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Short Communication

Co-ordinate Expression of GTP Cyclohydrolase I and Inducible Nitric Oxide Synthase in Rat Mesangial Cells

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Introduction

The synthesis of NO from L-arginine by NO synthase (NOS) is an important intracellular and intercellular signalling pathway originally discovered in macrophages and endothelial cells (1). Molecular cloning and sequence analyses revealed the existence of at least 3 main types of NOS isoforms. The brain and endothelial enzmes are constitutively expressed and are regulated by Ca^{2+}-mobilizing agonists that trigger an increase in intracellular free Ca^{2+}, which binds to calmodulin, and thus activates NOS. A third type of NOS has been cloned from murine macrophages and is not constitutively expressed, but is induced by γ-interferon and lipopolysaccharide, and is Ca^{2+}-independent. The macrophage NOS is regulated on a transcriptional level and once induced is active for hours and days. Not surprisingly, therefore, the inducible macrophage enzyme produces amounts of NO that are several hundred times higher than the ones produced by the constitutive endothelial cell and brain enzymes which are only active during the short periods of elevated intracellular Ca^{2+} observed after exposure of cells to Ca^{2+}-mobilizing agonists (1).

We have shown that cytokines, such as interleukin 1 (IL-1) and tumour necrosis factor α (TNFα), induce a macrophage type of NOS in mesangial cells with subsequent elevation of cellular cGMP concentrations (3,4). The excessive formation of NO and cGMP in mesangial cells not only alters corractile responses of the cells, but may also cause tissue injury and thus contribute to the pathogenesis of certain forms of glomerulonephritis. Inducible 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 IL-1β or TNFα and agents that elevate cellular levels of cAMP (6,7).

Tetrahydrobiopterin (BH_4) is an essential cofactor for inducible NOS and its biosynthesis occurs via two distinct pathways: a de novo pathway which uses GTP as a substrate and a salvage pathway using the intracellular pool of preexisting dihydropterins (8). Cytokines like γ-interferon or TNFα have been reported to potently stimulate the *de novo* synthesis of BH_4 in human and murine macrophages, fibroblasts and endothelial cells (8). This increased production of BH_4 may be requried in order to ensure a sufficient supply of cofactor for long-term synthesis of NO via the inducible NOS (8). We have shown previously that mesangial cells predominantly synthesize BH_4 by de novo synthesis from GTP. BH_4 synthesis is an absolute requirement for, and limits IL-1 β induction of NO synthesis in mesangial cells (9). Since GTP cyclohydrolase I (GTP-CH) is the rate-limiting enzyme for BH_4 synthesis in mesangial cells (9), we investigated whether the identified stimulators of inducible NOS

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expression in mesangial cells co-ordinately induce GTP-CH expression.

Materials and Methods

Rat glomerular mesangial cells were cultured as described previously (3,4). The GTP-CH cDNA probe was constructed by subcloning a 189-nuceotide fragment (Ncol/Notl) from the cDNA clone GTP-CH (a gift from Dr. Hatakeyama, Osaka, Japan) into the pSPTBM21 transcription vector (Boerhinger Mannheim). pGTP-CH corresponds to nucleotide 1-189 of the rat GTP-CH cDNA sequence (10).

GTP-CH mRNA was determined by nuclease protection assay. 20 µg of total RNA was hvbridized at 45℃ for 16h with 1 fmol of GTP-CH antisense probe transcribed from the plasmid pGTP-CH containing 19 base pairs of GTP-CH coding sequence and 0.5 fmol of ^{32}P-labelled GAPDH antisense probe transcribed from the pasmid pGAPDH containing 159 base pairs of GAPDH coding sequence. Nonhybridized single-stranded radiolabeled probe was then digested with 25 units of T1 and 0.5 ng of A ribonuclease for 1h at 30℃ and then protein digested with 50 µg of proteinase K for 30 min at 37℃. Samples were then electrophoresed in denaturing 8% polyacryamide gels containing 8M urea. Gels were dried and quantified by phosphor imaging. GTP-CH mRNA levels were expressed as % of control.

GTP-CH activity was assessed as previously described (11). Mesangial cells were disrupted by rapid freeze-thawing, extracts were centrifuged at 10,000 ×g for 10 min and aliquots of the supernatant were freed from low molecular weight compounds by chromatography on sephadex G-25. The sephadex G-25 eluate (5-6 mg of cell protein) was incubated with 2 mM GTP for 90 min at 37℃ in a total volume of 100 µl. The dihydroneopterin triphosphate thus formed was then oxidized to neopterin triphosphate with iodine solution, containing 2.5% (w/v) I_2, 5% (w/v) KI, in 20 mM HCl, Excessive iodine was destroyed with 0.5% (w/v) ascorbic acid. For HPLC analysis, the sample was applied to a Partisil-10 SAX column (120 mm × 6 mm). Neopterin triphosphate was eluted with a solvent of 250 mM $NH_4H_2PO_4$, 250 mM KCl, and 50 mM $Na_4P_2O_7$, pH 5.9, at a flow rate of 1 ml/min, and the products were detected fluorometrically (excitation 350 nm; emission 450 nm).

Results

Incubation of mesangial cells for 24 h with IL-1β (2 nM) or N^6, O-2'-dibutyryladenosine 3',5'-phosphate (Bt_2 cAMP, 0.5 mM) resulted in a 1.6-fold and 1.2-fold increase in GTP-CH activity, respectivly. Combination of IL-1β and forskolin (10 µM), a compound which increases cellular levels of cAMP by directly activating the catalytic moiety of adenylate cyclase, revealed a strong synergy in terms of GTP-CH activation-(5.1-fold increase). Because GTP-CH

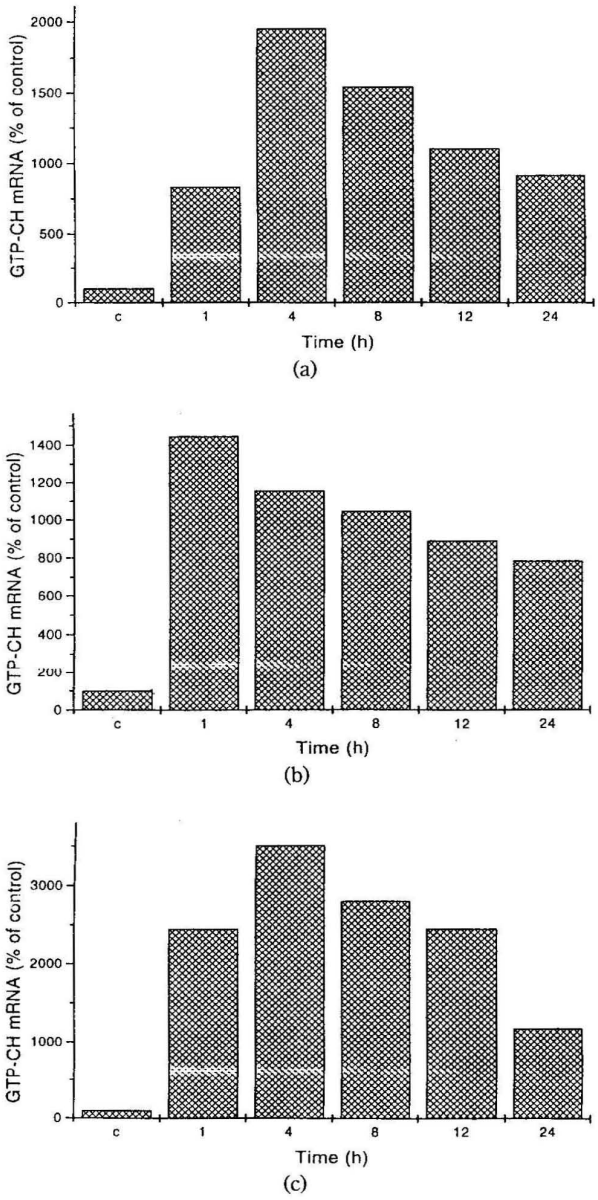

Figure 1. Time course of induction of GTP-CH mRNA in mesangial cells Mesangial cells were treated with IL-1β(2 nM) (A) Bt_2 cAMP (0.5mM), (B) or a combination of IL-1β (2 nM) plus forskolin(10 µM) (C) for the indicated time periods. RNA was extracted and GTP-CH mRNA was quantified by nuclease protection assay as described in Materials and Methods.

is the rate-limiting enzyme in BH_4 biosynthesis in mesangial cells (9), the changes in GTP-CH activity reported here likely reflect an increase in GTP-CH enzyme levels. As we do not have access to an antibody for quantitative Westernblot analysis we measured next GTP-CH mRNA steady-state levels in mesangial cells. With the cloning of a cDNA encoding rat GTP-CH (10) we were able to set up a highly sensitive and quantitative nuclease protection assay for GTP-CH mRNA. As shown in Figure 1, exposure of mesangial cells to IL-1β(A) or Bt_2 cAMP (B) caused a marked increase in GTP-CH mRNA levels as early as 1h after addition of the stimuli. Maximal effects were seen after 4h for IL-1β and after 1h for Bt_2 cAMP. Stimulation of mesangial cells with a combination of IL-1β plus forskolin (C) reveals an additive induction profile.

Discussion

Previously, we reported that the inflammatory cytokines IL-1β and TNFα as well as agents that elevate cellular concentrations of cAMP are capable of inducing NOS expression in mesangial cells (6,7). In the present study, we obseved that IL-1β and cAMP-induced NOS expression is associated with an increase in GTP-CH mRNA and GTP-CH activity. These data are compatible with the view that an increased *de novo* synthesis of GTP-CH is essential for optimal activity of inducible NOS in mesangial cells (9). Nuclear run-on transcription experiments suggested that IL-1β and cAMP synergistically interact to incerase inducible NOS gene expression at the transcriptional level. Furthermore, cAMP exposure markedly prolonged the half-life of inducible NOS mRNA (7). Comparable experiments have to be performed to assess the exact molecular mechanism of IL-1β and cAMP induction of GTP-CH in mesangial cell. However, an important difference between GTP-CH and inducible NOS regulation can already be derived from our present data (Figure 1A-C). Whereas IL-1β and cAMP cause a strong synergistic induction of inducible NOS mRNA and activity (6,7) there is only an additive effect on GTP-CH mRNA which is however combined with a synergistic stimulation of GTP-CH activity. This may suggest that in addition to transcriptional regulation there is a prominent posttranscriptional or even posttranslational modulation of GTP-CH expression and activity. This hypothesis is under evaluation in our laboratory.

Acknowledgments

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