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Bacterial Lipopolysaccharide Down-regulates Expression of GTP Cyclohydrolase I Feedback Regulatory Protein*

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GTP cyclohydrolase I feedback regulatory protein (GFRP) is a 9.7-kDa protein regulating GTP cyclohydrolase I activity in dependence of tetrahydrobiopterin and phenylalanine concentrations, thus enabling stimulation of tetrahydrobiopterin biosynthesis by phenylalanine to ensure its efficient metabolism by phenylalanine hydroxylase. Here, we were interested in regulation of GFRP expression by proinflammatory cytokines and stimuli, which are known to induce GTP cyclohydrolase I expression. Recombinant human GFRP stimulated recombinant human GTP cyclohydrolase I in the presence of phenylalanine and mediated feedback inhibition by tetrahydrobiopterin. Levels of GFRP mRNA in human myelomonocytoma (THP-1) cells remained unaltered by treatment of cells with interferon-γ or interleukin-1β, but were significantly down-regulated by bacterial lipopolysaccharide (LPS, 1 μg/ml), without or with cotreatment by interferon-γ, which strongly up-regulated GTP cyclohydrolase I expression and activity. GFRP expression was also suppressed in human umbilical vein endothelial cells treated with 1 μg/ml LPS, as well as in rat tissues 7 h post intraperitoneal injection of 10 mg/kg LPS. THP-1 cells stimulated with interferon-γ alone showed increased pteridine synthesis by addition of phenylalanine to the culture medium. Cells stimulated with interferon-γ plus LPS, in contrast, showed phenylalanine-independent pteridine synthesis. These results demonstrate that LPS down-regulates expression of GFRP, thus rendering pteridine synthesis independent of metabolic control by phenylalanine.

GTP cyclohydrolase I (EC 3.5.4.16) is the first and key enzyme of tetrahydrobiopterin biosynthesis. Tetrahydrobiopterin is required as cofactor for certain hydroxylases, including phenylalanine-4-, tyrosine-3-, tryptophan-5-, and glyceryl ether-monooxygenase, as well as nitric-oxide synthase (1). To ensure efficient degradation of phenylalanine by phenylalanine hydroxylase, GTP cyclohydrolase I activity and hence tetrahydrobiopterin biosynthesis is stimulated by phenylalanine, but not by other substrates of tetrahydrobiopterin-dependent enzymes. On a molecular level, this regulation is achieved by a small (9.7 kDa) regulatory protein associated with GTP cyclohydrolase I (2–4), which has been termed GTP cyclohydrolase I feedback regulatory protein (GFRP).[1] Thus, the tetrahydrobiopterin requirement for phenylalanine metabolism, which on a quantitative basis is more demanding than for *e.g.* nitric-oxide synthesis (5), is met by this finely tuned system.

In addition to the metabolic roles mentioned above, GTP cyclohydrolase I is induced by proinflammatory cytokines in a number of cells and tissues (6, 7). On a molecular level, this regulation is achieved by increased transcription/translation, yielding increased GTP cyclohydrolase I protein levels (8, 9). A role for this induction of GTP cyclohydrolase I by proinflammatory stimuli is the supply of sufficient tetrahydrobiopterin for the abundant formation of nitric oxide by the inducible isoform of nitric-oxide synthase, which is induced in parallel (7, 10).

In the present work we investigated the role of GFRP in cytokine-induced tetrahydrobiopterin biosynthesis. We present evidence that GFRP is down-regulated by bacterial lipopolysaccharide (LPS) in cultured human cells and in rats *in vivo*, thus rendering tetrahydrobiopterin biosynthesis independent of metabolic control by phenylalanine.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Human GFRP—A cDNA was prepared from total RNA of human myelomonocytoma (THP-1) cells (ATCC, Manassas, VA) using oligo(dT) as primer and Superscript II (Invitrogen, Carlsbad, CA) reverse transcriptase. From this cDNA the reading frame of human GFRP was obtained by polymerase chain reaction (PCR) using primers gfrp1 5′ ATGCCCTACCTGCTCATC 3′ and gfrp2 5′ TCATTCCTTGTGCAGACA 3′, *Taq* DNA polymerase (Promega, Heidelberg, Germany) and 120 s at 95 °C, followed by 30 cycles of 30 s at 94 °C, 60 s at 60 °C, 60 s at 72 °C. The resulting 255-bp product was cloned (TA cloning kit, Invitrogen), sequenced, and found identical to the sequence predicted from the human GFRP gene (GenBank™ accession number U78190) and used as a probe for Northern blots. For expression, an *Nde*I site was introduced by PCR (primer gfrpmut1 5′ GAATTCGGCATATGCCCTAC 3′ and gfrp2), and the GFRP reading frame was subcloned via the *Nde*I/*Xho*I sites into the pET21a vector (Novagen, Madison, WI). Expression was achieved in *Escherichia coli* BL21DE3 cells (Novagen) by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Bacteria were then collected by centrifugation, and the bacterial pellets were opened by incubation with lysozyme/DNase I, centrifuged, and freed from low molecular mass substances by gel filtration on NAP-5 columns (Amersham Biosciences, Inc., Uppsala, Sweden) using GTP cyclohydrolase I assay buffer (see below). Control bacterial extracts were prepared with GFRP-pet21a without IPTG and with pET21a vector containing no insert but treated with IPTG.

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[1] The abbreviations used are: GFRP, GTP cyclohydrolase I feedback regulatory protein; LPS, lipopolysaccharide; IPTG, isopropyl-β-D-thiogalactopyranoside; HUVEC, human umbilical vein endothelial cells; HPLC, high performance liquid chromatography.

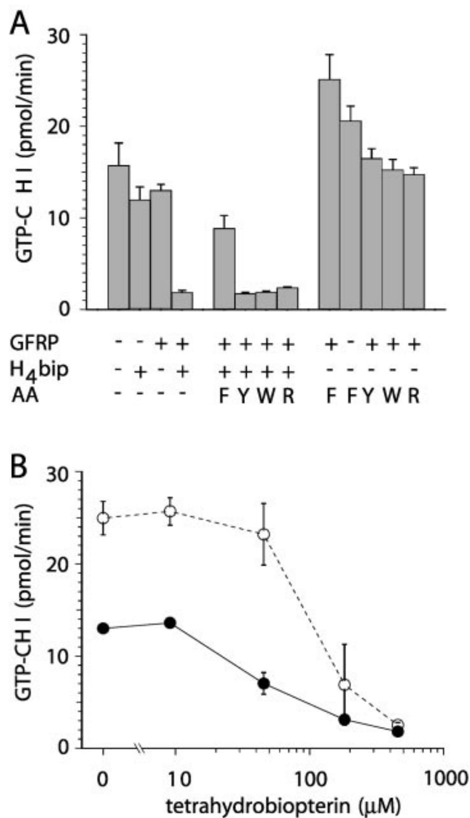

FIG. 1. **Effect of human GFRP on the activity of human recombinant GTP cyclohydrolase I.** GTP cyclohydrolase I activity in the respective incubations was deterimed as neopterin by HPLC and fluorescence detection after acidic iodine oxidation and dephosphorylation as detailed under "Experimental Procedures." *A*, effect of GFRP, tetrahydrobiopterin and amino acids on GTP cyclohydrolase I activity. Human recombinant GTP cyclohydrolase I (16 nmol/min) was incubated with protein fractions of bacterial lysates expressing GFRP, with tetrahydrobiopterin (*i.e.* (6*R*)-5,6,7,8-tetrahydro-L-*erythro* biopterin) (H_4bip, 45.5 μM) and with the following amino acids (*AA*, 2 mM) as indicated by the *plus* or *minus sign* and the letters (single-letter code: *F*, L-phenylalanine; *Y*, L-tyrosine; *W*, L-tryptophan; *R*, L-arginine). *B*, effect of the addition of L-phenylalanine on the feedback inhibition of human GTP cyclohydrolase I by tetrahydrobiopterin in presence of GFRP. *Filled circles* with *solid line*, no L-phenylalanine added; *open circles* with *dotted line*, 2 mM L-phenylalanine added. The figure shows mean ± S.D. values of measurements from three parallel incubations. Statistical differences (see text) were calculated by the Student's *t* test.

Analysis of Influence of Human Recombinant GFRP on the Activity of Human Recombinant GTP Cyclohydrolase I—Human recombinant GTP cyclohydrolase I was obtained by expression in pET16b and purification with DEAE-cellulose and hydroxylapatite columns as described previously (9). For GTP cyclohydrolase I incubations, standard GTP cyclohydrolase I assay buffer (11), *i.e.* 0.05 M Tris·HCl, pH 7.8, containing 0.3 M KCl, 2.5. mM EDTA, and 10% (v/v) glycerol was used. In a total volume of 110 μl, 2 mM GTP was incubated with 16 pmol/min GTP cyclohydrolase I and 40 μg of bacterial lysate containing human recombinant GFRP (see above), with or without addition of 9.1–455 μM tetrahydrobiopterin. In some experiments L-phenylalanine, L-tyrosine, L-tryptophan, or L-arginine was added (2 mM each). After incubation for 45 min at 37 °C, the incubation mixture was oxidized with acidic iodine, centrifuged, neutralized, incubated with alkaline phosphatase, and the resulting neopterin determined by HPLC with fluorescence detection as detailed elsewhere (11).

Cell Culture—Human THP-1 myelomoncytoma cells were grown in RPMI 1640 (Biochrom, Berlin, Germany) with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum in a humidified atmosphere containing 5% CO_2. In some experiments, 2 mM L-phenylalanine, L-tyrosine, L-tryptophan, or L-arginine was added to the culture medium with or without addition of 1–100 μM L-sepiapterin (Schircks Laboratories, Jona, Switzerland). Cells were treated for 48 h with 1250 units/ml human recombinant interferon-γ (Gammaferon, kindly provided by R. E. Rentschler Biotechnologie, Laupheim, Germany), LPS (1 μg/ml, *E. coli* O55:B5, Sigma, Vienna, Austria), or interleukin-1β (2.5 ng/ml, R & D Systems, Abingdon, UK). GTP cyclohydrolase I activity in intact cells was determined by quantification of the amount of neopterin accumulated in the culture medium by HPLC (11). For RNA isolation, the cells were collected 7 h post-stimulation, unless indicated otherwise. For mRNA stabilty measurements, THP-1 cells were incubated with or without 1 μg/ml LPS for 10 h. Actinomycin-D (5 μg/ml) was then added, and the cells were incubated for a further 1.5–24 h. Total RNA was prepared and GFRP mRNA quantified by real-time PCR as outlined below. Cell viability was assayed with a formazan dye reduction assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Promega, Heidelberg, Germany). Human umbilical vein endothelial cells (HUVEC) were isolated using 0.05% collagenase; cultivated in M199 with 15% fetal calf serum, 5% human serum, and 7.5 μg/ml endothelial growth supplement; stimulated with the indicated agents for 6 h; and collected as described previously (12). RNA was then prepared as detailed below.

Animal Experiments—The experimental protocol was approved by the local Committee on Animal Experiments of the Viennese government, and the care and handling of the animals were undertaken in accordance with the National Institutes of Health guidelines. Male Sprague-Dawley rats (240–260 g) were used. All animals had free access to normal food and water throughout the experiment. Six animals per group were used. Endotoxin from *E. coli* O26:B6 (Difco) was dissolved in 0.9% (w/v) NaCl and injected intraperitoneally (10 mg/kg); controls received no treatment. 7 h after endotoxin administration, the animals were killed with an overdose of pentobarbital sodium. Tissues were prepared, immediately frozen in liquid nitrogen, and stored at −80 °C until isolation of RNA.

Isolation of RNA, Northern Blots, and Real-time PCR—RNA was isolated from THP-1 cell pellets and rat tissues, which were ground under liquid nitrogen using a mortar, with the guanidium thiocyanate method (13). RNA from HUVEC was isolated by cesium chloride centrifugation. Total RNA (20 μg) was then resolved with 1% (w/v) agarose, 6% (v/v) formaldehyde gels; blotted to Duralon UV nylon membranes (Stratagene, La Jolla, CA) by means of vacuum; and cross-linked by UV irradiation (Stratalinker, Stratagene). Blots were hybridized overnight with 10^6 cpm/ml of [^{32}P]dCTP-labeled probes at 65 °C and exposed to Bio-Max MS or X-Omat autoradiography films (Eastman Kodak Co.) or to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). As probes the 255-bp GFRP reading frame PCR product (see above) and a 578-bp human GTP cyclohydrolase I PCR product (9) were applied. For quantitative, real-time PCR, 0.5 μg of total RNA was transcribed to cDNA using Superscript II reverse transciptase (Invitrogen) and random hexamer primers (Microsynth, Balgach, Switzerland). For quantification, the Taqman technology, an ABI Prism 7700 sequence detector (ABI Biosystems, Vienna, Austria), and the brilliant core PCR kit (Stratagene) were used. Probes, all 5′ 6-carboxyfluorescein- and 3′ 6-carboxytetramethylrhodamine-labeled, were obtained from Microsynth. Primers and probes had the following sequences: human GFRP, primer forward, 5′ TCATCAGCACCCAGATCCG 3′; primer reverse, 5′ TCTGATCCGACTGTTCATGC 3′; probe, 5′ CACCATAGTGGGGCCCACCTCCA 3′; rat GFRP, primer forward, 5′ AGCCCCAGCCACTCACC 3′; primer reverse, 5′ CCGAGTGCTCATCACCCAC 3′; probe, 5′ CAGATCCGTATGGAAGTGGGTCCCAC 3′; 18 S (rat and human), primer forward, 5′ CCATTCGAACGTCTGCCCTAT 3′; primer reverse, 5′ TCACCCGTGGTCACCATG 3′; probe, 5′ ACTTTCGATGGTAGTCGCCGTGCCT 3′. mRNA concentrations are expressed as numbers of copies of mRNA per numbers of copies 18 S RNA, which are both quantified in the same cDNA preparation.

RESULTS

L-Phenylalanine Stimulates Human Recombinant GTP Cyclohydrolase I in the Presence of GFRP—Since all work on the action of GFRP had been done thus far with rat enzymes, and since we wanted to study GFRP expression in human cells, we first cloned and expressed human GFRP and studied its action on human recombinant GTP cyclohydrolase I. As is shown in Fig. 1*A*, tetrahydrobiopterin inhibited GTP cyclohydrolase I only in the presence of GFRP. L-Phenylalanine, but not L-tyrosine, L-tryptophan, or L-arginine was able to overcome this feedback inhibition. L-Phenylalanine stimulated GTP cyclohydrolase I 2.40 ± 0.61-fold over the whole range of tetrahydrobiopterin concentrations studied, with the exception of 455 μM, which resulted in almost complete inhibition of GTP cyclohy-

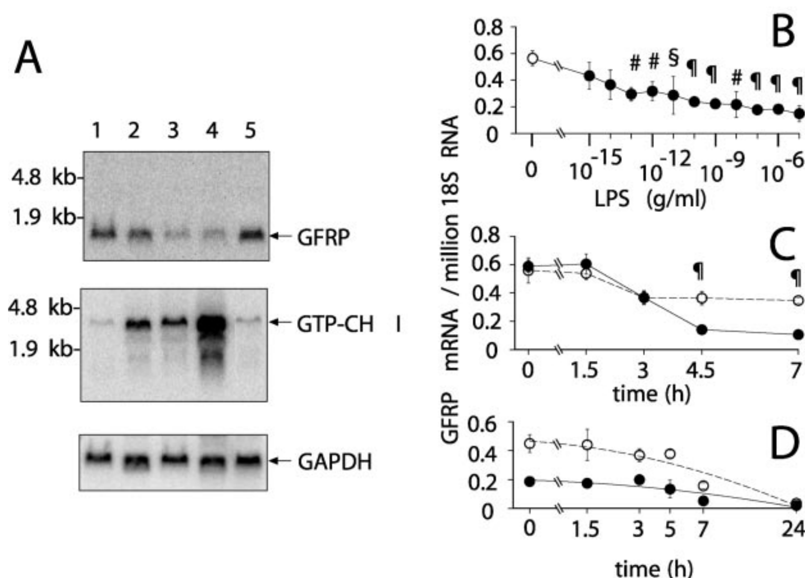

FIG. 2. **LPS down-regulates GFRP mRNA in human myelomonocytoma (THP-1) cells.** *A*, Northern blots. THP-1 cells were cultivated for 7 h in the presence of the following stimuli: control (*lane 1*), interferon-γ (1250 units/ml) (*lane 2*), LPS (1 μg/ml) (*lane 3*), interferon-γ plus LPS (*lane 4*), interleukin-1β (2.5 ng/ml) (*lane 5*). Cells were collected, RNA prepared, and Northern blots carried out as detailed under "Experimental Procedures." The same blot was probed first for GFRP, then for GTP cyclohydrolase I (*GTP-CH I*), and finally with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The size markers indicate the position of the two ribosomal RNA bands stained with ethidium bromide in the gel before blotting. One of three similar blots is shown. *B*, dose dependence. THP-1 cells were incubated with the indicated doses of LPS for 7 h; total RNA was prepared and GFRP mRNA quantified in relation to 18 S RNA by real-time PCR as described under "Experimental Procedures." *C*, time course. THP-1 cells were incubated with 1 μg/ml LPS (*filled circles* with *solid line*) or without LPS (*open circles* with *dashed line*) for the indicated time points, and GFRP mRNA was determined by real-time PCR in relation to 18 S RNA. *D*, mRNA stability. THP-1 cells were treated for 10 h with 1 μg/ml LPS (*filled circles* with *solid line*) or without LPS (*open circles* with *dashed line*). Actinomycin-D was then added, and the cells were harvested at the indicated time points post-actinomycin-D treatment, and GFRP mRNA was quantified in relation to 18 S RNA by real-time PCR as described under "Experimental Procedures." Statistical differences from untreated controls (see also text) were calculated by the Student's *t* test (§, $p < 0.05$; #, $p < 0.01$; ¶, $p < 0.001$).

drolase I irrespective of phenylalanine addition (Fig. 1*B*). In absence of GFRP, L-phenylalanine still had some effect on GTP cyclohydrolase I (1.31 ± 0.07-fold, Fig. 1*A*), but this was significantly lower ($p < 0.05$, Student's *t* test) than in presence of GFRP. Bacterial lysates carrying the empty vector treated with IPTG had no effect on GTP cyclohydrolase I activities irrespective of tetrahydrobiopterin and/or phenylalanine addition (not shown). The residual *E. coli* GTP cyclohydrolase I activity in GFRP preparations was less than 3% of the recombinant human GTP cyclohydrolase I activity in the assay mixture. Serial dilution experiments showed that bacterial lysates of incubation with the GFRP containing expression plasmid, but without IPTG induction, mediated less than 10% of feedback inhibition compared with the respective IPTG-treated cultures (not shown).

LPS Down-regulates GFRP mRNA in THP-1 Cells, in HUVEC, and in Rat Tissues—Next we studied expression of GFRP mRNA in conditions known to induce GTP cyclohydrolase I by Northern blot in THP-1 cells in relation to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Fig. 2*A*). While interferon-γ alone had no effect on the expression of GFRP (97 ± 31% of control), LPS (37 ± 15% of control) and interferon-γ plus LPS (42 ± 16% of control) significantly ($p < 0.05$, Student's *t* test) suppressed GFRP expression in THP-1 cells. This effect of LPS appeared not to be mediated by interleukin-1β, which had no effect on GFRP expression in THP-1 cells (Fig. 2*A*). A dose of 0.1 pg/ml LPS was sufficient to cause a significant suppression of GFRP mRNA ($p < 0.01$, Student's *t* test). This effect was gradually increasing with higher doses of LPS (up to 10 μg/ml, Fig. 2*B*). GFRP mRNA levels remained similar in controls and in LPS-treated THP-1 cells up to 3 h and became significantly different after 4.5 and 7 h ($p < 0.001$ for both time points, Student's *t* test, Fig. 2*C*). The stability of GFRP mRNA appeared to be similar in control cells and in cells treated with 1 μg/ml LPS (Fig. 2*D*).

In HUVEC, comparable effects were observed with real-time PCR in relation to 18 S RNA. Interferon-γ alone did not significantly suppress GFRP mRNA (65 ± 28% of control). In contrast, in LPS-treated and in interferon-γ plus LPS-treated cultures a significant suppression of GFRP mRNAs was observed (44 ± 33% of control, $p < 0.05$ and 43 ± 19% of control $p < 0.02$, respectively, Student's *t* test). Interleukin-1 β, in contrast, had no significant effect on GFRP expression (not shown).

Next we determined GFRP mRNA levels in rats treated with LPS by real-time PCR in relation to 18 S RNA. GFRP mRNA in tissues from control animals was highest in liver (2.7 ± 0.5 RNA copies per million 18 S RNA copies), followed by spleen (0.48 ± 0.08), lung (0.045 ± 0.011), and brain (0.033 ± 0.011). Seven hours post-intraperitoneal LPS treatment, GFRP mRNA levels were significantly lower in liver, spleen, and lung ($p < 0.0001$ each, Student's *t* test, Fig. 3). The slight decrease of GFRP mRNA levels seen in brain was not statistically significant.

LPS Renders Interferon-γ-induced Neopterin Formation by THP-1 Cells Independent of L-Phenylalanine Concentrations—Finally, we wanted to see whether the down-regulation of GFRP mRNA had functional consequences for GTP cyclohydrolase I activities in THP-1 cells. For this purpose, we compared formation of neopterin by THP-1 cells stimulated with interferon-γ alone (unaltered GFRP expression, Fig. 2*A*) with THP-1 cells stimulation by interferon-γ plus LPS (reduced GFRP expression, Fig. 2*A*). Neopterin formation was studied in cells treated with increasing doses of sepiapterin, which leads to accumulation of intracellular tetrahydrobiopterin, with or without the addition of L-phenylalanine. L-Phenylalanine stimulated neopterin formation in THP-1 cells treated with inter-

feron-γ alone ($p < 0.001$, Student's t test, Fig. 4*A*), whereas it had no stimulatory effect in cells treated with interferon-γ plus LPS (Fig. 4*B*). L-Phenylalanine had no effect on the cell viability in either type of stimulation (Fig. 4, *C* and *D*). The other amino acids tested, *i.e.* L-tyrosine, L-tryptophan, and L-arginine, had no effect on formation of neopterin by THP-1 cells (not shown).

DISCUSSION

In the present paper we studied the influence of proinflammatory stimuli on the expression of GFRP. We show that LPS down-regulates expression of GFRP in two cultured human cells as well as in rats treated with LPS *in vivo*. This finding was unexpected, since we had anticipated that GFRP would be induced together with the protein it regulates, *i.e.* with GTP cyclohydrolase I. In the case of LPS stimulation, just the opposite is the case. Parallel to the strong induction of GTP cyclohydrolase I, GFRP mRNA is down-regulated. This renders GTP cyclohydrolase I activity in the cells independent of the metabolic control by L-phenylalanine. A goal of the induction of GTP cyclohydrolase I by proinflammatory stimuli is to provide sufficient tetrahydrobiopterin for optimal formation of nitric oxide from L-arginine by the inducible isoform of nitric-oxide synthase (10), which is induced in parallel to GTP cyclohydrolase I (7). By down-regulation of GFRP, accumulation of tetrahydrobiopterin in cells is potentially maximized due to down-regulation of the feedback inhibitory mechanism. In addition, the metabolic control by phenylalanine, which is not required in the context of immunologically triggered nitric oxide formation, is switched off.

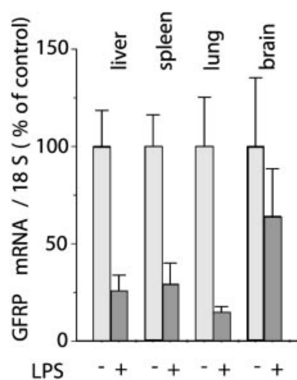

FIG. 3. **Expression of GFRP mRNA is decreased in tissues of rats treated with LPS.** Rats were treated intraperitoneally with 10 mg/kg LPS or remained untreated. After 7 h, tissues were prepared. RNA was isolated, transcribed to cDNA, and analyzed with real-time PCR (Taqman technology) as detailed under "Experimental Procedures." The figure shows mean and S.D. values of mRNA levels per 18 S RNA expressed as percentage of untreated controls. Tissues were prepared and quantifications performed in six individual animals per group. GFRP mRNA per 18 S RNA in the individual tissues of untreated controls were: liver, $2.7 \cdot 10^{-6}$; spleen, $0.48 \cdot 10^{-6}$; lung, $0.045 \cdot 10^{-6}$; brain, $0.033 \cdot 10^{-6}$. These levels were set 100% in the respective tissues. Upon LPS treatment, these were reduced to: liver, $0.7 \cdot 10^{-6}$; spleen, $0.13 \cdot 10^{-6}$; lung, $0.013 \cdot 10^{-6}$; brain, $0.021 \cdot 10^{-6}$, which are displayed as percentages of the above mentioned control levels. Statistical differences (see text) were calculated by the Student's t test.

The time course of the down-regulation of GFRP mRNA by LPS (Fig. 2*C*) and mRNA stability data (Fig. 2*D*) suggest that this down-regulation is caused by decreased transcription of GFRP mRNA. Unfortunately, the expression of GFRP in the cells is too low to allow for a direct examination of transcription rates by nuclear run on assays due to sensitivity limitations. In our rat experiments, we found significant decreases of GFRP upon LPS treatment in all organs investigated except for the brain. The reason for this exception might be that LPS is applied intraperitoneally to the animals, and that the blood-brain barrier protects the brain from most of this peripherally administered LPS.

This paper is the first to describe regulation of human GTP-cyclohydrolase I by human GFRP, all other studies had been done with the corresponding rat proteins. Consistent with our observations of neopterin formation by intact THP-1 cells, we find that phenylalanine not only overcomes feedback inhibition by tetrahydrobiopterin, but is also a stimulator of GTP cyclohydrolase I activity in the absence of added tetrahydrobiopterin. This is in line with observations on GFRP expressing serotonin neurons (14), as well as on purified rat proteins (15). Compared with previous studies (2–4) we needed somewhat higher tetrahydrobiopterin concentrations to achieve feedback inhibition of GTP cyclohydrolase I, which may be explained by different experimental conditions rather than by differences in the enzymatic properties. In contrast to these previous studies (2–4), we used standard GTP cyclohydrolase I assay conditions, *i.e.* saturating levels of GTP (2 mM instead of 0.2 mM) and higher salt concentrations (0.3 M KCl instead of 0.1 M KCl) to ensure a specific interaction of the components and to avoid artifacts due to GTP limitation.

While treatment of THP-1 cells with LPS in addition to interferon-γ abolished the stimulating effect of phenylalanine,

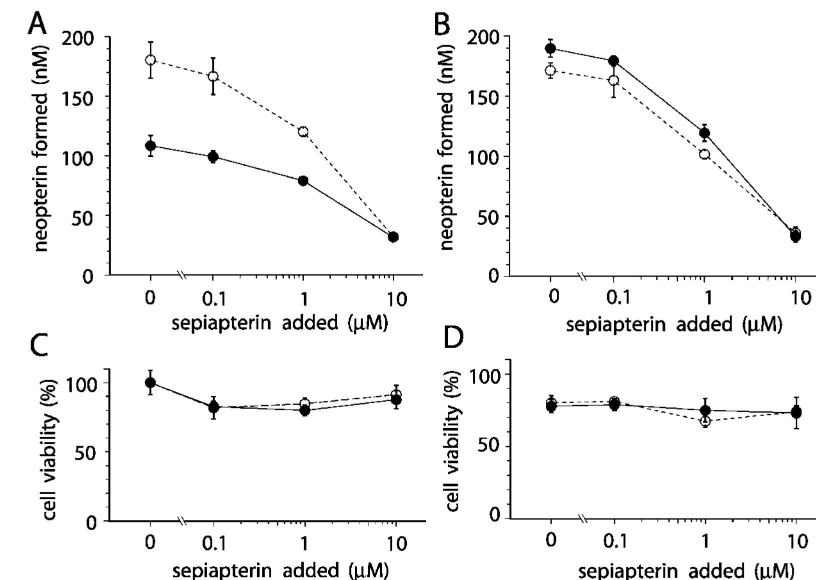

FIG. 4. **Influence of L-phenylalanine on neopterin formation by THP-1 cells treated with interferon-γ with and without LPS.** Human myelomoncytoma (THP-1) cells were stimulated with interferon-γ alone (*A*) or stimulated with interferon-γ plus LPS (*B*) and cultivated for 48 h in presence of the indicated concentrations of sepiapterin with or without L-phenylalanine. Neopterin was determined in supernatants by reversed phase HPLC with fluorescence detection (11). Immediately after collection of supernatants, cell viability was determined by a formazan dye reduction assay as detailed under "Experimental Procedures." *C*, cells stimulated with interferon-γ alone; *D*, cells stimulated with interferon-γ plus LPS). *Filled circles* with *solid line*, no addition of L-phenylalanine; *open circles* with *dashed line*, 2 mM L-phenylalanine added. The figure shows mean ± S.D. of measurements derived from four parallel incubations. Statistical differences (see text) were calculated by the Student's t test.

the feedback inhibition by sepiapterin apparently did not change. Similarly, dopamine neurons, which did not express GFRP and showed tetrahydrobiopterin levels unaltered by phenylalanine, were still susceptible to feedback inhibition by dihydroneopterin (14). Thus, either low levels of GFRP still mediated feedback inhibition by pterins but can no longer mediate phenylalanine effects, or yet unknown proteins different from GFRP may be responsible for this effect.

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