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ENDOTOXAEMIA IN RATS: ROLE OF LEUKOCYTE SEQUESTRATION IN RAPID PULMONARY NITRIC OXIDE SYNTHASE-2 EXPRESSION

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Nitric oxide (NO), depending on the amount, time and source of generation may exert both, protective and deleterious actions during endotoxic acute lung injury (ALI). Evaluation of the expression and localization of NOS isoforms in the lung of lipopolysaccharide (LPS) - treated rats may contribute to understanding the role of NO in pathogenesis of ALI.

Tissue samples (lung, heart, liver, kidney and spleen) as well as peripheral blood polymorphonuclear cells (PMNs) were collected from control male Wistar rats and LPS - treated animals, 15, 30, 60, 120 and 180 min after LPS injection (2 mg kg^{-1} min^{-1} for 10 minutes, i.v.). Levels of NOS-2 and NOS-3 mRNA and protein in tissues and PMNs were estimated by RT-PCR, Northern blotting and Western blotting. Additionally, myeloperoxidase (MPO) activity in tissue samples was assayed. NOS-3 mRNA as well as protein were detected in lungs of control animals; pulmonary NOS-3 expression was not influenced by LPS. The induction of NOS-2 mRNA in rat lungs and in PMNs isolated from peripheral blood was observed 15 minutes after LPS challenge. In contrast, increase of NOS-2 mRNA in the heart, kidneys, liver and spleen was observed 2-3 hours after LPS injection. In all tissues rise in NOS-2 mRNA was followed after 1-2 hours by increase of NOS-2 protein. Importantly, progressive leukocyte sequestration in the lung parenchyma that started as early as 15 min after LPS injection was revealed only in the lungs; in other organs no significant changes in MPO activity were detected up to 180 min after LPS injection. In conclusion, infusion of LPS caused much more rapid expression of NOS-2 in lungs as compared to the heart, kidneys, liver and spleen. Early induction of NOS-2 may depend on the LPS-stimulated rapid neutrophil sequestration within lung vasculature and fast induction of NOS-2 in sequestrated neutrophils.

K e y w o r d s : *nitric oxide synthase, lipopolysaccharide, leukocytes, acute lung injury*

INTRODUCTION

Sepsis-induced acute lung injury (ALI) remains a major clinical problem with significant morbidity and mortality (1). Leukocyte sequestration within pulmonary vasculature and their subsequent infiltration in lung tissue seems to be prerequisite to the development of ALI (2-4). Injection of lipopolysaccharide (LPS), a component of the wall of Gram-negative bacteria (5) causes rapid pulmonary leukocyte entrapment and is widely used animal model of ALI (6, 7). It was shown that LPS rapidly stimulates release of pneumotoxic lipid mediators such as platelet activating factor (PAF) and thromboxane A_2 (TXA_2), accompanied by increase of pulmonary production of nitric oxide (NO), which at least initially seems to prevent LPS-induced lung injury (8-11). As it is believed, LPS-triggered increase of NO seems to be biphasic, with first phase related to the activation of NOS-3 and the second, delayed, which depends on the induction of NOS-2 (9, 12-15). Administration of non-selective NOS inhibitors caused a dramatic aggravation of ALI and death of LPS-treated rats within 20-40 min (8, 16). Thus, accordingly NOS-3 has been proposed to play a protective role in the rat lungs in the early phase of endotoxaemia whereas NOS-2 has been claimed to be responsible for delayed hypotension in this model (8).

The roles and cellular localization of NOS isoforms in ALI have gained recently increasing attention. Interestingly, some authors suggested beneficial effects of NOS-2 in lung tissue after LPS challenge. For example, NOS-2-derived NO decreased inflammatory response induced by airway administration of LPS (17); NOS-2-derived NO in lungs appeared to be vasoprotective by suppression of adhesion of platelets and leukocytes to endothelial cells as well as by inhibition of endothelial cell apoptosis (18, 19). It should be noted that vast majority of reports concerning roles of NOS-2 isoform in lungs focus on the NOS-2, which is detected several hours after LPS injection and is expressed mainly due to the influx of inflammatory cells (20-22). Importantly, our preliminary data showed rise of NOS-2 mRNA in rat lungs but not in other organs as early as 15 minutes after intravenous LPS administration (23).

In the present study we provide detailed information about the time course of expression of mRNA and protein of NOS-2 and NOS-3 in lungs and other rat tissues in early phase of endotoxaemia. Additionally, we assess the role of leukocyte sequestration in LPS-induced changes of NOS isoform levels in tissues of endotoxaemic rats.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-300 g (Lod: Wist BR from Animal Laboratory of Polish Mother's Memorial Research Institute Hospital, Lodz, Poland) were used for experiments,

according to a protocol accepted by the Permanent Committee for Bioethics in Animal Investigation of the Rector of the Jagiellonian University (permission number - 302/97). The animals were kept in community cages, under standard conditions, 12 h day/ night regime, and free access to food and water.

Reagents

LPS (*Escherichia coli* serotype O127:B8; dissolved in sterile saline), chloroform, O-dianisidine, hexadecylotrimethylammonium bromide (HTAB), hydrogen peroxide, isopropanol were purchased from Sigma Chemicals International; thiopentone sodium was from Biochemie GmbH, Austria.

Experimental protocol

All experiments were done under thiopentone anesthesia (Thiopental, 120 mg kg^{-1}). LPS was injected to rats as an intravenous infusion (2 mg kg^{-1} min^{-1} for 10 minutes). Tissue samples (lungs, heart, kidney, liver and spleen) and samples of blood were taken from untreated animals (control) as well as from LPS-treated rats, 15, 30, 60, 120 and 180 min after start of LPS infusion. Tissue samples were minced into small fragments, immediately frozen in liquid nitrogen, and stored at -70°C until assayed.

Isolation of leukocytes from peripheral blood

Peripheral blood polymorphonuclear leukocytes (PMNs) were obtained from untreated animals (control) as well as from LPS-treated rats, 15, 30, 60, 120 and 180 min after start of LPS infusion. PMNs were isolated by a single step centrifugation on Gradisol G gradient (Polfa, Kutno, Poland) and followed by hypotonic lysis of erythrocytes, as recommended by the manufacturer. Neutrophil content of the suspensions was 96 ± 2% as determined by May-Grunewald-Giemsa staining.

RT-PCR

Expression of mRNA for NOS-2 and NOS-3 was evaluated by semiquantitative RT-PCR after extraction of total RNA with the TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. The yield and quality of the RNA were determined as described earlier (24).

Total RNA (1 μg) from each sample was reverse-transcribed to complementary DNA (cDNA) using oligo$(dT)_{12\text{-}18}$ primer and MMLV reverse transcriptase (Gibco BRL, USA). The final RT reaction volume was 20 μl. The reaction was performed in a thermal cycler Perkin Elmer 9600 at 42°C during 2 hrs. After completion of first-strand cDNA synthesis, the reaction was stopped by heat inactivation (5 min, 99°C). The polymerase chain reactions were performed with 1 μl RT product (cDNA) in a 25- μl reaction volume containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 U HotStarTaq DNA Polymerase (Qiagen, USA) and 1 μM of each primer (*Tab. I*). After an initial enzyme activation step ("hot start") for 15 min at 95°C, PCR was carried out under following conditions: denaturation for 1 min at 94°C, primer annealing for 30 sec at 59°C and, primer extension for 30 sec at 72°C. PCR reactions were terminated with a 10-minute extension at 72°C. For each primer pair, control experiments were performed to determine the range of cycles in which a given amount of cDNA would be amplified in a linear fashion: 30, 35, and 20 cycles for NOS-2, NOS-3, and and β-actin, respectively. Semiquantitative image analyses of the PCR products on the ethidium bromide-stained gels (2% agarose) were performed with freeware Scion image computer programe (Scion Corporation, USA). The data were normalized to transcript levels for β-actin gene and expressed as ratio of optical density.

Table I. Oligonucleotide primers for Reverse Transcriptase-PCR

Primer sequences	Size of PCR product (bp)	Reference
NOS-2 sense: 5'-tgg ctt gcc ctt gga agt ttc tc antisense: 5'-tgt ctc tgg gtc ctc tgg tca aa	384	(42)
NOS-3 sense: 5'-tcc agt tgt tcc acg gcc ac antisense: 5'-acc cgc act tct gtg cct tt	511	(44)
β-actin sense: 5'-agc ggg aaa tcg tgc gtg antisense: 5'-agc ggg aaa tcg tgc gtg	308	(45)

Northern blot

The PCR-amplified NOS-2 and NOS-3 cDNA fragments and the rat glyceraldehyde 3-phosphate dehydrgenase (GAPDH) cDNA probe (Sigma, USA) were used for hybridization. The probes were labeled with [α-^{32}P]-dCTP using the Megaprime™ DNA labeling kit (Amersham, UK).

Equal amounts of total RNA (10 μg) were denatured and subjected to electrophoresis in a 1.2% agarose-formaldehyde gel. The RNA was then blotted onto nylon membranes (Nylon membranes, positively charged, Boehringer Mannheim GmbH, Germany) by overnight capillary transfer. The RNA was immobilized on the membranes by baking under vacuum at 80°C for 2 hrs. The membranes were pre-hybridized in QuickHyb® Hybridization Solution (Stratagene, USA) at 68°C for 20 min. Hybridization was carried out at 68°C for 1 hr in the same solution with [α-^{32}P]-labeled probe ($1.25x10^6$ cpm ml^{-1}). After hybridization, the blots were washed twice for 15 min at room temperature with 2xSSC containing 0.1% (w/v) SDS and once for 30 min at 60°C with 0.1xSSC containing 0.1% (w/v) SDS. The RNA molecules on the filter were first hybridized with NOS-2 or NOS-3 probe and after film exposure subsequently underwent re-hybridization with [α-^{32}P]-labeled GAPDH probe. Expression of GAPDH mRNA was used as an internal control in all experiments. In addition, the RNA load per lane was assessed by ethidium bromide staining of the original agarose gel before capillary transfer. The blots were exposed to Kodak BioMax MS-1 film (Sigma, USA) at -70°C. The Northern blot autoradiographs were scanned and analyzed with Scion image computer programe (Scion Corporation, USA).

Immunoblotting

Tissue homogenates as well as PMNs pellets were lysed in buffer containing 1% Triton X-100, 0.1% SDS in PBS containing 1 mM PMSF, 100 μM leupeptin, 50 μM pepstatin A. Protein concentrations in lysates were determined using Bradford method. Samples, containing equal amounts of total protein were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg ml^{-1} bromophenol blue) in a ratio 4:1 (v/v) and boiled (4 min). Then samples (30-50 μg of total protein per lane) were separated on 7.5% SDS-polyacrylamide gels (Mini Protean II, Bio Rad, USA) using Laemmli buffer system and proteins were semi-dry transferred to nitrocelulose membranes (Bio Rad, USA). Non-specific binding sites were blocked overnight in 4°C with 5% (w/v) non-fat dried milk and the blots were incubated 2 hrs in RT with rabbit polyclonal antibody to NOS-2 (1: 1000) (Cayman, USA) or to

NOS-3 (1:250) (Transduction Laboratories, USA). Bands were detected with alkaline phosphatase-conjugated secondary antibody (1 hr in RT, 1:5000, Sigma, USA) and developed with BCIP and NBT (Sigma, USA). Rainbow markers (Amersham, USA) were used for molecular weight determinations. Protein bands were scanned and analyzed with Scion image freeware programe (Scion Corporation, USA).

Assay of myeloperoxidase (MPO) activity

Tissue samples were thawed, weighted and homogenized using Omni Mixer Homogenizer (Camlab USA) in glass homogenizing tubes containing ice cold 0.5% hexadecyltrimethylammonium bromide (HTAB) dissolved in 50 mM phosphate buffer pH 6.0 (200 mg tissue ml^{-1} KH_2PO_4-Na_2HPO_4). After centrifugation at 12000 g for 15 min at 4°C, the supernatants were harvested and assayed for MPO activity using the technique described by Bradley and coworkers (25). Briefly, 0.1 ml aliquots of the supernatants were mixed with 2.9 ml 50 mM phosphate buffer (pH 6.0) containing 0.167 mg ml^{-1} O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm per gram of wet lung tissue ($\Delta A\ min^{-1}\ gram^{-1}$) was measured every 1 sec for 30 sec in 23°C using Beckman DU 640 BV diode array spectrophotometer equipped with kinetic software package. Results were expressed as % of control MPO activity.

Statistics

Results are presented as mean ± SEM (Standard Error of the Means) for n experiments. Unpaired Student's t-test was used for evaluation of differences between the groups. A value of $p<0.05$ was accepted to be significant.

RESULTS

Expression of NOS-3 and NOS-2 in tissues of LPS-treated rats

NOS-3, but not NOS-2 mRNA and protein were detected in lungs of control rats (*Fig. 1*). The levels of NOS-3 mRNA and NOS-3 protein remained stable in all indicated time intervals after LPS administration (*Fig. 1*). However, LPS caused rapid time-dependent increase in NOS-2 mRNA expression in the lungs, observed using RT-PCR and Northern blot hybridization as early as after 15 min after LPS infusion (*Fig. 1*). The rise of NOS-2 mRNA was followed by increase in NOS-2 protein expression, which started as early as 60 min after LPS challenge and gradually increased up to 180 min (*Fig. 1*).

Importantly, significant increase of NOS-2 mRNA in heart, kidney, liver, and spleen was observed much later than in the lungs, i.e. 120-180 min after LPS challenge (*Fig. 2*). In these tissues induction of NOS-2 protein was observed 1-2 hours after the rise of mRNA (data not shown).

Induction of NOS-2 in leukocytes isolated from blood of LPS-treated rats

The NOS-2 mRNA and protein were undetectable in PMNs isolated from untreated rats (*Fig. 3*). LPS (2 mg kg^{-1} min^{-1} given i.v. for 10 minutes) caused

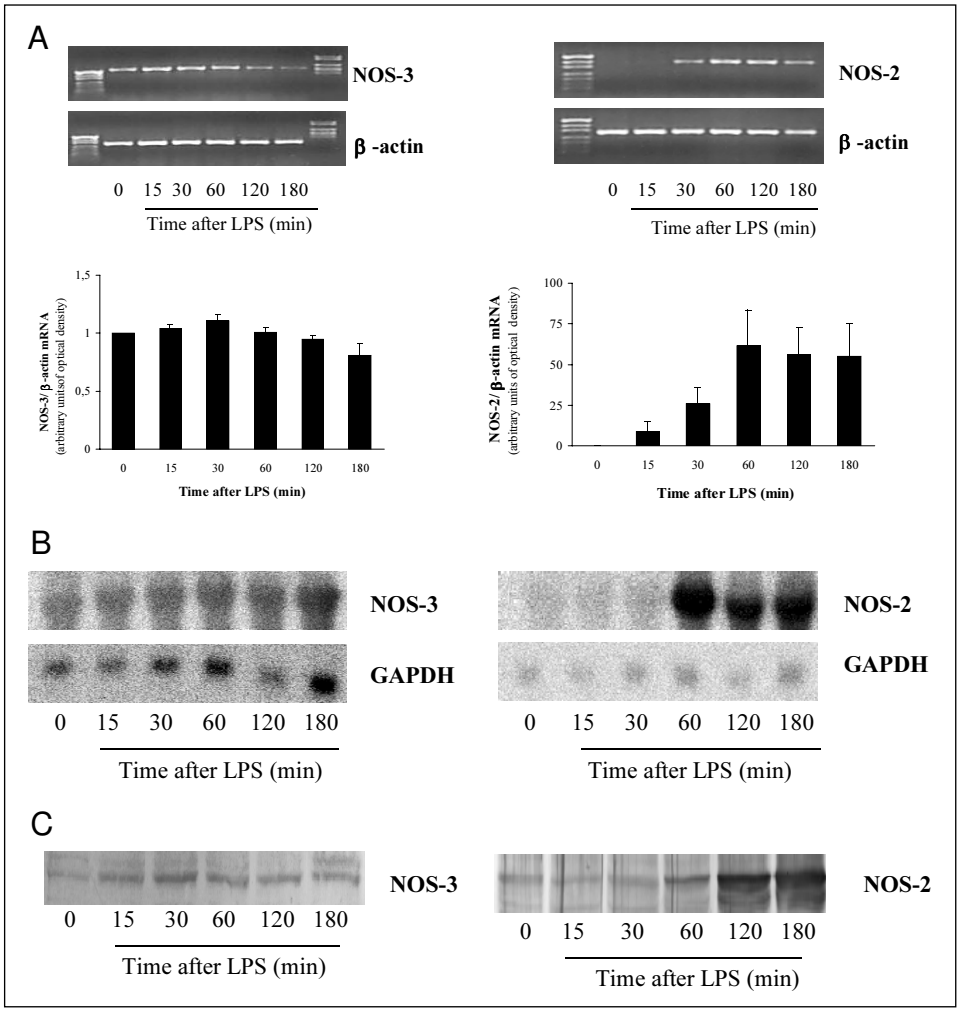

Fig. 1. (**A**) A representative RT-PCR analysis of lung NOS-3 and NOS-2 mRNA extracts from control and LPS-treated rats. NOS-3 and NOS-2 mRNA levels were normalized to the signal for β-actin mRNA. (**B**) Representative autoradiograms of Northern blot showing NOS-3 and NOS-2 mRNA expression in the rat lung in response to LPS. GAPDH mRNA served as internal control. (**C**) Representative Western immunoblots of lung samples for NOS-3 and NOS-2 protein expression in animals injected with LPS. Results are representative of 3 experiments on 3 separate animals at each time point.

rapid time-dependent increase in NOS-2 mRNA expression in circulating PMNs, as evidenced by RT-PCR and Northern hybridization 15 and 30 min after LPS challenge, respectively (*Fig. 3*). Noteworthy, increase in NOS-2 protein in PMNs was observed 60 min after LPS challenge (*Fig. 3*).

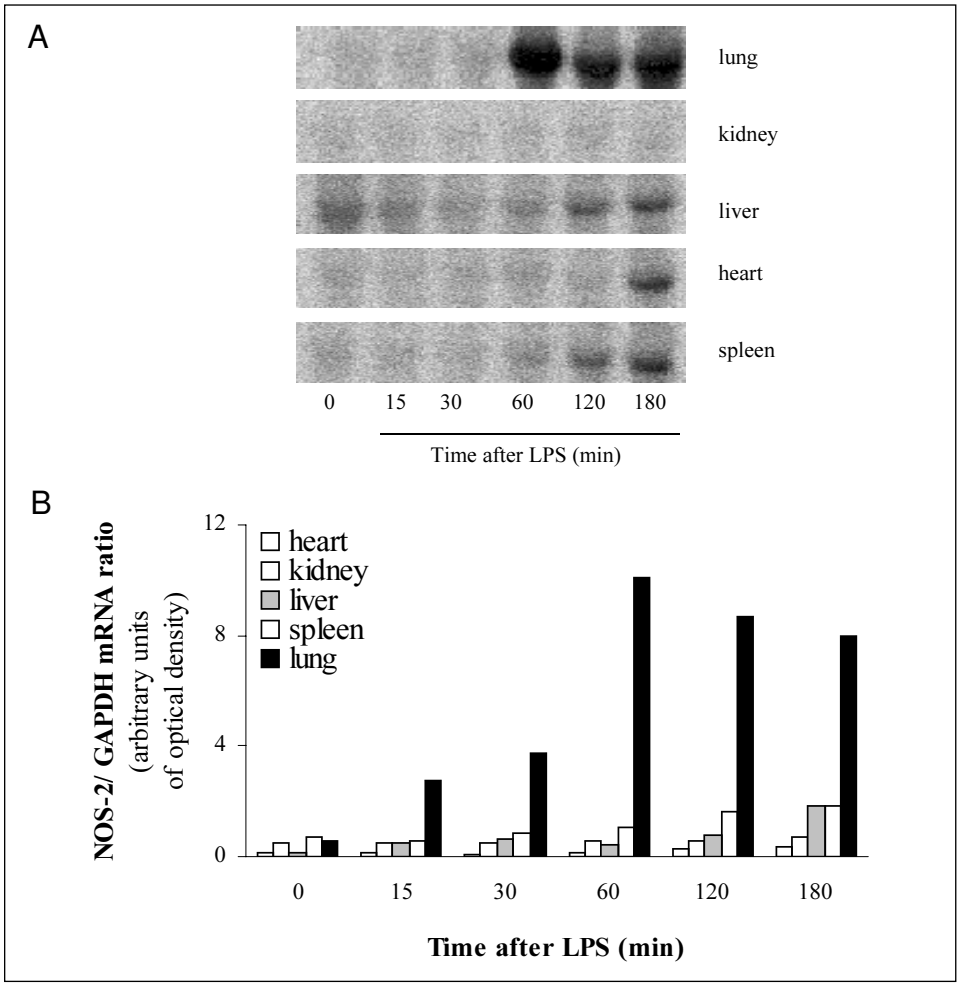

Fig. 2. (**A**) A representative Northern blot analysis of NOS-2 mRNA from various organs of control and LPS-treated rats. (**B**) Quantitation of NOS-2 mRNA levels normalized to the signal for GAPDH mRNA.

MPO activity in tissues of LPS-treated rats

There was marked increase in lung MPO activity as early as at 15 min after start of LPS infusion (*Fig. 4*), however, it reached statistical significance 60 min after LPS (*Fig. 4*). Lung MPO activity peaked after 120 min and remained stable up to 180 minutes after LPS challenge (*Fig. 4*).

Importantly, MPO activity in heart, kidney, liver and spleen in rats receiving LPS did not change as compared to untreated animals at any indicated time intervals after LPS infusion (*Fig. 4*).

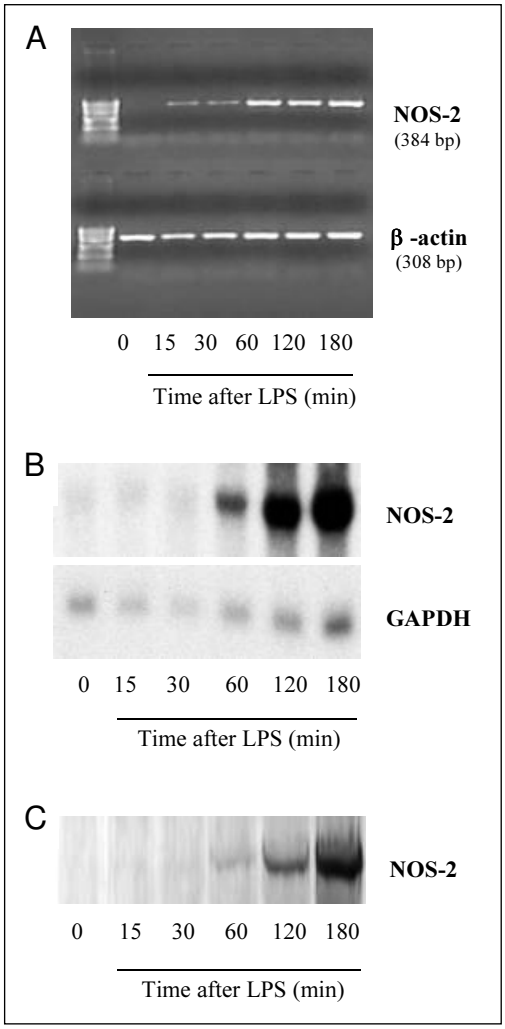

Fig. 3. (**A**) A representative RT-PCR analysis of LPS-induced NOS-2 mRNA expression in rat PMNs isolated from the blood. β-actin was used as control for the amount of NOS-2 cDNA present in each sample. (**B**) Representative autoradiograms of Northern blot analysis of NOS-2 mRNA expression in circulating PMNs. GAPDH mRNA served as internal control. (**C**) Representative Western blot immunoblot of NOS-2 protein extracts from rat PMNs. Results are representative of 3 separate experiments.

DISCUSSION

There are two main findings in this study, pointing to the lungs as a very unique organ in endotoxaemia. First, infusion of LPS caused much more rapid expression of NOS-2 in lungs as compared to the heart, kidneys, liver and spleen. Second, lungs, in contrast to other organs, represent the major site of rapid neutrophil sequestration. Furthermore, there is a striking correlation between the expression of NOS-2 and neutrophil content in lung tissue.

Consistent with previous studies (26, 27), we showed high levels of NOS-3 mRNA and NOS-3 protein in lungs of healthy animals. Unchanged pulmonary

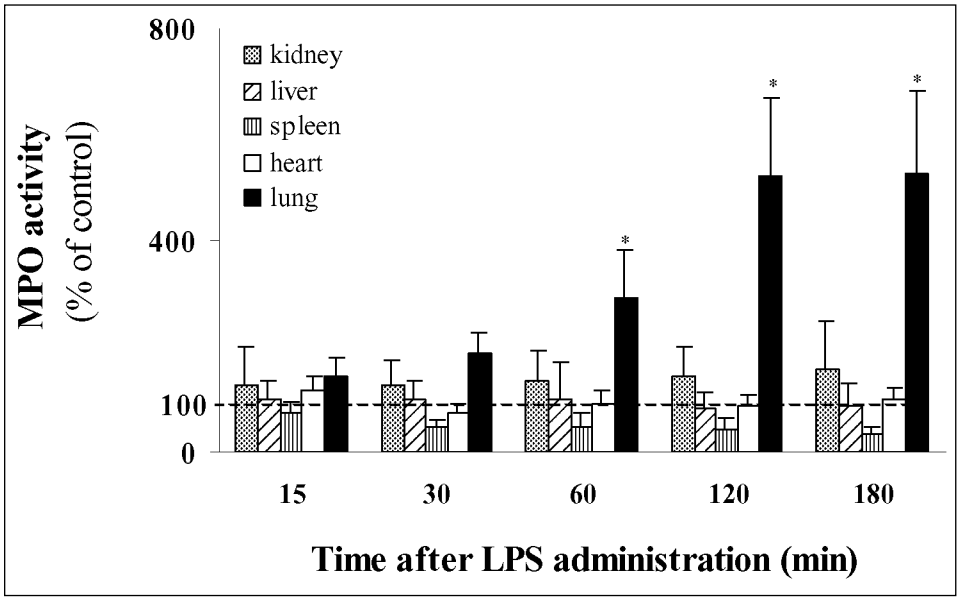

Fig. 4. MPO activity in rat tissues in response to LPS. Measurements were done at indicated time points after LPS injection. Data are expressed as % of control (mean ± SEM for n = 4-5 rats in each group).

NOS-3 expression after LPS administration is in line with Skidgel and coworkers which reported in mouse lungs stable levels of NOS-3 up to 6 h after i.p. injection of *Escherichia coli* (28). However, in this work we demonstrated for the first time immediate induction of NOS-2 mRNA in lung tissue of endotoxaemic rats. Previous studies, using various models of endotoxaemia showed induction of NOS-2 mRNA in the lung tissue at least 3 h (21), 4 h (29-30), or 5-6 h (31-32) after endotoxin challenge. By far, in such a short timescale reported by us, only the beginning of NF-κB activation was observed in lungs of mice and rats treated by *E. coli* and *Salmonella enteritidis* LPS (28, 33). In our study, in lung tissue NOS-2 protein was significantly elevated 1 hour after infusion of LPS. Moreover, this enzyme showed significant activity, as evidenced by citrulline assay (unpublished data). Majority of previous studies concerning LPS-treated rats showed that pulmonary NOS-2 protein requires at least several hours (32, 34-35). Our study demonstrates that inducible NOS-2, next to constitutively expressed NOS-3 appears in lungs in early phase of endotoxaemia. Interestingly, such a rapid NOS-2 induction in response to LPS injection seems to be unique for lung tissue. In our study, there were striking differences in time course of NOS-2 induction between lungs and other organs. Consistent with previous reports (29-30, 32, 34), we observed induction of NOS-2 mRNA in rat liver, spleen, kidney and heart not earlier than 2-3 hours after LPS administration. The rise of mRNA

was followed by increase of NOS-2 protein with delay of 1 hour (unpublished data). Thus, the question arises, why NOS-2 expression in lungs appeared to be much faster than in other tissues?

A consistent finding in rodent models of sepsis and septic patients is that, regardless of the organ in which the sepsis originates, the lungs are generally the first to fail (8-9, 16, 36). A key event that, in part, is thought to explain this pathology is the LPS-induced rapid accumulation of neutrophils within the lung (6-7, 37). Indeed, in our model of endotoxaemia, administration of LPS led to very rapid sequestration of neutrophils in lung tissue, as evidenced by measurement of tissue MPO activity. Although increase of MPO activity in lungs reached statistical significance after 60 minutes, it tended to be elevated as early as 15 min after LPS infusion. Likewise, Erzurum and coworkers noted an increase in pulmonary neutrophil content at 15 min after LPS administration (38). Importantly, in our model lungs appeared to be unique organ as far as the accumulation of neutrophils after LPS challenge is concerned. Moreover, there was a striking correlation between kinetics of the expression of NOS-2 mRNA and increase of MPO activity in lung tissue. Is it possible that the infiltrating neutrophils are responsible for the rapid increase in NOS-2 gene observed in lung tissue of LPS-treated rats?

All cell types are able to express NOS-2 in lungs following exposure to LPS. It was shown that to the late expression of lung NOS-2 contribute parenchymal cells, airway and vascular smooth muscle cells, pneumocytes, vascular endothelial cells, and alveolar macrophages (26, 33, 39-41).

Interestingly, several reports showed increase of lung NOS-2 levels mainly due to the influx of NOS-2 expressing inflammatory cells, which occurred 3 hours (21), and 4 hours (22) after LPS injection. In this work, we were not able to discriminate the cellular source of NOS-2 in lung tissue, however, our previous studies carried out in the same model pointed to infiltrating cells as a source of NOS-2 mRNA in lungs, as it was evidenced by hybridization *in situ* technique 0.5 hr after LPS challenge (42). Moreover, in our model, induction of NOS-2 in PMNs isolated from peripheral blood of LPS-treated animals showed similar time course to that observed in lung tissue. Thus, in our model it may well be that the very early expression of pulmonary NOS-2 mRNA is associated with massive LPS-induced sequestration of blood leukocytes, rather than with the induction of NOS-2 in the lung parenchyma.

We can only speculate, whether early expression of NOS-2 in endotoxaemia may play in lung tissue protective or deleterious role. Could leukocyte-derived NOS-2 oppose the pneumotoxic actions of PAF and TXA_2 (8, 10, 43), as it was demonstrated for NOS-3? It remains attractive hypothesis to be tested.

Summing up, we demonstrated that in endotoxaemia in rats infusion of LPS caused much more rapid expression of NOS-2 in lungs as compared to the heart, kidneys, liver and spleen. Early induction of NOS-2 may depend on the LPS-stimulated rapid neutrophil sequestration within lung vasculature and fast

induction of NOS-2 in sequestrated neutrophils. Pathophysiological role of leukocyte-derived NOS-2 in lungs in early phase of endotoxaemia requires further investigation.

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