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Induction of GTP cyclohydrolase I by bacterial lipopolysaccharide in the rat

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A 2- to 3-fold increase of GTP cyclohydrolase I (E.C. 3.5.4.16), the key enzyme of tetrahydrobiopterin biosynthesis from GTP, was observed in cerebellum, remaining brain, liver, spleen, and adrenal gland of rats treated with a single dose of lipopolysaccharide (LPS). This led to increased biopterin levels in tissues but not in plasma. Parallel induction of nitric oxide (NO) synthase was indicated by a 10- to 100-fold increase of plasma nitrate levels 6 and 12 hours after injection of LPS. Furthermore, systolic blood pressure was reduced significantly by 23%. Our results demonstrate induction of tetrahydrobiopterin biosynthesis after LPS treatment in vivo.

Tetrahydrobiopterin; GTP cyclohydrolase I; Nitric oxide; Lipopolysaccharide

1. INTRODUCTION

Three enzymes are involved in biosynthesis of tetrahydrobiopterin from GTP, i.e. GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase (EC 1.1.1.153) [1]. In human cells in vitro, an up to 100-fold stimulation of GTP cyclohydrolase I has been observed upon treatment with cytokines such as interferon-γ or tumour necrosis factor-α in a variety of cell types, including macrophages, dermal fibroblasts and tumour lines from different organs, and the virus-transformed T-cell line MT-2 (for review see [2]). Lipopolysaccharide (LPS) acts as a costimulator of interferon-γ but also exerts induction of GTP cyclohydrolase I as a single stimulus in macrophages and in endothelial cells [2,3]. In cells of rodent origin, cytokine-induced stimulation of GTP cyclohydrolase I was also seen in murine macrophages, which already express high enzyme activity when unstimulated, and in murine dermal fibroblasts [2,4]. As a result, intracellular tetrahydrobiopterin levels are increased in human and murine cells after cytokine or LPS treatment [2–4]. Increase in intracellular tetrahydrobiopterin levels upon cytokine or LPS treatment was recently also reported for rat vascular smooth muscle cells [5].

In humans, cytokine-induced stimulation of GTP cyclohydrolase I can be easily detected in vivo by measuring neopterin in body fluids. Neopterin is cleaved from the first intermediate of tetrahydrobiopterin synthesis, i.e. 7,8-dihydroneopterin triphosphate, by phosphatases. 7,8-Dihydroneopterin triphosphate accumulates in human but not in murine cells due to the comparatively low 6-pyruvoyl tetrahydropterin synthase activity found in human cells [4,6], thus explaining excretion of neopterin in humans but not in rodents [7]. Among human cells, the ratio of GTP cyclohydrolase I to 6-pyruvoyl tetrahydropterin synthase after cytokine treatment is highest in macrophages which leads to production of an about 50-fold excess of neopterin over biopterin [6,8]. Determination of neopterin in body fluids of humans has turned out to be a valuable marker for monitoring the immune status in patients suffering from diseases which involve activation of cell-mediated immunity (for review see [9]). Excretion of biopterin was only slightly elevated under these conditions [10].

In contrast to humans, in vivo induction of GTP cyclohydrolase I in the course of immune activation has thus far not been reported for rodents. However, the observation of a 2-fold increase of urinary biopterin excretion in tumour-bearing rats [11] suggests that mechanisms similar to humans also operate in rodents. We therefore were interested whether pteridine synthesis is induced in vivo in rodents challenged with an immune stimulus such as LPS.

Under these conditions, widespread induction of nitric oxide (NO) synthase, an enzyme which requires tetrahydrobiopterin for full activity (reviewed in [12]), is well documented [13–15]. Here, we report that LPS increases GTP cyclohydrolase I activities and tetrahydrobiopterin levels in various tissues of rats which is paralleled by highly elevated plasma nitrate levels and

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Abbreviations: HPLC, high-performance liquid chromatography; LPS, lipopolysaccharide; NO, nitric oxide.

a drop in systolic blood pressure but no increase of plasma biopterin concentrations.

2. MATERIALS AND METHODS

2.1. *Treatment of rats and preparation of tissue homogenates and plasma*

Male Sprague–Dawley rats (200–250 g, Himberg, Austria), fed ad libitum, were treated with 6 mg/kg of LPS (phenolic extract from *Escherichia coli* 055 : B5, Sigma, Munich, Germany) or vehicle (pyrogen-free phosphate-buffered saline, pH 7.4, containing 130 mM NaCl, 2 mM KCl, 6 mM Na_2HPO_4, and 1 mM KH_2PO_4) by intraperitoneal injection. In some experiments, systolic blood pressure was measured plethysmographically at the tail of restrained animals with an Infraton-Tensiomat (Boucke, Tübingen, Germany) between 6 and 7 h after injection of LPS. Tissues were removed after decapitation 7 and 12 h after LPS injection. Plasma was obtained by centrifugation of heparinized (50 IE/ml heparin, Novo Industrials, Bagsvaerd, Denmark) blood. Tissues were immediately homogenized in 5 ml 0.1 M Tris-HCl buffer, pH 7.8, containing 5 mM EDTA, 0.3 M KCl, 10% (v/v) glycerol, 100 μM phenylmethanesulphonyl fluoride (Serva, Heidelberg, Germany), and 5 mM 1,4-dithioerythritol (Merck, Darmstadt, Germany), using an Ultraturrax. Homogenates were then centrifuged for 5 min at 10,000 × *g* and the supernatants were used for further assays.

2.2. *Pteridine determination*

Homogenates or plasma were immediately treated with iodine in acidic or basic media according to Fukushima and Nixon [16]. Briefly, 500 μl of homogenate or plasma were mixed with 50 μl 1 N HCl (total biopterin) or 50 μl 1 N NaOH (alkali-stable 7,8-dihydrobiopterin and fully oxidized biopterin) and 50 μl 0.1 M iodine in 0.25 M potassium iodide. Mixtures were incubated at room temperature in the dark for 1 h. Alkaline incubations were acidified with 100 μl 1 N HCl. Insoluble material from alkaline and acidic incubations was removed by centrifugation at 10,000 × *g* for 2 min and excess iodine was reduced by 50 μl of 0.1 M ascorbic acid. Incubations were applied to solid phase cartridges (SCX, Varian, Palo Alto, CA, USA) and then directly eluted onto a reversed phase C-18 column (Lichrospher, 250 mm × 4 mm i.d., 5 μm particle size, Merck) using an AASP module (Varian) as described [17]. After elution with 0.015 M potassium phosphate buffer, pH 6.0, at a flow rate of 0.8 ml/min, pteridines were detected by fluorescence (excitation 350 nm, emission 440 nm).

2.3. *Determination of GTP cyclohydrolase I*

GTP cyclohydrolase I activity was quantified by a method modified from Viveros et al. [18] as previously described [6]. Freshly prepared homogenates were freed from low molecular mass compounds by Sephadex G-25 separation (NAP-5, Pharmacia, Uppsala, Sweden). The protein fraction was eluted with 0.1 M Tris-HCl, pH 7.8, 5 mM EDTA, 0.3 M KCl, 10% (v/v) glycerol, 100 μM phenylmethanesulfonyl fluoride, and 5 mM 1,4-dithioerythritol. Eluates were kept on ice until assayed for GTP cyclohydrolase I within 4 h of preparation. Briefly, 250 μl of protein fractions were mixed with 2 mM GTP in a final volume of 300 μl and incubated for 90 min at 37°C in the dark. The reaction was stopped by addition of 0.1 M HCl and 0.01 M I_2. After oxidation of 7,8-dihydroneopterin triphosphate to neopterin triphosphate for 1 h at room temperature in the dark, excess iodine was reduced with 0.1 M ascorbic acid, incubations were neutralized using 1 N NaOH, and neopterin triphosphate was cleaved to neopterin by incubation with alkaline phosphatase (8 U/ml, from calf intestine, Serva) for 1 h at 37°C. Neopterin was finally quantified by HPLC as outlined above according to a previously described method [17]. Protein was determined according to Bradford [19] (Bio-Rad, Richmond, CA, USA) with pure bovine serum albumine (Serva) as a standard.

2.4. *Determination of plasma nitrate levels*

Nitrate in plasma was detected according to Green et al. [20] with minor modifications. Briefly, 100 μl of plasma were treated with 20 μl of 35% sulfosalicylic acid (Merck), the protein precipitate was removed by centrifugation at 10,000 × *g* for 2 min and the acid was neutralized with 1 N NaOH. Samples (50 μl) were then applied to a reversed phase C-18 column (Lichrospher, 250 mm × 4 mm i.d., 5 μm particle size, Merck) and eluted with 5% NH_4Cl, pH 7.0. Nitrate was reduced to nitrite by a cadmium reactor (cadmium, 0.3–0.8 mm, 20–50 mesh ASTM, Merck, washed with 0.1 N HCl and packed into a Pharmacia HR 5/5 glass column), and nitrite was finally quantified after post-column mixing with the stable Griess-Ilosvay reagent from Merck by measurement of UV absorption at 546 nm.

3. RESULTS AND DISCUSSION

Table I shows GTP cyclohydrolase I activities and biopterin levels found in tissues of rats either untreated or treated with LPS for 7 and 12 h, respectively. For untreated animals, highest GTP cyclohydrolase I activities were found in liver, spleen, and adrenal gland, and activities detected here are within the same order of magnitude as was reported for Balb/c mice [7]. After 7 h of LPS treatment, a significant 2-fold increase of GTP cyclohydrolase I could be seen only in liver and spleen which was not further enhanced after longer LPS treatment. Increase in activity, however, becomes significant also in the other tissues tested after 12 h of LPS treatment, suggesting that induction of GTP cyclohydrolase I becomes generalized after longer LPS treatment.

In parallel, biopterin concentrations are significantly increased 2- to 10-fold in all tissues but liver. In previous work we found that biopterin levels of bile were 30-fold higher than biopterin levels from blood of various adjacent blood vessels [21], suggesting excretion of biopterin via bile. This may explain why liver biopterin concentrations were not found to be elevated despite enhanced GTP cyclohydrolase I activity. Biopterin levels of adrenal gland were far higher than those obtained with other tissues after LPS treatment for 12 h (Table I). The major portion of biopterin was found in its tetrahydro-form and variation of this amount most probably occurs due to instability of tetrahydrobiopterin after homogenization of the tissue.

In mammals, tetrahydrobiopterin acts as a hydroxylation cofactor in monooxygenation of phenylalanine [22], tyrosine [23], and tryptophan [24], and in the cleavage of glyceryl ethers in rat liver microsomes [25,26]. It was further shown to be required for full activity of both, cytokine-inducible and constitutive forms of NO synthase, an enzyme which further depends on NADPH and flavins, and catalyzes the oxygenation of L-arginine to NO and L-citrulline. NO has been shown to be involved in vasorelaxation, neurotransmission and in cytotoxicity (for review see [12]). Using intact cells, it has been demonstrated that intracellular tetrahydrobiopterin levels modulate the amount of NO formed in murine fibroblasts [27], endothelial and smooth muscle cells [28,5], as well as in porcine and human endothelial cells [29,3].

As previously reported, an about 10-fold increase in

urinary nitrate excretion has been observed in rats treated with LPS from Escherichia coli within 24 h after injection [30]. Further, induction of NO synthase in rats has been demonstrated for various tissues 6 h after LPS injection, yielding highest activities in lung, liver and spleen [13,15]. As can be seen from Table II, a 10- and 100-fold raise in plasma nitrate levels 7 h and 12 h after injection of LPS was observed in our experiments, indicating induction of NO synthase. In parallel, systolic blood pressure was reduced from 137±8 mm Hg (untreated controls) to 106±6 mm Hg (mean of 5 animals ± S.D., $P < 0.0001$, Student's t-test) as determined 6 h after LPS injection. This is consistent with the observation, that induction of NO synthase is involved in hypotension induced by LPS, tumour necrosis factor-α, and interleukin-1-α [31–33].

Interestingly, biopterin concentrations of plasma were not significantly increased after 7 h or 12 h of LPS treatment (Table II), suggesting that tetrahydrobiopterin is kept effectively within the tissues. This may explain why biopterin levels, which are strongly increased by cytokines in human cells in vitro [2,3,8], are only slightly increased in body fluids of patients with activated cellular immunity in vivo [10].

Our data clearly demonstrate that treatment with LPS, which may act directly but also by induction of interferon-γ and tumour necrosis factor-α [34], leads to increased activity of GTP cyclohydrolase I and therefore to increased tetrahydrobiopterin levels in various tissues. A similar increase has been reported previously for adrenal gland from rats treated with insulin and reserpine [18,35]. Inhibition of the effects of insulin and reserpine by cycloheximide suggested de novo synthesis of GTP cyclohydrolase I in this case [18,35]. In hepatocytes obtained from rats treated with *Corynebacterium parvum* a 2-fold increase of GTP cyclohydrolase I mRNA has been reported [36], thus indicating that also induction of GTP cyclohydrolase I by LPS may be regulated by protein de novo synthesis. It was suggested previously that increased tetrahydrobiopterin tissue levels may participate in the regulation of tyrosine hydroxylase and also tryptophan and phenylalanine hydroxylase, which are not saturated with tetrahydrobiopterin under normal conditions [18,35]. Administration of tetrahydrobiopterin increased tyrosine hydroxylation in rat striatum in vivo and in a number of in vitro systems including striatal synaptosomes and cultured sympathetic neurons (outlined in [35]). Results indicating that also NO synthase is not saturated with tetrahydrobiopterin but can be further stimulated by exogenous addi-

Table I

Induction of GTP cyclohydrolase I and formation of tetrahydrobiopterin in tissues of LPS-treated rats.

Tissue	LPS	Time	GTP-CHI ($pmol \cdot mg^{-1} \cdot min^{-1}$)	Total biopterin ($pmol \cdot mg^{-1}$)	H_4-biopterin (%)
Cerebellum	−	7 h	0.024 ± 0.005	7.2 ± 2.2	71 ± 27
	+	7 h	0.032 ± 0.010	9.3 ± 4.5	87 ± 18
	−	12 h	0.030 ± 0.011	9.4 ± 2.6	48 ± 14
	+	12 h	0.088 ± 0.025***	19.2 ± 5.1**	66 ± 14
Remaining brain	−	7 h	0.117 ± 0.090	12.0 ± 5.2	59 ± 26
	+	7 h	0.114 ± 0.020	10.0 ± 2.9	75 ± 8
	−	12 h	0.118 ± 0.011	12.3 ± 2.5	81 ± 5
	+	12 h	0.207 ± 0.061**	20.5 ± 4.7**	83 ± 7
Liver	−	7 h	1.520 ± 0.260	63.1 ± 30.4	54 ± 18
	+	7 h	3.351 ± 1.051**	76.3 ± 15.7	59 ± 7
	−	12 h	1.948 ± 0.576	78.8 ± 31.8	94 ± 9
	+	12 h	3.803 ± 0.760***	77.4 ± 19.1	84 ± 17
Spleen	−	7 h	0.572 ± 0.260	47.2 ± 28.4	70 ± 8
	+	7 h	1.101 ± 0.790*	61.2 ± 26.0	70 ± 7
	−	12 h	0.683 ± 0.179	42.0 ± 15.3	73 ± 17
	+	12 h	1.135 ± 0.441*	84.9 ± 17.5**	73 ± 16
Adrenal gland	−	7 h	0.535 ± 0.027	29.9 ± 11.0	82 ± 4
	+	7 h	1.040 ± 0.531	43.1 ± 14.6	68 ± 29
	−	12 h	0.688 ± 0.179	19.9 ± 9.1	49 ± 24
	+	12 h	2.270 ± 0.536***	182.0 ± 39.3***	37 ± 23

The data are means ± S.D. from 5 (7 h) or 6 (12 h) animals. Rats were treated with vehicle (phosphate-buffered saline) or 6 mg/ml LPS from *Escherichia coli* for 7 or 12 h. For GTP cyclohydrolase I and biopterin determination see section 2. Activity of GTP cyclohydrolase I is given as pmol neopterin formed per mg total protein per min. The percentage of tetrahydrobiopterin (H_4-biopterin) was calculated from the difference of total and alkali-stable 7,8-dihydro and oxidized biopterin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from vehicle-treated rats (Student's t-test).

Table II

Biopterin and plasma nitrate levels in LPS-treated rats.

Treatment	Total biopterin ($nmol \cdot l^{-1}$)	H_4-biopterin (%)	Nitrate ($\mu mol \cdot l^{-1}$)	Protein ($g \cdot l^{-1}$)
Control 7 h	53.1 ± 11.0	59 ± 6.7	15.7 ± 2.7	46.7 ± 6.3
LPS 7 h	53.7 ± 23.1	62 ± 20.4	190.1 ± 93.4**	46.4 ± 10.6
Control 12 h	56.6 ± 21.2	71 ± 13	14.3 ± 2.6	47.5 ± 5.2
LPS 12 h	69.3 ± 15.1	60 ± 17	1378.5 ± 416.2***	46.5 ± 4.6

The data are means ± S.D. from 5 (7 h) or 6 (12 h) animals. Rats were treated with vehicle (phosphate-buffered saline) or 6 mg/kg LPS from *Escherichia coli* for 7 or 12 h. Biopterin was detected after oxidation with iodine in acidic (total biopterin) or alkaline (7,8-dihydro- and oxidized biopterin) media by HPLC with fluorescence detection. The percentage of tetrahydrobiopterin (H_4-biopterin) was calculated from the difference of total and alkali-stable biopterin. Nitrate was detected after reduction to nitrite by means of a cadmium reactor by incubation with Griess-Ilosvay reagent. $^{**}P < 0.01$, $^{***}P < 0.001$; significantly different from vehicle-treated rats (Student's *t*-test).

tion of the cofactor were obtained for cytokine-inducible NO synthase [5,27,28] as well as for constitutive NO synthase from human endothelial cells [3]. Thus, increased tetrahydrobiopterin levels following LPS treatment may act on NO formation as well as on biosynthesis of catecholamines and serotonin. It remains to be seen whether there is a ranking order in stimulation of the various tetrahydrobiopterin-dependent enzymes by LPS- or cytokine-induced raise in tetrahydrobiopterin and to what relative extent these pathways contribute to LPS- and cytokine-mediated effects such as e.g. hypotension [31–33].

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