RESEARCH COMMUNICATION

Dexamethasone differentially affects interleukin 1 β - and cyclic AMP-induced nitric oxide synthase mRNA expression in renal mesangial cells

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Inducible nitric oxide synthase (iNOS) is expressed in renal mesangial cells in response to two principal classes of activating signals that interact in a synergistic fashion. These two groups of activators comprise inflammatory cytokines such as interleukin (IL)-1 β or tumour necrosis factor α and agents that elevate cellular levels of cyclic AMP (cAMP). We examined whether dexamethasone differentially affects iNOS induction in response to IL-1 β and a membrane-permeable cAMP analogue, *N*⁶,*O*-2'-dibutyryl adenosine 3',5'-phosphate (Bt₂cAMP). Nanomolar con-

centrations of dexamethasone suppress IL-1 β - as well as Bt₂cAMP-induced iNOS protein expression and production of nitrite, the stable end product of nitric oxide (NO) formation. In contrast, dexamethasone prevents induction of iNOS mRNA in response to Bt₂cAMP without affecting IL-1 β -triggered increase in iNOS mRNA levels. These data suggest that dexamethasone acts at different levels, depending on the stimulus used to suppress iNOS induction in mesangial cells.

INTRODUCTION

Nitric oxide (NO), a free-radical gas produced by many cell types, mediates blood-vessel relaxation, functions as a neurotransmitter in the central and peripheral nervous systems, mediates macrophage cytotoxicity during host defence and leads to tissue injury in some inflammatory and autoimmune diseases [1–3]. Increasing evidence indicates that NO orchestrates acute and chronic inflammatory processes and contributes to the pathogenesis of several diseases [4–6]. The careful control of production or activity of this extremely reactive molecule is essential for the prevention of deleterious inflammatory reactions. Whereas the activity of the constitutive NO synthases in brain and endothelial cells is regulated by increases in intracellular free [Ca²⁺] and phosphorylation [7] events in a time frame of seconds and minutes, the inducible NO synthase (iNOS) is regulated primarily at a transcriptional level [8]. Once induced, the latter enzyme produces NO for periods of hours and days [1,3,8].

We have used renal mesangial cells, a specialized type of vascular smooth muscle cell that contracts upon stimulation with vasoactive hormones like angiotensin II and thus contributes to the regulation of the glomerular filtration rate [9]. In co-incubation experiments, it has been shown that NO release from glomerular endothelial cells increases cyclic GMP within mesangial cells and thereby inhibits angiotensin-II-stimulated cell contraction [10]. Moreover, mesangial cells not only act as a target cell for NO but also have themselves the capacity to synthesize NO by expressing a macrophage type of iNOS when exposed to inflammatory cytokines [11–13] or agents that elevate cellular levels of cyclic AMP (cAMP) [14,15]. The excessive formation of NO in mesangial cells not only blocks the contractile responsiveness of the cells [12] but may also contribute to tissue injury observed in the pathogenesis of certain forms of

glomerulonephritis [6,16]. Previously we have reported that several commonly used glucocorticoids inhibit interleukin (IL)-1 β -induced iNOS activity in mesangial cells at concentrations that correspond well with their anti-inflammatory potencies [11,17]. In the present study we report that dexamethasone differentially inhibits IL- β - and cAMP-induced iNOS mRNA expression in mesangial cells.

MATERIALS AND METHODS

Materials

Recombinant human IL- β was generously supplied by Dr. Klaus Vosbeck (Ciba-Geigy AG, Basel, Switzerland); *N*⁶,*O*-2'-dibutyryl adenosine 3',5'-phosphate (Bt₂cAMP) dexamethasone and adenosine 2',5'-diphosphate were from Sigma (Buchs, Switzerland); the cDNA clone pMac-NOS, which encodes the inducible macrophage NO synthase was kindly provided by Dr. J. Cunningham (Boston, MA, U.S.A.); the cDNA clone pEX6, which encodes human β -actin, was a gift from Dr. U. Aepli (Basel, Switzerland); nylon membranes (Gene Screen) were purchased from DuPont de Nemours International (Regensdorf, Switzerland); [³²P]dATP (specific radioactivity 3000 Ci/mmol) was from Amersham (Dübendorf, Switzerland); cell-culture media and nutrients were from Gibco BRL (Basel, Switzerland); all other chemicals were either from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Antibodies specific for iNOS were generated using the multiple antigen peptide (MAP) system [18]. The peptide antigen based on an N-terminal sequence of mouse iNOS (NVPESLDKLHVT) [19] is assembled stepwise on the MAP core resin. After complete peptide assembly, the MAP system is cleaved from the resin support and after dialysis used for immunization.

Abbreviations used: cAMP, cyclic AMP; CRE, cAMP response element; NO, nitric oxide; iNOS, inducible NO synthase; IL, interleukin; Bt₂cAMP, *N*⁶,*O*-2'-dibutyryl adenosine 3',5'-phosphate; MAP, multiple antigen peptide; DMEM, Dulbecco's modified Eagle's medium.

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Cell culture

Rat renal mesangial cells were cultured as described previously [12]. In a second step, single cells were cloned by limited dilution using 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) fetal calf serum, penicillin (100 i.u./ml), streptomycin (100 µg/ml) and bovine insulin (0.66 i.u./ml). For the experiments, passages 9–11 of mesangial cells were used.

Nitrite analysis

NOS activity was measured as nitrite production in rat glomerular mesangial cells [12,13]. Confluent mesangial cells were cultured in 16 mm diameter dishes. For stimulation, cells were washed twice with PBS and incubated in Dulbecco's modified Eagle's medium (DMEM) without Phenol Red and supplemented with 0.1 mg/ml fatty-acid-free BSA (Sigma), with or without agents for 24 h. Thereafter, the medium was withdrawn and centrifuged for 10 min at 900 *g* in an Eppendorf laboratory centrifuge. Nitrite was measured by mixing 150 µl of the supernatant with an equal volume of Griess reagent. The A_{560} was measured and the nitrite concentration was determined using a calibration curve with sodium nitrite standards.

Northern blot analysis

Confluent mesangial cells were incubated in DMEM without Phenol Red, supplemented with 0.1 mg/ml fatty-acid-free BSA (Sigma), with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested using a rubber policeman. After centrifugation for 10 min at 550 *g* in a Heraeus sepatec laboratory centrifuge, the supernatant was removed and the pellet frozen in liquid nitrogen and stored until isolation of RNA was performed. Total cellular RNA was extracted from the cell pellets using the guanidinium thiocyanate/caesium chloride method [15]. Samples of RNA (20 mg) were separated on 0.8% (w/v) agarose gels containing 0.65 M formaldehyde before transfer to Gene Screen membranes. After baking at 80 °C for 2 h and prehybridization for 4 h, the filters were hybridized for 16–18 h to a 32 P-labelled *Sma*I cDNA insert from pMac-NOS [19]. To correct for variations in RNA amount, the NOS probe was stripped with boiling 0.1 × SSPE/1% (w/v) SDS (1 × SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA) and the blots were rehybridized to either the 32 P-labelled *Bam*HI/*Sal*I cDNA insert from clone pEX 6 coding for β -actin or to the 32 P-labelled *Eco*RI insert coding for 28S rRNA. DNA probes (radioactivity $\sim 2 \times 10^6$ c.p.m./ml) were radioactively labelled with [α - 32 P]dATP by random priming (Boehringer-Mannheim). Hybridization reactions were performed in 50% (v/v) formamide, 5 × SSPE, 10 × Denhardt's solution, 0.5% (w/v) SDS and 250 µg/ml salmon sperm DNA. Filters were washed three times in 2 × SSPE, 0.1% SDS at room temperature for 30 min, and then in 2 × SSPE, 2% SDS at 65 °C for 30 min. Filters were exposed for 6–48 h to Kodak X-Omat XAR film using intensifying screens.

Western blot analysis

Confluent mesangial cells were stimulated with different cytokines for 24 h and then washed with PBS and scraped into 1 ml of buffer A (50 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 µM leupeptin, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol). Cells were homogenized in a Dounce homogenizer and centrifuged at 200 000 *g* for 30 min at 4 °C.

Protein concentration was determined by the method of Bradford [20]. The supernatants (3 mg of protein) were incubated for 30 min at 4 °C with 400 µl of a 1:1 (v/v) slurry of 2',5'-ADP-agarose in buffer A with gentle agitation. After centrifugation for 10 min at 3000 *g* the pellets were washed twice with buffer A, once with buffer A containing 0.5 M NaCl, and finally with buffer A only. The proteins were solubilized from the beads by heating for 5 min at 95 °C in 0.1 ml of SDS/PAGE sample buffer [3.6% (w/v) SDS, 15% (w/v) glycerol, 120 mM Tris/HCl, pH 6.8, 0.125 M dithiothreitol] and subjected to SDS/PAGE [7.5% (w/v) acrylamide gel]. Immunoblotting was performed as described [21] using the anti-iNOS antibody at a dilution of 1:1000 (v/v).

RESULTS

Incubation of mesangial cells for 24 h with IL-1 β (1 nM) or Bt $_2$ cAMP (1 mM) increases the production of nitrite, one of the end-products of NO formation, from 14 ± 7 nmol/mg of protein in unstimulated cells, to 617 ± 49 and 462 ± 28 nmol/mg of protein (means $\pm$ S.D., $n = 4$) respectively. Simultaneous incubation of cells with IL-1 β or Bt $_2$ cAMP together with dexamethasone dose-dependently inhibits nitrite production as shown in Figures 1(a) and 1(b). Half-maximal inhibition of IL-1 β - and Bt $_2$ cAMP-stimulated nitrite formation is observed at 4.5 ± 1.0 nM and 1.8 ± 0.8 nM dexamethasone (means $\pm$ S.D., $n = 4$) respectively.

Western blot analysis with a specific polyclonal antibody demonstrates that IL-1 β and Bt $_2$ cAMP treatment of mesangial cells dramatically up-regulates the appearance of a single band of ~ 130 kDa, consistent with iNOS protein (lanes 2 in Figures 2a

Figure 1 Inhibition of IL-1 β - and Bt $_2$ cAMP-stimulated nitrite formation in mesangial cells by dexamethasone

Mesangial cells were incubated for 24 h with (a) IL-1 β (1 nM) or (b) Bt $_2$ cAMP (1 mM) together with the indicated concentrations of dexamethasone. Thereafter, the medium was removed and used for nitrite determination. Data represent means $\pm$ S.D. ($n = 4$).

Figure 2 Inhibition of (a) IL-1 β - and (b) Bt₂cAMP-stimulated iNOS protein expression in mesangial cells by dexamethasone

Mesangial cells were incubated for 16 h with vehicle (control, lane 1), (a) IL-1 β (1 nM, lane 2) or (b) Bt₂cAMP (1 mM, lane 2), alone or together with dexamethasone [100 nM (lane 4), 1 μ M (lane 5) or 10 μ M (lane 6)]. The case for dexamethasone (10 μ M) alone is depicted in lane 3. Samples were analysed by SDS/PAGE and transferred to a nitrocellulose filter. Immunoblots were developed by using antiserum against iNOS at a dilution of 1:1000. Similar results were obtained from three independent experiments.

and 2b). This band is not detected in unstimulated cells (lanes 1 in Figures 2a and b). Co-incubation of IL-1 β or Bt₂cAMP with increasing concentrations of dexamethasone prevents iNOS protein expression as shown in Figure 2. The induction of iNOS protein by Bt₂cAMP turned out to be most sensitive to the action of dexamethasone, as even the lowest concentration of dexamethasone tested (100 nM) completely inhibits iNOS protein expression (the blot shown in Figure 2b has been exposed for a much longer time period than the one shown in Figure 2a). These data suggest that the events inhibited by dexamethasone precede the synthesis of iNOS protein. Comparable results have been obtained using three additional antibodies: one iNOS-specific monoclonal antibody (Affiniti Research Products, Nottingham, U.K.) and two polyclonal antibodies raised in rabbits against a 196-amino-acid N-terminal polypeptide of murine iNOS expressed in *Escherichia coli* M15 and a 60 kDa haem-binding domain of iNOS expressed in *E. coli* JM 109 (N. Ghilardi and J. Pfeilschifter, unpublished work). All antibodies used demonstrated immunoreactivity against a single protein band with a molecular mass of 130–135 kDa present in stimulated mesangial cells and RAW 264.7 cells but not in control cells (results not shown).

In order to assess whether the suppression of iNOS synthesis by dexamethasone is associated with changes in the mRNA levels coding for iNOS, we performed Northern blot analyses. As shown in Figure 3, dexamethasone at concentrations of 10 nM completely abolishes Bt₂cAMP-induced elevation of iNOS mRNA steady-state level. In contrast, as shown in Figure 4, dexamethasone in concentrations up to 10 μ M does not inhibit IL-1 β -induced accumulation of iNOS mRNA, although the protein-blotting data and nitrite measurements (Figures 1a and 2a) demonstrate that dexamethasone at concentrations of ≥ 10 nM severely suppresses iNOS expression and activity. In

Figure 3 Inhibition of Bt₂cAMP-stimulated induction of iNOS mRNA in mesangial cells by dexamethasone

Mesangial cells were incubated for 24 h with vehicle (control, lane 1), Bt₂cAMP (1 mM, lane 2) alone or together with dexamethasone [1 nM (lane 3), 3 nM (lane 4), 6 nM (lane 5) or 10 nM (lane 6)]. Total RNA (20 ng) was successively hybridized to ³²P-labelled iNOS and 28S ribosomal cDNA probes as described in the Materials and methods section. Similar results were obtained from four independent experiments.

Figure 4 Inhibition of IL-1 β -stimulated induction of iNOS mRNA in mesangial cells by dexamethasone

Mesangial cells were incubated for 24 h with vehicle (control, lane 1), IL-1 β (1 nM, lane 2) alone or together with dexamethasone [100 nM (lane 4), 1 μ M (lane 5) or 10 μ M (lane 6)]. The case for dexamethasone (10 μ M) alone is depicted in lane 3. Total RNA (20 μ g) was successively hybridized to ³²P-labelled iNOS and β -actin cDNA probes as described in the Materials and methods section. Similar results were obtained from three independent experiments.

contrast, 10 μ M dexamethasone consistently increased IL-1 β -induced iNOS mRNA levels (Figure 4, lane 6).

DISCUSSION

In the present study we demonstrate for the first time that glucocorticoids differentially suppress the expression of iNOS mRNA in IL-1 β - and cAMP-stimulated mesangial cells. The Northern-blot analyses reveal that dexamethasone prevents cAMP-induced increase in iNOS mRNA level without suppressing IL-1 β -induced elevations. We have reported that the level of iNOS mRNA is controlled by at least two different signalling pathways: one involving cAMP and the other being triggered by cytokines such as IL-1 β or tumour necrosis factor α . Both pathways act synergistically and thus potentially up-regulate the expression of iNOS mRNA in mesangial cells [15]. Recently, we reported that pyrrolidine dithiocarbamate, an inhibitor of the nuclear factor κ B activation, potently suppresses IL-1 β - but not cAMP-induced iNOS mRNA expression, indicating that different

transcription factors mediate cytokine and cAMP regulation of iNOS gene expression [22]. The diverse susceptibilities of the two pathways to glucocorticoids reported in the present study further prove that cAMP and cytokines stimulate iNOS gene expression via different signalling cascades. The mechanisms of iNOS gene regulation has not been well characterized. The 5'-flanking region of mouse iNOS gene contains at least 24 oligonucleotide elements similar in sequence to consensus sequences for the binding of transcription factors that may mediate inducibility of iNOS by cytokines and other stimuli [8]. iNOS induction in lipopolysaccharide-treated macrophages and in IL-1 β -stimulated mesangial cells depends on nuclear factor κ B activation [22,23]. Inhibition of IL-1 β -induced iNOS protein expression by dexamethasone without inhibition of mRNA levels suggests that dexamethasone blocks iNOS expression at a post-transcriptional level, possibly by interfering with iNOS mRNA translation or iNOS protein degradation. Moreover, high concentrations of dexamethasone (10 μ M) consistently augmented IL-1 β -induced iNOS mRNA levels (Figure 4). Whether this is attributable to increased iNOS gene expression or mRNA stabilization remains to be evaluated.

Similarly, Cetkovic-Cvrlje et al. [24] observed that dexamethasone decreased IL-1 β -induced nitrite production in insulin-producing RIN m5F cells without affecting iNOS mRNA expression. These observations contrast with the findings of Perrella et al. [25], who described that dexamethasone does not inhibit nitrite production in IL-1 β -induced smooth-muscle cells, although it modestly decreased the transcriptional rate and increases the iNOS mRNA half-life. Further work is required to establish the exact mechanism of glucocorticoid action on iNOS mRNA expression, and such work may reveal cell-specific transcriptional as well as post-transcriptional regulatory activities.

Recently, we have shown that cAMP induces iNOS gene transcription in mesangial cells and, in addition, prolongs the half-life of iNOS mRNA [15]. Induction of iNOS by cAMP has also been reported in vascular smooth-muscle cells [26,27]. Many of the cAMP-inducible genes possess common DNA elements in their 5'-flanking regions termed the cAMP response element (CRE) or activator protein 2 element [28]. It has not yet been proven whether the iNOS gene belongs to this family of cAMP-regulated genes and contains the corresponding classical DNA elements in its 5'-upstream region. Negative and positive regulation of cAMP-induced genes by glucocorticoids have been reported in several instances. Inhibition of the cAMP stimulation of the human glycoprotein hormone α -subunit gene involves DNA binding of the hormone receptor to a glucocorticoid response element overlapping the CRE [29]. An interaction between the glucocorticoid receptor and the CRE-binding protein has been shown to account for the response of the phosphoenolpyruvate carboxykinase gene in rat hepatoma cells [30]. Although the data presented here do not address the molecular mechanism by which dexamethasone prevents cAMP-induced increases in iNOS mRNA steady-state levels, the examples above

described highlight that several levels of control exist must be considered in further investigations. Glucocorticoid hormones inhibit inflammatory processes and limit the proliferative response of cells in chronic destructive diseases [31]. Inhibition of cytokine and cAMP induction of iNOS in mesangial cells may be one aspect of the beneficial glucocorticoid action seen in certain renal diseases.

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