

# Lipopolysaccharide treatment in vivo induces tissue expression of GTP cyclohydrolase I mRNA

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**Abstract** A significant induction of GTP cyclohydrolase I (GTPCH) mRNA was observed in lung, heart and kidney of rats treated with lipopolysaccharide (LPS; 10 mg/kg i.v.). GTPCH mRNA levels in liver were high even in untreated rats, and remained elevated after LPS treatment. Parallel induction of nitric oxide synthase (NOS) mRNA was observed in these tissues of LPS-treated rats. Our results demonstrate induction of GTPCH mRNA after LPS treatment in vivo and provide molecular evidence for the increased GTPCH activity which may up-regulate NOS activity in vivo.

**Key words:** GTP cyclohydrolase I; mRNA; Lipopolysaccharide; Nitric oxide synthase

## 1. Introduction

Tetrahydrobiopterin (BH4) is an essential cofactor of all isoforms of nitric oxide (NO) synthase (NOS) [1]. It was first demonstrated that the increase in BH4 levels was crucial for cytokine-induced NO production in murine fibroblasts [2]. The causal relationship between increased BH4 levels and cytokine-induced increase in NO production was confirmed in rat vascular smooth muscle cells [3] and a murine macrophage cell line [4]. Moreover, recent reports demonstrated that inflammatory cytokines increase NOS activity in human endothelial cells selectively by increasing BH4 levels [5,6]. Thus, NOS can be regulated through the availability of BH4. Under these conditions, cytokines increase GTP cyclohydrolase I (GTPCH) activity [3–6], the rate-limiting enzyme for de novo BH4 synthesis [7]. Increased GTPCH activity has been also demonstrated in tissues of bacterial lipopolysaccharide (LPS)-treated rats [8] in which widespread induction of NOS is well documented [9–11]. We recently showed that LPS and interferon- $\gamma$  increased steady state levels of mRNA for GTPCH in rat vascular smooth muscle cells [12]. This cannot be extrapolated as the mechanism for the increase in GTPCH activity of all cell types, but similar regulation appears possible in other cell types and in vivo. In the present study, we investigated whether LPS treatment in vivo induces the expression of GTPCH mRNA to obtain molecular evidence responsible for the increased GTPCH activity which may up-regulate NOS activity in vivo.

## 2. Materials and methods

### 2.1. Animal treatment and extraction of RNA

Male Wistar rats (250–300 g) were injected intravenously with either LPS (*E. coli* type, Serotype 0111:B4, 10 mg/kg; Sigma, St. Louis, MO)

or saline (1 ml/kg) for endotoxin and control group, respectively. Three hours later, the animals were sacrificed by exsanguination, and various organs were removed. The organs were frozen in liquid nitrogen immediately after excision and stored at  $-70^{\circ}\text{C}$  before RNA extraction. Total RNA was extracted from lung, heart, liver and kidney using the guanidinium isothiocyanate/acid phenol method [13].

### 2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed by standard methods using 1  $\mu\text{g}$  total RNA. The first strand cDNA was synthesized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA) followed by PCR amplification using synthetic gene-specific primers for rat GTP cyclohydrolase I [12] and murine-inducible NOS (iNOS) [14]. Primers used were: GTPCH forward 21-mer, 5'-GGATACCAGGAGACCA-TCTCA-3'; GTPCH reverse 21-mer, 5'-TAGCATGGTGCTAGTGA-CAGT-3'; iNOS forward 21-mer, 5'-CTGCAGGTCTTTGACGCT-CGG-3'; iNOS reverse 21-mer, 5'-GTGGAACACAGGGGTGAT-GCT-3'. PCR amplification was performed using a DNA PCR kit (Perkin Elmer, Norwalk, CT, USA) according to the following schedule: denaturation, annealing, and elongation at 95, 55 and 72°C for 30 s, 30 s, and 1 min, respectively, for 30 cycles. Parallel amplification of rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was performed for reference using primers as described [15]. The possible contamination of any PCR component was excluded by performing a PCR reaction with these components in the absence of RT product in each set of experiment (negative control). To verify that the amplification did not proceed from residual genomic DNA, a control PCR reaction was done for each RNA using RNA as template.

### 2.3. Analysis of PCR products

PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. The intensity of the PCR bands on the negative film of the gel photographs was quantified by use of a video densitometer linked to a computer analysis system (ACI, Kanagawa, Japan). All PCR reactions resulted in the amplification of a single product of the predicted size for GTPCH (372bp) and iNOS (741bp).

### 2.4. Statistical analysis

Data are expressed as mean  $\pm$  S.E.M. of three determinations. A Student's unpaired *t*-test was used to compare mean differences. A level of  $P < 0.05$  was accepted as statistically significant.

## 3. Results

The PCR products using GTPCH and iNOS specific primers showed clear bands at predicted size of 372 and 742 bp, respectively. These bands were absent in the PCR-amplified products using RNA as template or lacking a cDNA template. This indicates that the 372 and 741 bp bands originated from mRNA, but not from genomic DNA or other contamination.

As shown Fig. 1, the GTPCH signal was absent or negligible in control lung, heart and kidney. In contrast, a large GTPCH signal was detected in control liver as well as LPS-treated lung, heart, liver and kidney. While the iNOS signal was absent in RNAs from control lung, heart, liver and kidney, a large iNOS signal was detected in all RNAs from LPS treated-rats.

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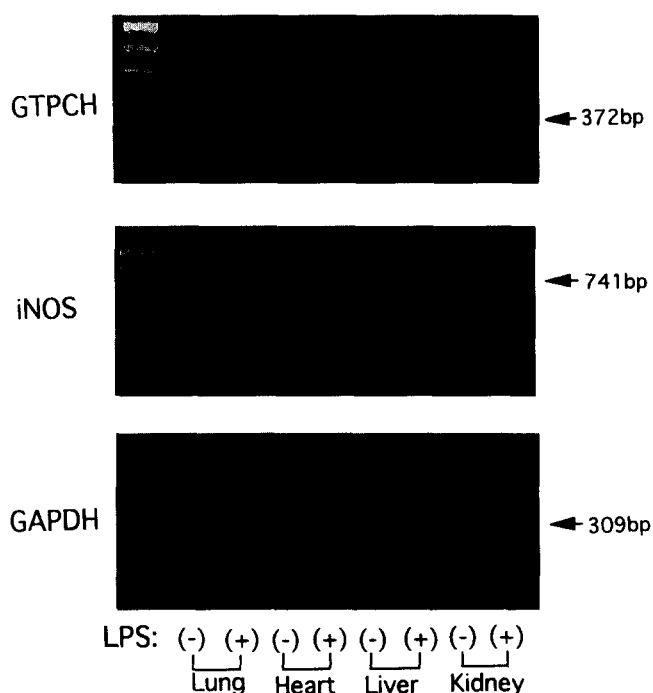


Fig. 1. Effects of LPS treatment in vivo on GTPCH and iNOS mRNA expression in lung, heart, liver and kidney. Total RNA was prepared from tissues of rats 3 h after intravenous administration of saline (control: LPS(-)) or LPS (10 mg/kg: LPS(+)), and assayed by RT-PCR using gene-specific primers. Results using primers specific for GAPDH are shown for comparison. DNA size markers in the left lanes correspond to: 2000, 1200, 800, 400, 200, and 100 bp. Arrows indicate the predicted size of PCR products.

Our preliminary study showed a linear relationship between the quantity of total cDNA for PCR and the intensity of the PCR product for GTPCH in LPS-treated rat liver. Since the quantity of GTPCH cDNA for PCR amplification in each sample is considered to vary with the concentration of GTPCH mRNA, the PCR signal for GTPCH was regarded as the relative abundance of GTPCH mRNA. Moreover, we normalized the GTPCH signal relative to corresponding GAPDH signal from the same RNA, which was expressed as GTPCH/GAPDH ratio. As shown in Fig. 2, the GTPCH/GAPDH ratio in lung, heart and kidney was substantially increased by treatment of rats with LPS. In contrast, there was no significant difference in GTPCH/GAPDH ratio between control and LPS-treated liver.

#### 4. Discussion

This is the first report to show that LPS treatment in vivo induces GTPCH mRNA. Increased GTPCH activity and therefore increased BH4 levels in tissues have previously been shown in the rat treated with LPS [8]. This suggests that either LPS-induced biopterin synthesis arises from post-translational activation of pre-existing GTPCH or GTPCH induction is regulated by protein de novo synthesis. Our data strongly support the latter possibility. LPS and interferon- $\gamma$  increases GTPCH mRNA abundance in cultured vascular smooth muscle cells in the presence of a protein synthesis inhibitor, indicating no requirement for intermediary protein synthesis [12]. Therefore,

LPS may act directly to induce GTPCH mRNA in vivo as well. LPS also increases interferon- $\gamma$  and tumor necrosis factor- $\alpha$  in vivo [16], that in turn, may lead to induction of GTPCH mRNA.

LPS induces iNOS in vivo as well as in various cell types which produce large quantities of NO and profound vasodilation; this process has been implicated as the cause of Gram-negative septic shock [1,17,18]. Widespread induction of iNOS in LPS-treated rat is well documented [9–11]. In the present study, we also confirmed a significant induction of iNOS mRNA in the rat tissues, which is accompanied by increased  $\text{NO}_2^-/\text{NO}_3^-$  levels in rat plasma ( $58.2 \pm 3.7$  vs.  $167.7 \pm 24.8$   $\mu\text{M}$ : mean  $\pm$  S.E.M.,  $n = 3$ ) and an induction of  $\text{Ca}^{2+}$ -independent NOS activity in the lung homogenates three hours after LPS administration (10 mg/kg, i.v.) [19]. We previously showed using rat vascular smooth muscle that immunostimulant-evoked BH4 synthesis occurs with a time course that parallels that for coinduced NO synthesis and is preceded by an increase in GTPCH mRNA [12]. Under these conditions, the intracellular concentration of BH4 appears to be rate limiting for NO synthesis. Indeed, a significant up-regulation of NO synthesis in vascular smooth muscle is observed with administration of excess BH4, and NO synthesis can be prevented by inhibitors of enzymes, such as GTPCH, that participate in BH4 synthesis [3,12]. Similar results have been shown in cultured murine macrophages [4]. Furthermore, it has recently been shown that inflammatory cytokines increase NOS activity in cultured human endothelial cells by increasing BH4 levels even in the face of falling total enzyme [6]. These observations suggest a key role for BH4 in regulation of NO production by both constitutive and inducible isoforms of NOS.

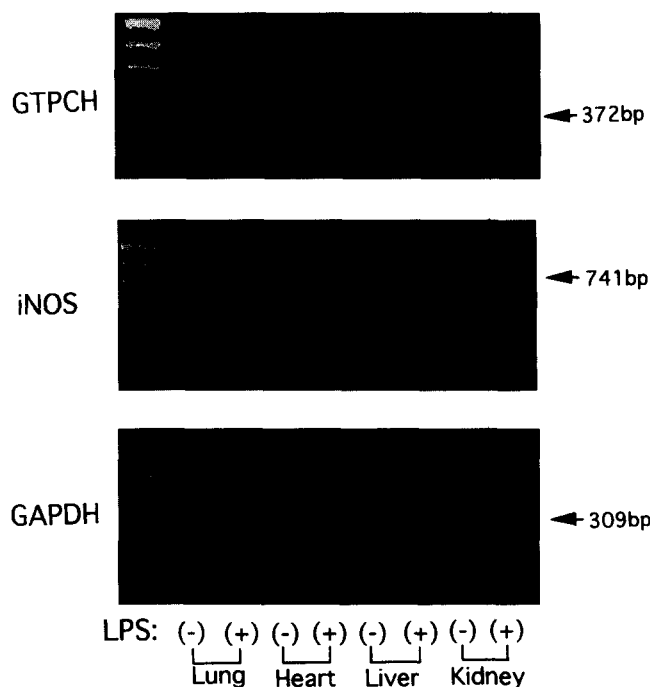


Fig. 2. Effects of LPS on GTPCH mRNA expression in various tissues. The intensity ratio of GTPCH to GAPDH was shown. Data are mean  $\pm$  S.E.M. of 3 animals (control: open columns, LPS-treated: closed columns). GTPCH/GAPDH in control lung, heart and kidney was not shown as the GTPCH signal was too low to be quantified.

Werner-Felmayer et al. reported that biopterin concentrations of plasma were not significantly increased despite a significant increase in biopterin in tissues of LPS-treated rat [8]. They suggested that BH4 is kept effectively within the cells. However, this suggestion contradicts a recent report showing that BH4 is a secretory product of vascular endothelial cells [20]. Moreover, the same authors reported that endothelial cells secrete BH4 vectorially into the direction of vascular smooth muscle [21]. We also observed the secretion of substantial portion of BH4 synthesized by LPS-activated vascular smooth muscle cells and macrophages (unpublished data). Thus, BH4 may play a key role in a autocrine/paracrine fashion for supporting NOS activity. GTPCH activity appears low levels in tissues under normal conditions [8,22]. Therefore, an increase in GTPCH activity by activation of GTPCH gene expression may be necessary for NOS to exert full activity. Conversely, inhibition of GTPCH induction may become an important target for pharmacological interventions for NO overproduction, i.e. septic and cytokine-induced shock.

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