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# PYRROLIDINE DITHIOCARBAMATE DIFFERENTIALLY AFFECTS INTERLEUKIN 1β- AND cAMP-INDUCED NITRIC OXIDE SYNTHASE EXPRESSION IN RAT RENAL MESANGIAL CELLS

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Inducible nitric oxide synthase (NOS) 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 1 (IL-1) or tumour necrosis factor α and agents that elevate cellular levels of cAMP. We have used pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of nuclear factor κB (NFκB), to determine its role in IL-1β- and cAMP-triggered NOS expression. Micromolar amounts of PDTC suppress IL-1β-, but not cAMP-stimulated nitrite production, the stable end product of NO formation in mesangial cells. Furthermore, PDTC completely inhibited the increase of NOS mRNA in response to IL-1β, while only marginally affecting cAMP-induced NOS mRNA levels. Our data suggest that NFκB activation is an essential component of the IL-1β signalling pathway responsible for NOS gene activation and that cAMP triggers a separate signalling cascade not involving NFκB. These observations may provide a basis for the synergistic stimulation of NOS expression by cytokines and cAMP in mesangial cells. 

Nitric oxide (NO) is a highly versatile intracellular and intercellular messenger in most mammalian cellular systems and participates in blood vessel relaxation, modulation of synaptic transmission and tumouricidal and bactericidal activities of macrophages (1,2). Three main types of NOS isoforms have been characterized by molecular cloning and sequencing analyses (3-6). The brain and endothelial forms of NOS are constitutively expressed and are regulated by short-term elevations of intracellular free $Ca^{2+}$ concentration in response to hormone or neurotransmitter

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***Abbreviations***: NOS, nitric oxide synthase, IL-1, interleukin 1, PDTC, pyrrolidine dithiocarbamate, NFκB, nuclear factor κB, NO, nitric oxide, $Bt_2$ cAMP, $N^6$,0-2'-dibutyryladenosine 3',5'-phosphate.

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stimulation (1,2). A third type of NOS has been cloned from macrophages and proved to be approximately 50% identical to the brain and endothelial enzymes (5,6). In contrast to the brain end endothelial isoforms, the macrophage NOS is not constitutively expressed, but is induced by lipopolysaccharides and γ-interferon, and is not regulated by $Ca^{2+}$.

Glomerular mesangial cells are a specialized type of vascular smooth muscle cells and participate in the regulation of the glomerular filtration rate (7). These cells respond to endothelial cell-derived NO with cGMP formation and subsequent relaxation (8,9). We and others have shown that inflammatory cytokines such as IL-1 or tumour necrosis factor α induce a macrophage-type of NOS in mesangial cells with subsequent increase in cellular cGMP concentration (10-12). The excessive formation of NO and cGMP in mesangial cells not only blocks contractile responsiveness of the cells (12), but may also contribute to tissue injury observed in the pathogenesis of certain forms of glomerulonephritis (13,14). Recently we have observed that not only inflammatory cytokines but also cAMP up-regulates NOS expression and subsequent nitrite formation in mesangial cells (15). Furthermore, forskolin, an activator of adenylate cyclase, interacted synergistically with IL-1β and tumour necrosis factor α to induce NOS gene transcription and nitrite formation in mesangial cells (15,16). In addition message stability studies suggest that cAMP prolongs the half-life of NOS mRNA in mesangial cells (16), thus suggesting that multiple signalling pathways interact to regulate NOS expression in mesangial cells. The nuclear factors involved in regulation of transcription of the NOS gene are poorly defined. In the present study we have used pyrrolidine dithiocarbamate, an inhibitor of NFκB activation (17), to determine whether NFκB is required for cytokine- and cAMP-induced NOS gene transcription in mesangial cells.

## *Materials and Methods*

### Cell culture

Rat glomerular mesangial cells were cultured as described previously (12). In a second step, single cells were cloned by limited dilution using 96-micro-well plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, negative staining for factor VIII-related antigen and cytokeratin excluded endothelial and epithelial contaminations, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II $AT_1$ receptor was used as a functional criterion for characterizing the cloned cell line. The cells were grown in RPMI 1640

supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and bovine insulin at 0.66 U/ml (Sigma). For the experiments passages 9-16 were used.

**Nitrite analysis**

Nitrite production by rat glomerular mesangial cells was measured as a read-out for NO synthase activity as described previously (18). Confluent mesangial cells in 24-well plates were washed twice with PBS and incubated in DMEM without phenol red and supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Thereafter, the medium was withdrawn and nitrite was measured by mixing 200 µl of the supernatant with 100 µl of Griess reagent. The absorbance at 550 mm was measured and the nitrite concentration was determined using a calibration curve with sodium nitrite standards.

**Northern blot analysis**

Confluent mesangial cells were cultured in 150 mm diameter culture dishes. For stimulation, cells were washed twice with PBS and incubated in DMEM without phenol red, supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium thiocyanate/caesiumchloride method (19). Samples of 20 µg RNA were separated on 1% agarose gels containing 0.66 M formaldehyde prior to transfer to gene screen membranes (New England Nuclear). 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 (6). To correct for variations in RNA amount, the NOS-probe was stripped with boiling 0.1 x SSPE/ 1% SDS and the blots were rehybridized to the 32P-labelled BamHI/SalI cDNA insert from clone pEX 6 coding for β-actin. DNA-Probes (≈ $0.5x10^6$ cpm /ml) were radioactively labelled with $\alpha$-$^{32}P$-dATP by random priming (Boehringer-Mannheim). Hybridization reactions were performed in 50%(v/v) Formamide, 5x SSC, 5x Denhardt's solution, 1% (w/v) SDS, 10% (w/v) dextran sulfate and 250 µg/ml salmon sperm DNA. Filters were washed 3 times in 2xSSC, 0.1%SDS at room temperature for 30 min, and then in 2xSSC, 2% SDS at 65°C for 30 min. Filters were exposed for 6 - 48 h to Kodak X-Omat XAR-film using intensifying screens.

**Chemicals**

Recombinant human IL-1β was generously supplied by Dr. Klaus Vosbeck, Ciba-Geigy Ltd., Basel, Switzerland; $Bt_2$ cAMP and PDTC were from Sigma, Buchs, Switzerland. The cDNA clone pMac-Nos, coding for the inducible macrophage NO synthase was kindly provided by Dr. J. Cunningham, Boston, MA; the cDNA clone pEX6, coding for human β-actin, was a gift from Dr. U. Aebi, Basel, Switzerland, nylon membranes (Gene Screen) were purchased from DuPont de Nemours International, Regensdorf, Switzerland; $^{32}P$-dATP (specific activity 3000 Ci/mmol) was from Amersham, Dübendorf; Switzerland; cell culture media and nutrients were from Gibco BRL, Basel, Switzerland and all other chemicals were either from Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland.

## *Results*

Incubation of mesangial cells for 20h with either IL-1β (2 nM) or the membrane-permeable cAMP analogue, $N^6$, 0-2'-dibutyryladenoside 3',5'-phosphate ($Bt_2$ cAMP, 5mM) increased the production of nitrite as shown in Fig. 1. Nitrite formation was

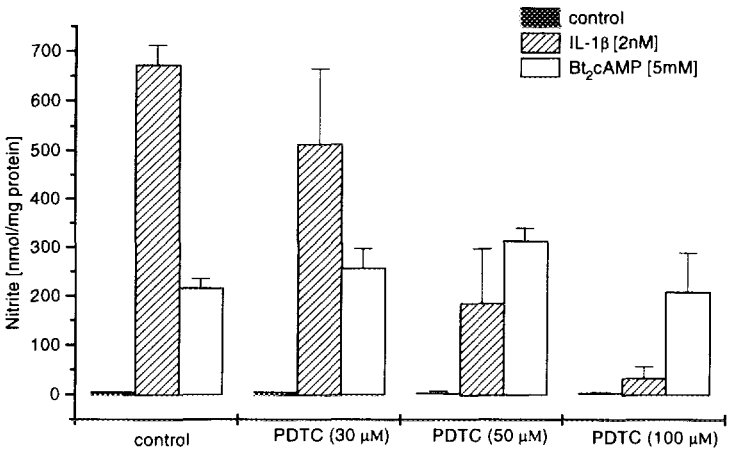


Fig. 1. Effects of pyrollidine dithiocarbamate (PDTC) or IL-1β- and $Bt_2$ cAMP-stimulated nitrite production. Confluent mesangial cells were incubated vehicle (control, black bars), IL-1β (2 nM, hatched bars) or $Bt_2$ cAMP (5 mM, open bars) plus the indicated concentrations of PDTC for 20 h. Thereafter, the medium was removed and used for nitrite determination. Results are means ± S.D. of 4 experiments.

determined as a read-out for NOS activity in mesangial cells as described previously (12,20). Basal levels of nitrite in unstimulated cells were 4±2 nmol/mg of protein (mean ± S.D., n=4) and IL-1β and $Bt_2$cAMP increased nitrite synthesis by 168-fold and 54-fold, respectively. Simultaneous incubation of IL-1β or $Bt_2$ cAMP with the NFκB inhibitor PDTC dose dependently inhibited nitrite generation in response to IL-1β but not to $Bt_2$ cAMP (Fig. 1). Half-maximal inhibition of IL-1β-stimulated nitrite synthesis was observed at 41 μM of PDTC. In contrast, $Bt_2$ cAMP-induced nitrite formation was not affected by PDTC up to concentrations of 100 μM (Fig. 1). In order to determine whether the IL-1β-stimulated NOS activity and its inhibition by PDTC are due to regulation of NOS gene expression, we performed Northern blot analyses. The data in Fig. 2 demonstrate up-regulation of NOS mRNA levels upon stimulation with IL-1β for 6 hrs (lane 3) and 12h (lane 5). NOS mRNA was present as a single band of approximately 4.5 kb. In unstimulated cells there was no detectable NOS mRNA (lane 1). Addition of PDTC alone had no effect on NOS mRNA levels (lane 2). Coincubation of mesangial cells with IL-1β plus PDTC caused a complete suppression of NOS induction after 6h incubation (lane 4) and even after 12h incubation only a weak induction of NOS mRNA was detectable in the presence of PDTC (lane 6). In contrast, PDTC did not or only slightly attenuate NOS mRNA increase observed after stimulation of mesangial cells with $Bt_2$ cAMP (Fig. 3).

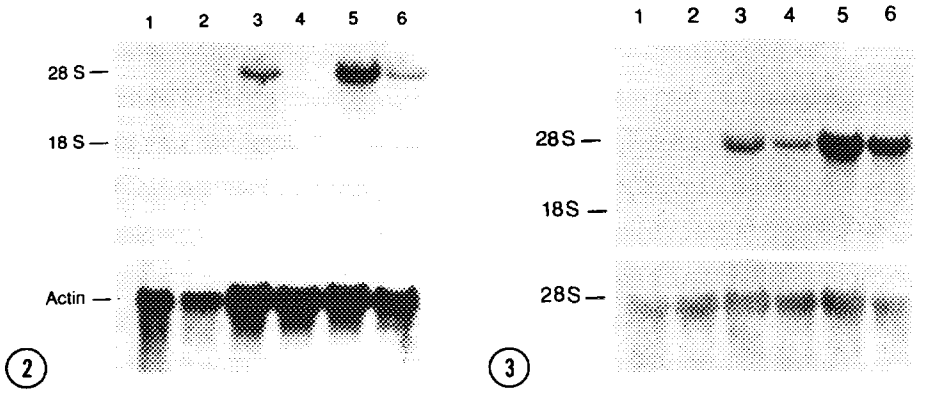


Fig. 2. Effects of pyrollidine dithiocarbamate (PDTC) on IL-1β-stimulated NOS mRNA accumulation. Confluent mesangial cells were incubated for 6 h with vehicle (control) (1), PDTC (100 μM) (2), IL-1β (2 nM) (3), or IL-1β plus PDTC (4), or for 12h with IL-1β (5) or IL-1β plus PDTC (6). Total cellular RNA (20 μg) was successively hybridized to $^{32}$P-labelled NOS and β-actin probes as described in the Materials and Methods section.

Fig. 3. Effects of pyrollidine dithiocarbamate (PDTC) on $Bt_2$ cAMP stimulated NOS mRNA accumulation. Confluent mesangial cells were incubated for 6 h with vehicle (control) (1), PDTC (100 μM) (2), $Bt_2$ cAMP (5 mM) (3), or $Bt_2$ cAMP plus PDTC (4), or for 12h with $Bt_2$ cAMP (5) or $Bt_2$ cAMP plus PDTC (6). Total cellular RNA (20 μg) was successively hybridized to $^{32}$P-labelled NOS and 28S ribosomal RNA probes as described in the Materials and Methods section.

### *Discussion*

Whereas the constitutive brain and endothelial NOS enzymes are controlled by $Ca^{2+}$-mobilizing agonists in a very transient and highly controlled fashion, the inducible macrophage-type NOS is regulated on a transcriptional level and once induced is active for hours and days. The inducible NOS has been cloned from mouse macrophages (5,6) and mediates some of the tumouricidal and bactericidal actions of macrophages activated by bacterial endotoxins and γ-interferon (2). Proinflammatory cytokines such as IL-1 or tumour necrosis factor α trigger the expression of a macrophage-type of NOS in vascular smooth muscle cells, hepatocytes, endothelial cells, mesangial cells and variety of other cell types (2). The control of transcription in response to inflammatory cytokines has been intensively studied, and it has become clear that a number of ubiquitous transcription factors are involved in many different cellular sytems. Very prominent under these factors is NFκB a multi-subunit transcription factor that is activated in response to IL-1β, tumour necrosis factor α, bacterial endotoxin and various other stimuli and plays a pivotal role in the development of the cellular immune and inflammatory responses (21). NFκB is constitutively present in cells as a

heterodimer, consisting of a p50 DNA-binding subunit and a p65 transactivating subunit. This NFκB complex is maintained in a latent cytoplasmic form by the physical association with an inhibitor IκB (21). The mechanism leading to NFκB activation has not been fully defined, but phosphorylation of IκB has been identified as one step in the activation process. Phosphorylation of IκB causes dissociation from NFκB and nuclear translocation of NFκB with subsequent specific gene transcription. NFκB has been suggested to mediate lipopolysaccharide and γ-interferon induction of NOS in rat alveolar macrophages (22) and murine bone marrow-derived macrophages (23). Furthermore, Xie et al. (24) reported on the presence of a potential NFκB-binding site in the 5'-flanking region of the inducible NOS gene. Recently we have shown that NOS expression in renal mesangial cells is controlled by at least two different signalling pathways, one involving cAMP and the other being triggered by cytokines such as IL-1β or tumour necrosis factor α. Both pathways act synergistically and thus potently up-regulate the expression of NOS in mesangial cells (15,16). Nuclear run-on transcription experiments suggested that IL-1β and cAMP synergistically interact to increase NOS gene expression at the transcriptional level. Furthermore, cAMP exposure markedly prolonged the half-life of NOS mRNA (16). In order to further clarify the mechanism of the synergistic interaction between IL-1β and cAMP we evaluated whether activation of NFκB participates in NOS induction by these two agents. For our experiments we took advantage of the fact that dithiocarbamates efficiently suppress the activation of NFκB in stimulated cells, while leaving the DNA-binding activity of other transcription factors such as SP-1, Oct and CREB unaffected (17). Expression of the inducible NOS gene in response to IL-1β stimultation is strongly suppressed by PDTC in a dose-dependent fashion (Fig. 2), suggesting that NFκB is involved in NOS transcription in mesangial cells. In contrast cAMP-triggered expression of the NOS gene is not affected by PDTC, indicating that cAMP activates other nuclear factors, most probably CREB or related proteins. This is particularly important as it has been shown recently for human myeloid cells that cAMP can activate NFκB (25).

From the data present in this study it is becoming obvious that the induction of NOS activity in mesangial cells is regulated by multiple signalling cascades which may act alone or synergize with each other when added in combination. It is clear from our results that PDTC can efficiently inhibit IL-1β but not cAMP- stimulated NOS expression in mesangial cells. Obviously IL-1β uses NFκB to trigger NOS

transcription whereas cAMP activates a transcription factor different from NFκB. Whether the cooperation between these two factors provides the basis for the synergistic activation of NOS transcription is under investigation in our laboratory.

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