British Journal of Pharmacology (1997) 122, 534–538

Differential effect of dexamethasone on interleukin 1β- and cyclic AMP-triggered expression of GTP cyclohydrolase I in rat renal mesangial cells

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1 Endogenous synthesis of tetrahydrobiopterin (BH_4) is an essential requirement for cytokine-stimulated nitric oxide (NO) synthesis in rat mesangial cells. GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis, is expressed in renal mesangial cells in response to two principal classes of activating signals. These two groups of activators comprise inflammatory cytokines such as interleukin (IL)-1β and agents that elevate cellular levels of cyclic AMP.

2 We examined the action of the potent anti-inflammatory drug dexamethasone on GTP cyclohydrolase I induction in response to IL-1β and a membrane-permeable cyclic AMP analogue, N^6, *O*-2′-dibutyryladenosine 3′-5′-phosphate (Bt_2cyclic AMP).

3 Nanomolar concentrations of dexamethasone markedly attenuated IL-1β-induced GTP cyclohydrolase I mRNA steady state level as well as IL-1β-induced GTP cyclohydrolase I protein expression and enzyme activity. In contrast, dexamethasone did not inhibit Bt_2cyclic AMP-triggered increase in GTP cyclohydrolase I mRNA level and protein expression, and low (1 nM) or high (1 and 10 μM) doses of dexamethasone consistently increased Bt_2cyclic AMP-induced GTP cyclohydrolase activity.

4 In summary, these results suggest that glucocorticoids act at several levels, critically dependent on the stimulus used, to control GTP cyclohydrolase I expression.

Keywords: Dexamethasone; tetrahydrobiopterin; GTP cyclohydrolase I; nitric oxide; interleukin 1; cyclic AMP; mesangial cells

Introduction

Guanosine 5′-triphosphate (GTP) cyclohydrolase I converts GTP to 7,8-dihydroneopterin triphosphate, which is further metabolized to tetrahydrobiopterin (BH_4) in mammals (Nichol *et al*., 1985). BH_4 is a cofactor of amino acid hydroxylases responsible for the metabolism of phenylalanine and the biosynthesis of neurotransmitters (Kaufman, 1993). In addition, BH_4 is an essential cofactor of the inducible nitric oxide synthase (iNOS). Cytokines like interferon γ or tumour necrosis factor α stimulate the activity of GTP cyclohydrolase I up to 100 fold (Werner *et al*., 1995), whereas the two further enzymes forming BH_4 from 7,8-dihydroneopterin, 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase, are constitutively expressed (Werner *et al*., 1990). Depending on the induced activity of GTP cyclohydrolase I, BH_4 accumulates in cytokine-treated cells (Werner *et al*., 1995). This increased production of BH_4 is required to ensure a sufficient supply of cofactor for long-term synthesis of NO by iNOS. Indeed, in cells the pathways for pteridine synthesis and NO production are tightly coupled and cytokines that stimulate iNOS expression simultaneously stimulate expression of GTP cyclohydrolase I (Werner *et al*., 1995). Mesangial cells are a specialized type of vascular smooth muscle cells that contract upon stimulation with vasoactive hormones like angiotensin II and thus contribute to the regulation of the glomerular filtration rate (Pfeilschifter, 1989). In coincubation experiments, it has been shown that NO release from glomerular endothelial cells increases guanosine 3′:5′-cyclic monophosphate (cyclic GMP) within mesangial cells and thereby inhibits angiotensin II-stimulated cell contraction (Marsden *et al*., 1990). Moreover, mesangial cells not only function as a target for NO but also themselves have the capacity to synthesize NO by expressing a macrophage type of iNOS when exposed to inflammatory cytokines like IL-1β (Pfeilschifter & Schwarzenbach, 1990; Pfeilschifter *et al*., 1992; Mühl *et al*., 1993) or agents that elevate cellular levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Mühl *et al*., 1994; Kunz *et al*., 1994a). The excessive formation of NO in mesangial cells not only blocks the contractile responsiveness of the cells but may also contribute to tissue injury observed in the pathogenesis of certain forms of glomerulonephritis (Pfeilschifter *et al*., 1993; Cattell & Cook, 1993). Previously we have shown that BH_4 synthesis is an absolute requirement for, and limits IL-1β induction of NO formation in mesangial cells (Mühl & Pfeilschifter, 1994). Furthermore, the identified stimulators of iNOS expression in mesangial cells, co-ordinately induce GTP cyclohydrolase I expression in the cells (Plüss *et al*., 1996). In the present study, we found that dexamethasone differential affects IL-1β- and cyclic AMP-induced GTP cyclohydrolase I expression in mesangial cells.

Methods

Cell culture

Rat renal mesangial cells were cultured as described previously (Pfeilschifter *et al*., 1992). In a second step, single cells were cloned by limited dilution with 96-microwell plates. Clones with apparent mesangial cell morphology were characterized and used for further processing. The cells were grown in RPMI 1640 supplemented with 10% (v/v) foetal calf serum, penicillin (100 uml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), and bovine insulin (0.66 uml^{-1}). For the experiments passages 9 to 14 of mesangial cells were used.

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Nuclease protection assay

Nuclease protection assay was performed as described previously (Plüss *et al*., 1996). Briefly, 20 μg of total RNA were hybridized at 45°C for 16 h with 1 fmol of GTPCH antisense probe transcribed from plasmid pGTPCH and 0.5 fmol of ^{32}P-labelled GAPDH antisense probe transcribed from plasmid pGAPDH. The GAPDH probe was used to correct for variations. Nuclease protection assay was performed as described in the Boehringer RNase Protection Kit manual. Samples were analysed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions and quantified by Phosphor Imager and MD ImageQuant Software Version 3.3 (Bucher, Basel, Switzerland).

Western blot analysis

Western blot was performed as described previously (Plüss *et al*., 1996). Briefly, confluent mesangial cells were stimulated with different cytokines for the times indicated, washed with PBS, and scraped into 1.5 ml of RiPa - buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% Na-deoxycholat, 1 μM leupeptin, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM pepstatin). Cells were homogenized with a Dounce homogenizer and centrifuged at 14 000 $\times g$ for 15 min at 4°C. Protein concentration was determined by the method of Bradford (1976). The supernatants (3 mg of protein) were incubated overnight at 4°C with 40 μl of a 50% (v/v) slurry of protein A-sepharose in H_2O and 15 μl GTP-CH antibody (1 : 100) with gentle agitation. After centrifugation for 1 min at 14 000 $\times g$ the pellets were washed three times with RiPa-buffer. Pellets were resuspended in 45 μl of sodium dodecyl sulphate (SDS)-PAGE sample buffer and proteins were subjected to SDS-PAGE (12%). Immunoblotting was performed as described, by use of the anti-GTP-CH antibody at a dilution of 1 : 800 (Plüss *et al*., 1996). The antiserum had been raised against purified rat GTP cyclohydrolase I expressed in *Escherichia coli* TB1 strain. The antiserum recognized a single band with a molecular mass of 34 kDa present in stimulated mesangial cells but not in control cells. Purified GTP cyclohydrolase I completely quenched the reactivity of the antiserum, as described in detail elsewhere (Plüss *et al*., 1996).

Preparation of cell extracts

An extract was prepared form $(4-5)\times10^7$ cells. Cells were harvested by trypsin treatment, washed once with culture medium containing 10% FCS and once with PBS. Cells were resuspended in H_2O containing 5 mM dithioerythrit and 0.1 mg ml^{-1} phenylmethylsulphonyl fluoride. After 2 passes through a 26G needle, extracts were centrifuged at 10 000 $\times g$ for 10 min. For enzyme activity determinations, aliquots of the supernatant were separated from low molecular weight compounds by chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden). The protein-containing fraction was then immediately used to assay for GTP-CH activity.

Determination of GTP cyclohydrolase I activity

GTP cyclohydrolase activity was assessed by a method modified from Viveros *et al*. (1981). Sephadex G-25 eluates were prepared in 0.1 M Tris/HCl, ph 7.8, 0.3 M KCl, 2.5 mM EDTA, and 10% (v/v) glycerin. The eluate was incubated with 2 mM GTP for 90 min at 37°C in the dark in a total volume of 300 μl. The dihydroneopterin triphosphate thus formed was then oxidized to neopterin triphosphate with 0.1 M HCl, 0.01 M I_2 for 1 h in the dark at room temperature. After removal of precipitate by centrifugation at 10 000 $\times g$ for 2 min, excessive iodine was destroyed with 0.1 M ascorbic acid and the acid was neutralized by addition of 1 M NaOH. Neopterin triphosphate was then cleaved to neopterin by incubation with 8 units ml^{-1} of alkaline phosphate for 1 h at 37°C. Neopterin was finally quantified by solid-phase extraction and on-line elution to reverse-phase high performance liquid chromatography (h.p.l.c.) with fluorescence detection (Werner *et al*., 1987) with a detection limit of 0.1 pmol.

Materials

Recombinant human IL-1β was generously supplied by Dr Christiane Rondorf (Ciba, Basel, Switzerland); N^6, *O*-2′-dibutyryladenosine 3′,5′-phosphate (Bt_2cyclic AMP) was from Sigma (Buchs, Switzerland); cDNA encoding rat GTP-CH in pBluescript KS was kindly provided by Dr K. Hatakeyama; [α-^{32}P]-UTP (800 Ci mmol^{-1}) was purchased from Amersham (Dübendorf, Switzerland); cell-culture media and nutrients were from Gibco BRL (Basel, Switzerland); restriction endonucleases, RNase A and T_1, DNA ligase T_4 and RNA polymerase SP6 were purchased from Boehringer (Mannheim, Germany). Additional chemicals were either from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Results

In order to assess the effect of dexamethasone on IL-1β or Bt_2cyclic AMP-induced GTP cyclohydrolase I mRNA steady state levels in mesangial cells, we performed nuclease protection assays. As shown in Figure 1a and Table 1, dexamethasone at concentrations $\geqslant$1 nM decreased IL-1β-induced elevation of GTP cyclohydrolase I mRNA steady-state level, by up to 36% at 100 nM dexamethasone. In contrast, as shown in Figure 1b and Table 1, dexamethasone at concentrations up to 10 μM did not inhibit Bt_2cyclic AMP-induced accumulation of GTP cyclohydrolase I mRNA.

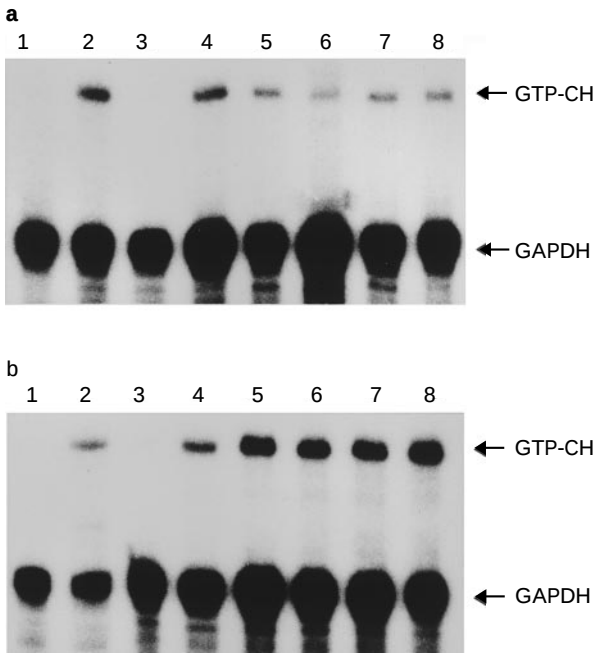

Figure 1 Inhibition of (a) IL-1β- and (b) BT_2cyclic AMP-stimulated induction of GTP cyclohydrolase I mRNA in mesangial cells by dexamethasone. (a) Mesangial cells were incubated for 24 h with vehicle (control, lane 1), IL-1β (1 nM, lane 2) alone or together with dexamethasone (1 nM (lane 4), 10 nM (lane 5), 100 nM (lane 6), 1 μM (lane 7) or 10 μM (lane 8). Dexamethasone (10 μM) alone is depicted in lane 3. (b) Mesangial cells were incubated for 24 h with vehicle (control, lane 1), Bt_2cyclic AMP (0.5 mM, lane 2) alone or together with dexamethasone (1 nM (lane 4), 10 nM (lane 5), 100 nM (lane 6) 1 μM (lane 7) or 10 μM (lane 8)). Dexamethasone (10 μM) alone is depicted in lane 3. Total cellular RNA (20 μg) was extracted and GTP cyclohydrolase I and GAPDH mRNA were detected by nuclease protection assay as described in Methods. Similar results were obtained from three independent experiments.

Table 1 Quantification of dexamethasone effect on IL-1β and Bt_2 cyclic AMP-stimulated induction of GTP cyclohydrolase I mRNA levels in mesangial cells

Addition	*24 h*
IL-1β	100
Dexamethasone (10 μM)	0
IL-1β + 1 nM Dex	85.4 ± 6.4
IL-1β + 10 nM Dex	75.0 ± 7.4
IL-1β + 100 nM Dex	64.0 ± 6.6
IL-1β + 1 μM Dex	70.6 ± 5.5
IL-1β + 10 μM Dex	73.4 ± 4.8
cAMP	100
Dexamethasone (10 μM)	0
cAMP + 1 nM Dex	104.9 ± 7.8
cAMP + 10 nM Dex	103.4 ± 4.7
cAMP + 100 nM Dex	102.1 ± 6.5
cAMP + 1 μM Dex	110.6 ± 5.4
cAMP + 10 μM Dex	108.5 ± 6.1

Mesangial cells were treated as described in Figure 1. The gels were dried and quantified by phosphor imaging (arbitrary units). The signal density of each RNA sample hybridized to GTP cyclohydrolase I probe was divided by that hybridized to the GAPDH probe. The corrected density of IL-1β and Bt_2cyclic AMP-stimulated cells was set as 100%. The data are expressed as means ± s.d. of three independent experiments. Dex, dexamethasone; cAMP, Bt_2cyclic AMP.

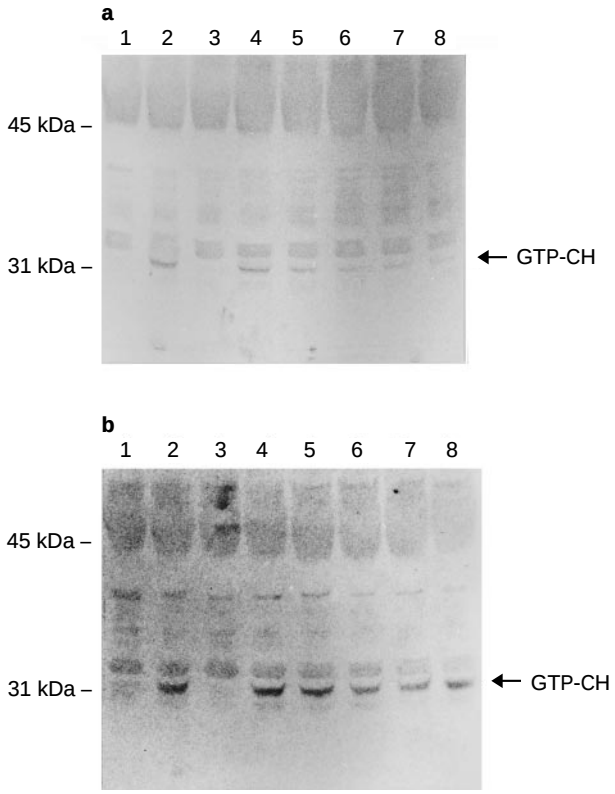

Figure 2 Inhibition of (a) IL-1β- and (b) Bt_2cyclic AMP-stimulated GTP cyclohydrolase I protein expression in mesangial cells by dexamethasone. (a) Mesangial cells were incubated for 24 h with vehicle (control, lane 1), IL-1β (1 nM, lane 2) alone or together with dexamethasone (1 nM (lane 4), 10 nM (lane 5), 100 nM (lane 6), 1 μM (lane 7) or 10 μM (lane 8)). Dexamethasone (10 μM) alone is depicted in lane 3. (b) Mesangial cells were incubated for 24 h with vehicle (control, lane 1), Bt_2cyclic AMP (0.5 mM, lane 2) alone or together with dexamethasone (1 nM (lane 4), 10 nM (lane 5), 100 nM (lane 6) 1 μM (lane 7) or 10 μM (lane 8)). Dexamethasone (10 μM) alone is depicted in lane 3. Samples were analysed by SDS/PAGE (12% gels) and transferred to a nitro-cellulose filter. Immunoblots were developed by using antiserum against GTP cyclohydrolase I at a dilution of 1 : 800. Bands were detected with alkaline phsosphatase. Similar results were obtained from three independent experiments.

Western blot analysis with a specific polyclonal antibody suggested that IL-1β and Bt_2cyclic AMP treatment of mesangial cells up-regulated the production of the GTP cyclohydrolase I protein (lanes 2 in Figures 2a and b). The band was clearly not present in unstimulated control cells (lanes 1 in Figures 2a and b. Co-incubation of IL-1β with dexamethasone concentration-dependently decreased enzyme levels as shown in Figure 2a. Maximal inhibition was observed at dexamethasone concentrations of 10 μM which blocked more than 85% of GTP cyclohydrolase protein levels when compared with the expression in cells treated with IL-1β only. The induction of GTP cyclohydrolase I by Bt_2cyclic AMP turned out to be intensive to dexamethasone and even elevated concentrations of dexamethasone (10 μM) only slightly affected protein expression (Figure 2b).

To evaluate the action of dexamethasone on IL-1β and Bt_2cyclic AMP-induced GTP cyclohydrolase I activity, enzyme activity measurements were performed as described previously (Werner *et al*., 1987; 1989). Incubation of mesangial cells for 24 h with IL-1β or Bt_2cyclic AMP led to an 8 and 4 fold increase in GTP cyclohydrolase I activity, respectively (Table 2). Simultaneous incubation of mesangial cells with IL-1β or Bt_2cyclic AMP together with dexamethasone differently affected the GTP cyclohydrolase I activity. As shown in Table 2, dexamethasone potently inhibited IL-1β-induced GTP cyclohydrolase I activity. Already at 1 nM dexamethasone, the lowest concentration tested, we observed more than 50% inhibition of GTP cyclohydrolase I activity and a maximal 85% inhibition was observed at 100 nM of the drug (Table 2). In contrast, as shown in Table 2, low (1 nM) or high (1 and 10 μM) doses of dexamethasone consistently increased Bt_2cyclic AMP-induced GTP cyclohydrolase I activity, although the nuclease protection assay and protein-blotting data (Table 1 and Figure 2b) demonstrated that dexamethasone did not, or only slightly, affect GTP cyclohydrolase mRNA accumulation and protein expression. Dexamethasone at intermediate concentrations of 10 and 100 nM tended to inhibit Bt_2cyclic AMP-induced GTP cyclohydrolase I activity weakly (Table 2).

Table 2 Inhibition of IL-1β- and Bt_2cyclic AMP-stimulated GTP cyclohydrolase I activity in mesangial cells by dexamethasone

Addition	*GTP cyclohydrolase I activity*	*GTP cyclohydrolase I activity* (% of stimulated cells)
Control	0.010; 0.011	–
IL-1β	0.198; 0.085	100, 100
Dexamethasone (10 μM)	0.017; 0.013	3.7, 2.7
IL-1β + 1nM Dex	0.077; 0.034	35.6, 31.1
IL-1β + 10 nM Dex	0.064; 0.020	28.7, 12.2
IL-1β + 100 nM Dex	0.052; 0.016	22.3, 6.8
IL-1β + 1 μM Dex	0.061; 0.024	27.1, 17.6
IL-1β + 10 μMDex	0.062; 0.029	27.7, 24.3
Control	0.026; 0.000	–
cAMP	0.094; 0.037	100, 100
Dexamethasone (10 μM)	0.026; 0.000	0, 0
cAMP + 1 nM Dex	0.112; 0.057	126.5, 154.1
cAMP + 10 nM Dex	0.080; 0.033	79.4, 89.2
cAMP + 100 nM Dex	0.086; 0.035	88.2, 94.6
cAMP + 1 μM Dex	0.134; 0.056	158.8, 151.4
cAMP + 10 μM Dex	0.148; 0.096	179.4, 259.5

Mesangial cells were treated with vehicle (control), IL-1β (1 nM), Bt_2cAMP (cAMP, 0.5 mM), combinations of IL-β plus dexamethasone (Dex) or Bt_2cAMP plus dexamethasone for 24 h. GTP cyclohydrolase I activity determinations were performed as described in the Methods section. The activity of GTP cyclohydrolase I is given as pmol of neopterin formed min^{-1} mg^{-1} total protein. The data are from two independent experiments.

Discussion

We have shown that the expression of GTP cyclohydrolase I is controlled by at least two different signalling pathways: one involving inflammatory cytokines such as IL-1β or tumour necrosis factor α and the other being triggered by agents that elevate cellular concentrations of cyclic AMP (Plüss *et al*., 1996). In the present study we demonstrate for the first time that dexamethasone differentially affects the expression and activity of GTP cyclohydrolase I in IL-1β- and cyclic AMP-stimulated mesangial cells. Dexamethasone markedly reduced the increase in GTP cyclohydrolase mRNA abundance and largely prevented the IL-1β-stimulated increase in enzyme protein and activity levels in mesangial cells. In contrast, the glucocorticoid did not inhibit the cyclic AMP-induced increase in GTP cyclohydrolase I mRNA levels, had little (if any) effect on enzyme protein levels and even augmented GTP cyclohydrolase I activity at high concentrations. These findings were unexpected as they contrast with dexamethasone regulation of iNOS expression (Mühl *et al*., 1994; Kunz *et al*., 1994b; 1996). We and others have shown that in cells the pathways for pteridine synthesis and NO production are tightly coupled, and cytokines and cyclic AMP that stimulate iNOS expression simultaneously stimulate the expression of GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis (Werner *et al*., 1995; Plüss *et al*., 1996). Although dexamethasone differentially affected IL-1β- and cyclic AMP-induced iNOS mRNA and protein expression this occurred in a way complementary to the effects on GTP cyclohydrolase I expression. The glucocorticoid suppressed IL-1β- and cyclic AMP-induced iNOS protein expression but only blocked cyclic AMP-induced iNOS mRNA levels without affecting IL-1β-triggered increase in iNOS mRNA levels (Kunz *et al*., 1994b). These data suggest that dexamethasone acts at different levels, depending on the stimulus used, to interfere with GTP cyclohydrolase I and iNOS expression. Moreover, the molecular pathways used by IL-1β and cyclic AMP to trigger iNOS and GTP cyclohydrolase I expression are at least partially separate.

Glucocorticoids repress the expression of a variety of genes by binding to the glucocorticoid receptor, which enables it to bind the regulatory elements in the 5′-flanking region of target genes and to block transcription of these genes (Barnes & Adcock, 1993). Alternatively, protein-protein interaction between the glucocorticoid receptor and other transcription factors such as nuclear factor κB (NFκB) or AP1 have been suggested to mediate gene repression by glucocorticoids (Barnes & Adcock, 1993). Such a negative cross-communication between transcription factors seems also be operative in dexamethasone inhibition of iNOS expression as the steroid diminishes the IL-1β-stimulated formation of NFκB complexes and this was reversed by the glucocorticoid receptor antagonist RU-486 (Kunz *et al*., 1996; Kleinert *et al*., 1996). The elucidation of GTP cyclohydrolase I gene expression by IL-1β and cyclic AMP and its modulation by dexamethasone will require molecular cloning and characterization of its promoter.

Besides the transcriptional effects, dexamethasone has to exert posttranscriptional and posttranslational actions to explain the data presented in this paper. Previously we have observed that dexamethasone increases iNOS mRNA half-life approximately 3 fold and, even more importantly, dexamethasone has pronounced inhibitory effects on iNOS protein synthesis and decreases iNOS protein stability (Kunz *et al*., 1996). Whether similar effects apply to GTP cyclohydrolase I mRNA stability, protein synthesis and stability must be considered in further investigations. The data in Table 2 when compared to the information in Figures 1 and 2 suggest a biphasic response to dexamethasone. This is particularly true for the data from cyclic AMP-stimulated cells, where low (1–10 nM) and high ($\geqslant$1 μM) concentrations of dexamethasone potentiated cyclic AMP-induced GTP cyclohydrolase I activity (Table 2). Similar observations have been obtained for cytokine-induction of secretory phospholipase A_2 which is also enhanced by low concentrations (1–10 nM) of dexamethasone and other glucocorticoids (Pfeilschifter *et al*., 1993). A bell-shaped dose-response curve was also observed for glucocorticoid-induced secretion of macrophage inhibitory factor (Calandra *et al*., 1995). This factor was found to override, in a concentration-dependent manner, glucocorticoid inhibition of cytokine production in macrophages, thus producing a bell-shaped dose-response curve. Whether such a counter-regulatory mediator is also produced in mesangial cells to balance the inhibitory effect of dexamethasone remains to be investigated.

In any case, the data presented in this paper highlight the fact that glucocorticoids control expression of inflammatory mediators at several levels. Dexamethasone not only suppresses the expression of iNOS but, in addition, it limits the availability of the essential iNOS cofactor BH_4 by blocking the expression of GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis. This multitude of actions may form the basis of the marked anti-inflammatory potency of glucocorticoids used to treat inflammatory renal diseases.

This work was supported by Swiss National Science Foundation grant 31-43090.95, by a grant from the Commission of the European Union (Biomed 2, PL 950979) to J.P., and by Austrian Research Funds zur Förderung der wissenschaftlichen Forschung (P11301) to E.R.W.; C.P. is supported by a grant from the Krebsliga beider Basel. We thank Dr Hatakeyama for kindly providing the rat GTP cyclohydrolase I cDNA clone, and Dr C. Rordorf for the generous donation of recombinant IL-1β.

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(Received December 2, 1996
Revised March 21, 1997
Accepted June 25, 1997)