg}$ , 1.5 h), pretreated or not with PTX (10 mg/kg, i.p. for seven consecutive days). A – Concentration of TNF in the blood plasma. B – Concentration of TNF released to culture medium by peritoneal leukocytes incubated *ex vivo* (100,000/ml) for 24 h. TNF was measured by ELISA. Each bar represents mean  $\pm$  SD of measurements for 5 animals. \* -  $P < 0.05$  in comparison with mice injected with LPS alone.

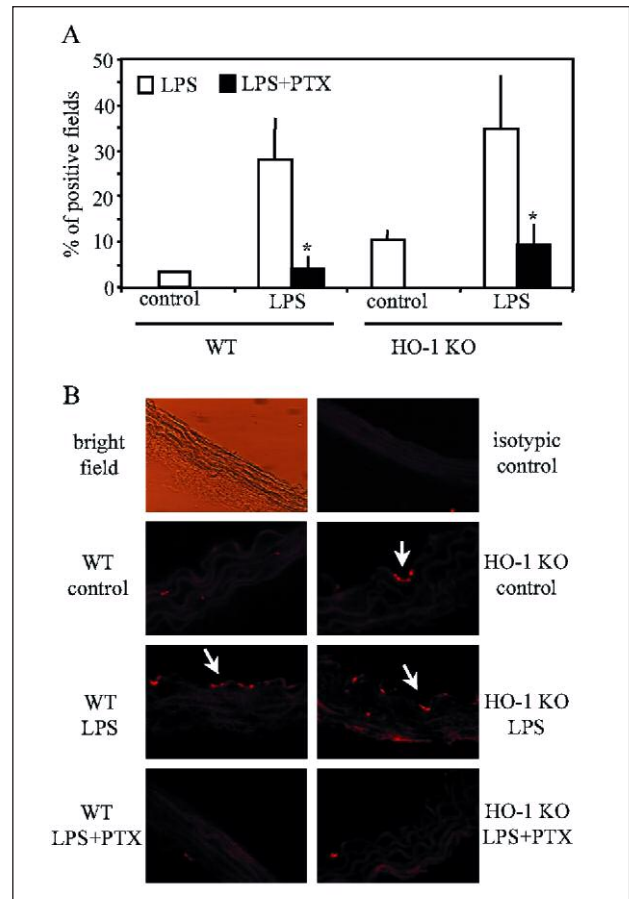
HO-1 WT cells.

Finally, we checked the effect of PTX on the inflammatory reaction in the blood vessels of LPS-injected mice. To this aim we collected the aortas of intact or LPS-injected animals of both genotypes to perform an immunohistochemical staining for VCAM-1 expression. As shown in *Fig. 9*, lack of HO-1 gene was associated with stronger expression of VCAM-1 in intact animals. At 1.5 h after i.p. injection with LPS, VCAM-1 in aortal intima was upregulated, to similar values in HO-1 deficient and wild-type mice. PTX very potently reduced expression of VCAM-1. Like in all previous experiments, its activity was independent of HO-1 and the extent of reduction was comparable in mice of both HO-1 genotypes (*Fig. 9*).

## DISCUSSION

Starting point to our study was the hypothesis based on experiments done in L292 fibroblasts, that PTX in the range of 0.1 mM to 1.0 mM strongly induces expression of HO-1 and that cytoprotective action of PTX is mediated *via* HO-1 pathway, possibly through elevated synthesis of CO (20). Our aim was to investigate the role of HO-1 in anti-inflammatory and cytoprotective effects of PTX in monocytes and endothelial cells.

HO-1, a cytoprotective, anti-inflammatory and proangiogenic enzyme, can be induced by numerous factors, including important drugs, such as rapamycin, statins, dopamine, probucol, or even aspirin (31-33). It has been suggested in many papers that activation of HO-1 pathway, leading to removal of prooxidative heme and increased



**Fig. 9.** Effect of PTX on expression of VCAM-1 protein in aorta of wild type and HO-1 deficient mice injected with LPS (5  $\mu\text{g}/\text{kg}$ , 1.5 h), pretreated or not with PTX (10 mg/kg, i.p. for seven consecutive days). Immunohistochemical staining. A – Percent of fields with positive staining per one specimen. Each bar represents mean  $\pm$  SD of measurements for 5 animals. \* -  $P < 0.05$  in comparison with mice injected with LPS alone. B – representative pictures.

production of CO and biliverdin, may contribute to therapeutic effects of these medicines (34). In most cases, however, these presumptions derived from the cell culture experiments only, where relatively high doses of the drugs were used. Thus, studies in animal models, especially employing HO-1 deficient mice are desired to validate the actual role of HO-1.

Therapeutic plasma levels of PTX and its active metabolites range from  $10^{-6}$  to  $10^{-4}$  M (5). Therefore, *in vitro* we used the concentrations of  $10^{-7}$  –  $10^{-3}$  M, while *in vivo* we applied PTX at the dose of 10 mg/kg, relevant to clinical trials (35). First, we decided to check the effect of PTX on expression of HO-1 in cultured murine and human monocytes and endothelial cells, and in several organs of PTX-injected mice.

The obtained results show that PTX slightly decreases expression of HO-1 in all cell lines studied, both at mRNA and protein levels. Inhibitory effects of PTX was observed also if HO-1 was induced by hemin, CoPPIX or 15d-PGJ<sub>2</sub>. We detected an upregulation of HO-1 only in some experiments, when very high, cytotoxic doses of PTX were used (data not shown). The reason for such discrepancy with results published earlier (20) is not clear and may be associated with the different cell types used.

It seems, however, that PTX-mediated inhibition of HO-1 expression is not surprising, as the promoter of HO-1 contains

binding sites for NF $\kappa$ B and AP-1 (36), the transcription factors attenuated by PTX (18, 19), as demonstrated also in our experimental settings. In accordance, PTX significantly decreased LPS-induced upregulation of placental HO-1 in mice (37). Similar inhibition of intestinal and lung HO-1 generation was also reported in rats treated with hypertonic saline/PTX infusion (38, 39). In our experiments PTX injected i.p. for seven days to the mice, although effective in reduction of TNF generation, did not influence HO-1 expression in kidney, liver, skin, spleen and heart.

Then, to investigate whether different levels of HO-1 activity may influence anti-inflammatory efficacy of PTX, we employed three experimental models: i) monocytes and endothelial cells incubated in the presence of pharmacological inducer or inhibitor of HO-1, ii) cells stably or transiently overexpressing HO-1, iii) mice with the knocked-out HO-1 gene. Results obtained in all models univocally evidence that HO-1 pathway does not play a role in PTX signaling.

Thus, in contrast to the results reported by Oh and colleagues (20), we found that preincubation of cells with the HO-1 activator hemin or with HO-1 inhibitor SnPP neither mock nor modulate activity of PTX. We also did not observe any influence of HO-1 overexpression on effects of PTX in cells stably transfected with pcDNA-HO-1 plasmid or transiently transduced with adenoviral vectors harboring HO-1 cDNA. In all experimental settings we demonstrated exactly the same inhibition of TNF production. We also checked the cytoprotective effects of PTX in endothelial cells treated with H<sub>2</sub>O<sub>2</sub> to investigate the potential role of HO-1 in this activity, but we did not obtain any protection by PTX, regardless of the HO-1 expression. Thus, we conclude that involvement of HO-1 in PTX action is rather limited to some cell lines only, and HO-1 cannot be regarded as a mediator of PTX signaling.

Importantly, this conclusion is confirmed by experiments carried out in the animal model of endotoxemia. Seven-day pretreatment with PTX strongly inhibited LPS-induced production of TNF, reduced endothelial expression of VCAM-1, and augmented peritoneal infiltration of leukocytes. The observed effects were very similar in HO-1 knock-out and wild type mice, giving strong evidence that HO-1 is not involved in PTX activities. Worth mentioning that apart from analysis of TNF we performed similar *in vitro* and *in vivo* measurements of IL-1 $\beta$ , IL-6, IL-8/KC, ICAM-1, and E-selectin. Again, we found that HO-1 did not modulate effects of PTX on expression of any cytokine (data not shown).

TNF, the major target in anti-inflammatory effects of PTX, is crucial in triggering the lethal effects of septic shock (40). PTX not only reduces TNF generation *in vitro* and *in vivo* (10, 18, 41), but also potently inhibits endotoxin-induced neutrophil degranulation (42), and decreases the binding of activated T cells to endothelial cells (43). As costimulation of neutrophils and cytokines may play an important role in organ injury in sepsis (42), PTX was shown to be of great benefit in different models of animal septic shock (11, 12, 18, 44). Pretreatment of mice or rats with PTX resulted also in the reduction of liver, kidney and lung injury during endotoxemia (12, 45, 46), and in prevention of hepatitis (47). Finally, experiments performed in rats suggested also that PTX may exert gastroprotective effects, both through inhibition of TNF synthesis and attenuation of oxidative stress, and through enhancement of gastric microcirculation (48, 49). Our study confirms anti-inflammatory effect of PTX in acute endotoxemia, as illustrated by decreased generation of TNF and VCAM-1 in mice.

Moreover, we found that pretreatment with PTX strongly increased number of cells recruited in the peritoneal cavity in response to LPS, either in the wild-type or in HO-1 deficient mice. Similar effects were observed earlier in mice after cecal

ligation and puncture, where PTX increased cell influx to the peritoneum (50). It is in agreement with opinion that PTX acts on the activated neutrophils, increasing their chemotaxis (51). In fact, PTX was shown to restore impaired chemotactic efficiency of neutrophils incubated with serum of patients subjected to IL-2 therapy (52). Our data suggest that PTX increases peritoneal infiltration of both granulocytes and lymphocytes 1.5 h after LPS stimulation. Very early tissue sequestration of neutrophils in response to LPS (even 10 minutes after intravenous injection) were observed earlier (53). Interestingly, number of infiltrating cells is lower in HO-1 knock-outs, what might be associated with weakened migratory capabilities of HO-1 deficient cells (54).

Today, PTX is widely used for treatment of occlusive arterial diseases and cerebrovascular disorders (1, 55, 56). It has been also suggested as a potential drug in treatment of sepsis. Actually, inhibition of TNF and better cardiopulmonary function was demonstrated in several clinical trials (57-61), and some reports showed even improved survival of septic patients (62). PTX was also reported to be helpful in preventing the systemic inflammation in patients undergoing cardiopulmonary bypass (63-65), and in reduction of TNF synthesis in patients with severe *Plasmodium falciparum* malaria (66). Clinical results are, however, ambiguous and some trials failed to show any beneficial effects of PTX in sepsis (67) and malaria (68) or even in decrease of TNF production (35).

According to earlier postulate (20) that PTX activity is mediated by HO-1 pathway, one could suppose that efficacy of PTX as an anti-inflammatory agent depends on polymorphism of HO-1 promoter resulting in different HO-1 activity in patients enrolled. This could be a possible explanation for the discrepant results of clinical trials. Our studies, performed in endothelial cells of different genotypes excluded this possibility, showing that PTX is an efficient anti-inflammatory compound, regardless of variants of HO-1 promoter.

In summary, we demonstrated that PTX dose-dependently decreases the expression of HO-1 in cultured human and murine monocytes and endothelial cells, whereas does not modulate significantly *in vivo* HO-1 expression in murine organs. Anti-inflammatory efficacy of PTX is independent of HO-1 pathway, as demonstrated in cells of different HO-1 levels and in HO-1 deficient mice.

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Author's address: Alicja Jozkowicz, PhD, DSc, Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland; Phone: +48-12-664-6411; Fax: +48-12-664-6411; e-mail: alicja.jozkowicz@uj.edu.pl