Nitric oxide synthase isoform III gene expression in rat liver is up-regulated by lipopolysaccharide and lipoteichoic acid

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Abstract This study was done to investigate the influence of Gram-negative and Gram-positive sepsis on the expression of the three isoforms of nitric oxide synthase (*NOS*) gene in rat liver and kidney. Male Sprague-Dawley rats were treated with lipopolysaccharide (LPS, 10 mg/kg i.v.) as an in vivo model for Gram-negative sepsis or lipoteichoic acid (LTA, 10 mg/kg i.v.) as an in vivo model for Gram-positive sepsis. Animals were killed 12 h and 24 h after i.v. treatment. NOS mRNA of the three isoforms was determined by RNase protection assay. *NOS II* gene expression was strongly induced after LPS or LTA treatment in rat liver and kidney, indicating the efficacy of this treatment to induce sepsis. We found no change of *NOS I* gene expression after LPS or LTA injection in rat liver and kidney. *NOS III* gene expression was increased about 8-fold 12 h and about 5-fold 24 h after induction of sepsis in the rat liver whereas in the kidney there was no significant increase in *NOS III* gene expression. After correction for length NOS III mRNA was about 4- and 40-fold more abundant 12 h and 24 h after LPS treatment than NOS II mRNA in the liver, respectively. Twelve and 24 h after LTA treatment NOS III mRNA was about 18- and 140-fold more abundant than NOS II in the liver. These findings suggest that NOS III is an even more potent source of NO than NOS II in the liver after stimulation with LPS or LTA.

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Key words: Nitric oxide synthase; Sepsis; Liver; Kidney; Gene expression; In vivo

1. Introduction

Nitric oxide (NO) is known to play an important role in acute inflammation or sepsis. NO is produced by three isoforms of NO synthases (NOS I, II and III). The isozymes of NOS are classified in two types: Ca^{2+}-dependent and Ca^{2+}-independent. The Ca^{2+}-dependent isoforms I and III are so far reported to be constitutive whereas the Ca^{2+}-independent isoform II is inducible. It is the isoform II also termed iNOS that is known to be predominant active during inflammation [1]. NOS isoform I also named ncNOS has not yet been reported to be regulated by inflammatory stimuli. There is still confusion concerning the role of NOS isoform III also known as ecNOS during sepsis. Oguchi et al. reported in 1992 the existence of a novel and distinct Ca^{2+}-dependent isozyme of NOS that is inducible by i.v. injection of LPS [2]. They found increased levels of this distinct NOS mRNA in various organs whereas it is not yet clear whether this thus reported novel NOS is an enzymatically active degradation product of inducible NOS isoform. On the other hand, NOS III mRNA has been reported to be down-regulated by TNF-α [3–5] or LPS

[6,7]. In view of these findings it appeared reasonable to us to investigate more systematically the expression of the three isoforms of NOS during sepsis. To this end, we injected animals LPS (10 mg/kg i.v.) as an in vivo model of Gram-negative sepsis or LTA (10 mg/kg) as an in vivo model for Gram-positive sepsis. mRNA levels for NOS I, NOS II and NOS III in the rat liver and kidney before and 12 h and 24 h after injection of LPS or LTA were determined by specific ribonuclease (RNase) protection. We compared the effects on the constitutively expressed isoforms of NOS (NOS I and III) with those on the expression of NOS isoform II, which is generally considered to be up-regulated during inflammation. There was no NOS I mRNA detectable in the liver before and after LPS or LTA treatment and there was no change of *NOS I* gene expression in the kidney during sepsis treatment. As expected *NOS II* gene expression was strongly induced after LPS or LTA injection. The most striking finding was that *NOS III* gene expression was up-regulated in the liver but not in the kidney after treatment with both LPS or LTA. This NOS III isozyme that has so far been reported to be constitutively expressed seems to be inducible in the liver but not in the kidney by injection of LPS or LTA and appears to be an even more potent source of NO than NOS II in the liver.

2. Materials and methods

2.1. Animal experiments

All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the German Law on the Protection for Animals.

Male Sprague-Dawley rats (300–350 g) that had free access to food and water were used for the experiments. Also, during the experiments, the animals had free access to food and water. Thereafter, different treatment groups were examined: (1) control animals receiving 2 ml of Ringer's solution i.v. (*n* = 6); (2) animals treated with LPS (from *Escherichia coli*; serotype 0111:B4; Sigma) 10 mg/kg i.v. and killed 12 h (*n* = 6) and 24 h (*n* = 6) after LPS treatment; (3) animals treated with LTA (from *Staphylococcus aureus*; Sigma) 10 mg/kg i.v. and killed 12 h (*n* = 6) and 24 h (*n* = 6) after LTA treatment.

For i.v. injection the animals were anesthetized for 15 min with isoflurane under controlled ventilation with a face mask. Therefore a Trajan 808 with a connected vaporizer (Dräger, Lübeck) and a ventilation pump (Rhema Electronics, Hofheim) were used.

At the end of the experiments, animals were killed by decapitation during isoflurane anesthesia, blood and urine were collected and organs were rapidly extirpated, weighed, frozen in liquid nitrogen and stored at −80°C until RNA extraction.

2.2. Extraction of RNA

Total RNA was extracted from the organs, which were stored at −80°C, according to the protocol of Chomczynski and Sacchi [8] by homogenization in Solution D (guanidine thiocyanate (4 M) containing 0.5% *N*-lauryl-sarcosinate, 10 mM ethylenediaminetetraacetic acid (EDTA), 25 mM sodium citrate and 700 mM β-mercaptoethanol) using a polytron homogenizer. Sequentially, 1 ml of 2 M sodium

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acetate (pH 4), 10 ml of phenol (water saturated) and 2 ml of chloroform were added to the homogenate, with thorough mixing after the addition of each reagent. After cooling on ice for 15 min, samples were centrifuged at $10\,000 \times g$ for 15 min at 4°C. RNA in the supernatant was precipitated with an equal volume of isopropanol at −20°C for at least 1 h. After centrifugation, RNA pellets were resuspended in 0.5 ml of solution D, again precipitated with an equal volume of isopropanol at −20°C and RNA pellets were finally dissolved in diethylpyrocarbonate-treated water and stored at −80°C till further processing in RNase protection assays.

2.3. Determination of NOS I, NOS II and NOS III mRNA by RNase protection assay

The abundance of NOS I, NOS II and NOS III mRNA in total RNA isolated from the organs was determined by RNase protection assay as described for NOS I [9]. A NOS I cRNA probe containing 324 bp of the NOS I antisense sequence was generated from a pAM18 vector carrying a HincII/KpnI restriction fragment of NOS I cDNA by transcription with SP6 RNA polymerase (Amersham International, UK). The NOS II and NOS III cRNA probes containing a 518 bp insert and a 184 bp insert, respectively, were generated from a pGEM-4Z vector carrying a BamHI/EcoRI restriction fragment of NOS II cDNA and a pGEM-4Z vector carrying a KpnI/PstI restriction fragment of NOS III cDNA, respectively. Transcripts were continuously labeled with [^{32}P]GTP (410 Ci/mmol or 15.17 TBq/mmol; Amersham International) and purified on a Sephadex G50 spun column. For hybridization total RNA was dissolved in a buffer containing 80% formamide, 40 mM piperazine, N,N'-bis(2-ethane sulphonic acid) 400 mM NaCl, 1 mM EDTA (pH 8). 100 µg of total RNA were hybridized in a volume of 50 µl at 60°C for 12 h with each 5×10^5 cpm radiolabeled NOS I, NOS III and NOS II probe, respectively. RNase digestion with RNase A and T1 was carried out at 20°C for 30 min and terminated by incubation with proteinase K (1 mg/ml) and sodium dodecyl sulphate (SDS 0.4%) at 37°C for 30 min.

Protected NOS I, NOS II and NOS III mRNA fragments were purified by phenol/chloroform extraction, ethanol precipitation and subsequent electrophoresis on a denaturing 6% polyacrylamide gel. After autoradiography of the dried gel at −80°C for 1 day, bands were excised from the gel and radioactivity was counted with a liquid scintillation counter (1500 CarbTm, Pachard Instrument Company, Downers Grove, IL). To estimate the relative proportion of NOS I, NOS II and NOS III mRNA in the different organs from the protection assays, the relative lengths of the protected fragments were taken into account.

2.4. Statistics

The ANOVA test was used for calculation of statistical significance and $P < 0.05$ was considered significant.

3. Results

3.1. NOS I gene expression before and after treatment with LPS or LTA

In the liver NOS I mRNA was not detectable either in the control group or after treatment with LPS or LTA.

In the kidney NOS I mRNA was detectable (0.5 ± 0.03 cpm/µg RNA) but there was no significant change in NOS I gene expression 12 h or 24 h after LPS or LTA treatment (Fig. 1).

3.2. NOS II gene expression before and after treatment with LPS or LTA

In the liver NOS II mRNA was not detectable in normal rats. Injection of LPS resulted in a time-dependent increase in NOS II gene expression (Fig. 2). NOS II mRNA was strongly elevated 12 h after injection of LPS (2.6 ± 0.2 cpm/µg RNA) and was decreased to 5% of the 12 h level at 24 h after LPS treatment (0.14 ± 0.02 cpm/µg RNA). Injection of LTA resulted also in a time-dependent increase in NOS II gene expression whereas the increase was smaller than after LPS

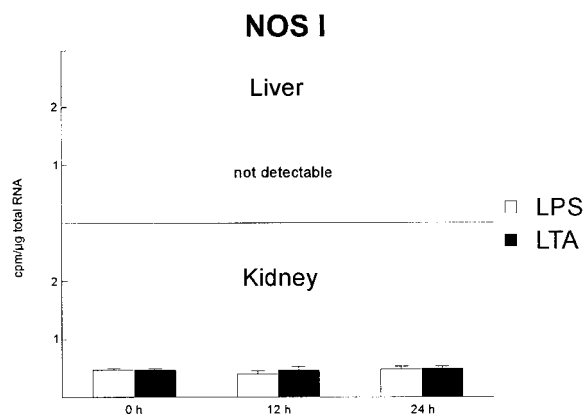Fig. 1. Effects of LPS (10 mg/kg) and LTA (10 mg/kg) on NOS I mRNA levels in rat liver and kidney 12 h and 24 h after i.v. injection. Total RNA (100 µg) were assayed for NOS I and hybridization signal for NOS I mRNA is given per µg RNA. Data are means ± SE of six animals in each group.

treatment. The decrease in NOS II gene expression between 12 h and 24 h after LTA treatment was smaller than after LPS treatment (0.47 ± 0.03 cpm/µg RNA after 12 h; 0.04 ± 0.005 cpm/µg RNA after 24 h).

In the kidney NOS II mRNA was also not detectable in normal rats. NOS II gene expression was also strongly induced 12 h after injection of LPS (0.67 ± 0.08 cpm/µg RNA) and was decreased to about 15% of the 12 h level at 24 h after LPS treatment (0.10 ± 0.015 cpm/µg RNA). Injection of LTA resulted also in a smaller, time-dependent increase in NOS II gene expression whereas the decrease between 12 h and 24 h after LTA treatment was also smaller than after LPS treatment (0.14 ± 0.015 cpm/µg RNA after 12 h; 0.05 ± 0.006 cpm/µg RNA after 24 h).

3.3. NOS III gene expression before and after treatment with LPS or LTA

As shown in Fig. 3 NOS III mRNA was detectable in the liver of normal rats (1.2 ± 0.1 cpm/µg RNA). NOS III mRNA was increased about 8-fold 12 h after LPS treatment (10 ± 1.1 cpm/µg RNA) and decreased to about 60% of the 12 h level at 24 h after LPS treatment (6.0 ± 0.3 cpm/µg RNA). Injection of LTA resulted in a similar increase in NOS III gene expression whereas the increase tended to be smaller (8.6 ± 0.8 cpm/µg RNA after 12 h; 5.7 ± 0.3 cpm/µg RNA after 24 h).

In the kidney NOS III mRNA was detectable in normal rats (6.4 ± 0.5 cpm/µg RNA) but there was no significant increase in NOS III gene expression either after LPS or LTA treatment.

4. Discussion

The aim of this study was to investigate the influence of Gram-negative and Gram-positive sepsis on the gene expression of NOS isoforms I and III in rat liver and kidney and to compare these effects with those on the expression of NOS isoform II, which is generally considered to be up-regulated during inflammation. Our findings show that NOS I gene is already substantially expressed in the kidney but not in the liver of normal animals. NOS II mRNA was not detectable in the liver and kidney of normal animals whereas NOS III gene

is expressed in the liver and kidney of normal rats. NOS III mRNA was more abundant in the kidney than in the liver. These findings are in accordance to the results of previous studies [9–12].

To induce sepsis, we used two manoeuvres, namely i.v. injection of LPS (10 mg/kg) for induction of Gram-negative sepsis and i.v. injection of LTA for induction of Gram-positive sepsis [13]. These two manoeuvres caused a time-dependent stimulation of NOS II gene expression in the liver and kidney, whereas up-regulation of NOS II mRNA was stronger after LPS treatment than after LTA treatment. It is well established that bacterial lipopolysaccharide induces widespread in vivo tissue expression of NOS II mRNA [10]. For LTA there are few studies reported so far concerning NOS II. It has been observed that LTA from Streptococcus viridans provokes the accumulation of NOS II mRNA in macrophages [13]. It has also been noticed that NOS II protein expression is increased by LTA from Staphylococcus aureus in the rat lung [14] and in macrophages [15]. In vitro analysis showed that cyclic GMP levels were elevated in vascular smooth muscle cells [16] and endothelium-free aortic rings [17] after incubation with LTA from Staphylococcus aureus. There is no prior investigation concerning the expression of NOS II mRNA in the rat liver and kidney after injection of LTA.

Our findings show that injection of LPS or LTA has no significant effect on NOS I gene expression in rat kidney 12 h and 24 h after injection. In the rat liver NOS I mRNA was not detectable before and after injection of LPS or LTA. Little information is available on the regulation of NOS I expression. There is evidence from structural investigations that the NOS I gene may be transcriptionally regulated [18]. For the rat brain it has been observed in vivo that NOS I mRNA is down-regulated 4 h after i.p. injection of 15 mg/kg LPS [17]. No previous study is accessible reporting a change in NOS I gene expression during sepsis in rat liver or kidney.

This study demonstrates that NOS III gene expression is induced in the liver of rats treated with LPS or LTA. There was no significant increase in NOS III gene expression in the kidney after LPS or LTA treatment. The NOS isoform III is known to be Ca^{2+}-dependent and has so far been considered
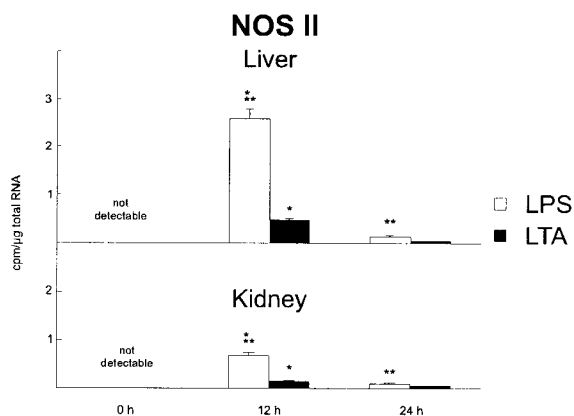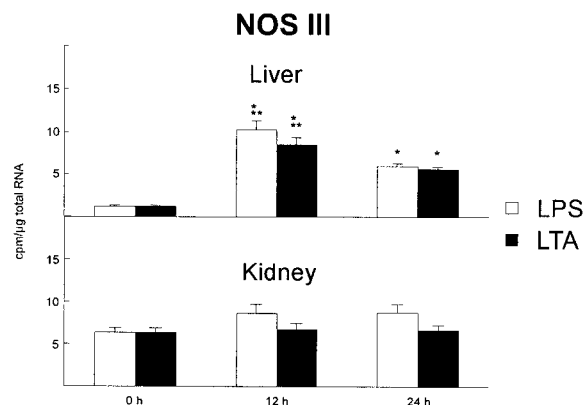

Fig. 3. Effects of LPS (10 mg/kg) and LTA (10 mg/kg) on NOS III mRNA levels in rat liver and kidney 12 h and 24 h after i.v. injection. Total RNA (100 μg) were assayed for NOS III and hybridization signal for NOS III mRNA is given per μg RNA. Data are means ± SE of six animals in each group. *$P < 0.01$ vs. control (0 h). **$P < 0.01$ vs. 24 h.

to be constitutively expressed. The NOS III promoter is known to be 'TATA-less' and exhibits promoter elements consistent with a constitutively expressed gene [19]. Recent studies have shown that the NOS III gene is actively regulated in response to various physiological or pathophysiological stimuli [3,20,21]. This is consistent with the finding that the 5'-flanking region of NOS III gene contains putative shear stress, AP-1, AP-2, NF-1 heavy metal, acute-phase response, and sterol-regulatory cis-acting DNA elements [19,22,23]. Oguchi et al. reported the existence of a novel and distinct Ca^{2+}-dependent isozyme of NOS that is inducible by i.v. injection of LPS [2]. They found increased levels of this distinct NOS mRNA in various organs (liver, lung, spleen, colon, ileum) by Northern blot analysis. As mentioned above Western blot analysis of this induced NOS showed cross-reactivity with the inducible NOS from rat liver. It is not yet clear whether this thus reported novel NOS is an enzymatically active degradation product of inducible NOS isoform. The findings of Oguchi et al. suggest that there might be another source of NO besides NOS II during sepsis. The results of the present study show that NOS III is another potent source of NO besides NOS II in rat liver during sepsis.

NO plays an important role in the control of vascular tone [24] and it has been established that NO has antimicrobial [25] and platelet inhibiting activity [26]. Consequently in the condition of septic shock the controlled over production of NO by NOS II and NOS III in the liver could be of relevance for maintaining an adequate blood flow and for inhibition of intravascular coagulation and also for antimicrobial defense.

It has been reported that NOS III mRNA was down-regulated in bovine coronary venular endothelial cells by LPS whereas the mechanism of action was ascribed to a post-transcriptional regulation via destabilization or increased degradation of NOS III mRNA with no effect on transcription [6]. Liu et al. found down-regulation of NOS III mRNA rat lung, heart and aorta after injection of LPS (15 mg/kg i.p.) by Northern blot analysis [7].

These findings are not in contrast to our results. Whether the effect of LPS or LTA on NOS III gene expression is specific to the liver remains to be investigated. Further explorations are necessary to characterize this NOS III isoform in the liver.

Fig. 2. Effects of LPS (10 mg/kg) and LTA (10 mg/kg) on NOS II mRNA levels in rat liver and kidney 12 h and 24 h after i.v. injection. Total RNA (100 μg) were assayed for NOS II and hybridization signal for NOS II mRNA is given per μg RNA. Data are means ± SE of six animals in each group. *$P < 0.01$ vs. 24 h. **$P < 0.01$ vs. LTA.

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