Potentiation of nitric oxide synthase expression by superoxide in interleukin 1β-stimulated rat mesangial cells

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Abstract Exposure of mesangial cells to superoxide, generated by the hypoxanthine/xanthine oxidase system or by the redox cycler 2,3-dimethoxy-1,4-naphthoquinone caused a concentration-dependent amplification of interleukin (IL)-1β-stimulated nitrite production. The effect of superoxide was accompanied by an increase in inducible nitric oxide synthase (iNOS) protein and iNOS mRNA levels. Incubation of mesangial cells with superoxide alone did not induce iNOS expression. To elucidate whether the increase of iNOS expression is due to transcriptional upregulation we fused a 4.5-kb genomic iNOS fragment that contains the transcriptional start site of the rat iNOS gene to a luciferase reporter gene. In transient transfection studies, superoxide caused a 10-fold augmentation of iNOS promoter activity in IL-1β-challenged mesangial cells. Our data identify superoxide as a co-stimulatory factor amplifying cytokine-induced iNOS gene expression and subsequent nitric oxide (NO) synthesis.

Key words: Inducible nitric oxide synthase regulation; Superoxide; Nitric oxide; Mesangial cell; Rat

1. Introduction

Glomerular mesangial cells (MC) are smooth muscle-like pericytes that participate in the regulation of the glomerular filtration rate [1]. Due to their ability to produce inflammatory mediators such as cytokines [2], chemokines [3], nitric oxide (NO) [4], reactive oxygen species (ROS) [5] and products of arachidonic acid metabolism [6], MC are thought to play an important role during inflammatory glomerular diseases [7].

Interleukin-1β (IL-1β) leads to the transcription of the inducible form of NO-synthases (iNOS) in MC that results in enhanced formation of cGMP and subsequent inhibition of contractile responsiveness of MC [8]. The fact that the NOS-inhibitor L-NMMA reduces severity in a rat model of glomerular injury induced by anti-thymocyte serum suggests additional cytotoxic effects of NO [9,10]. We have recently reported that high amounts of exogenously delivered or endogenously produced NO leads to apoptosis of MC [11,12]. ROS are intermediates produced by infiltrating neutrophils and macrophages during inflammation. Radeke et al. [5] have shown that the superoxide radical (O_2^-) the primary product of ROS is also synthesized in human MC in response to IL-1 or TNF-α. Whereas the iNOS is the only enzyme responsible for high NO build-up in MC, the ROS system is more complex. This is due to the existence of several O_2^- synthesizing enzymes that use different substrates including NADPH oxidase, xanthine oxidase and cyclooxygenases. Moreover, other enzymes like superoxide dismutase, catalase, myeloperoxidase or non-enzymatic processes such as the Haber-Weiss reaction produce O_2^--derived ROS including hydrogen peroxide, hydroxyl radicals and hypohalous acids that exert diverse biological effects [13] including regulation of gene transcription [14]. Sandau et al. [15] reported that NO and O_2^- both are able to trigger apoptosis in rat MC. The effect was crucially determined by the ratio of NO and O_2^- indicating that both autacoids neutralize each other forming peroxynitrite which is ineffective in this regard.

We have been interested in the complex regulation of iNOS gene expression in MC [16–19] and have reported positive feedback regulation of iNOS expression by its product NO [20]. In this study we addressed the possible participation of ROS and in particular O_2^- in iNOS expression in MC.

2. Materials and methods

2.1. Reagents

Human recombinant IL-1β was kindly provided by Dr. Ch. Rordorf (Novartis, Pharma, Basel, Switzerland). A cDNA clone representing the murine inducible NO-synthase (pMac-NOS) was generously provided by Dr. J. Cunningham (Boston, MA, USA). Clone pEX6 that represents human β-actin cDNA was a gift from Dr. U. Aebi (Basel, Switzerland). [α-^{32}P]dATP and 'ready prime' DNA labelling kit were obtained from Amersham Buchler (Braunschweig, Germany). Nylon blotting membranes from Life Science (Schleicher&Schüll, Dassel, Germany) or Millipore (Eschborn, Germany). Tissue culture plastic was from Falcon (Becton-Dickinson, Heidelberg, Germany), media and sera from Gibco-BRL (Eggenstein, Germany). Xanthine oxidase was purchased from Boehringer Mannheim (Mannheim, Germany). 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) was from Calbiochem-Novabiochem (Bad Soden, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2. Cell culture

Rat glomerular MC were cultured as decribed previously [21]. MC were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamate, 5 ng/ml insulin, 100 U/ml penicillin and 1 μg/ml streptomycin. To obtain quiescent cells, MC were maintained in serum-free Dulbecco's minimal essential medium (DMEM) supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin for 24 h before cytokine treatment. MC were used between passages 8 and 19.

2.3. Nitrite analysis

Nitrite (NO_2^-), the stable end-product of NO, was measured in the culture medium with the Griess method using a ready to use reagent (Merck, Darmstadt, Germany) as described [22].

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Abbreviations: IL-1β, interleukin 1β; iNOS, inducible nitric oxide synthase; MC, mesangial cells; HXXO, 50 μM hypoxanthine+ xanthine oxidase; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone

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2.4. Northern blot analysis

Isolation of total cellular RNA from MC and Northern blotting was performed as described previously [18,23]. Filter-bound RNA was hybridized to the radiolabelled *Sma*I fragment of the murine cDNA clone pMac-NOS. Equivalent loading of the RNA probes was corrected after rehybridization of the filter with a cDNA probe for β-actin (pEX6).

2.5. Generation of the anti-iNOS N-terminal antibody

A cDNA-fragment that represents 196 amino acids of the iNOS N-terminus was cloned into the expression vector pDS56 and expressed in *E. coli* strain M15. The His_6-tagged polypeptide was purified using the XPress-kit (Invitrogen, Groningen, Netherlands). Female New Zealand rabbits were immunized and the antisera were tested in Western blots. A prominent iNOS band at the expected size of 130 kDa was obtained when protein was prepared from MC treated with IL-1β [24].

2.6. Western blot analysis

MC grown in 10-cm dishes were lysed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2 mM dithiothreitol, 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and left on ice for 15 min. Extracts were sonicated three times for 10 s and centrifuged at 13 000 rpm for 2 min. The protein concentration of the lysate was determined from the supernatant by use of the Bradford protein assay (Bio-Rad, München, Germany). 100 μg of total protein from each sample was subjected to SDS-PAGE (8% (w/v) acrylamide gel). Subsequently, the protein was blotted to a nylon membrane and immunoreactive protein was detected with the anti-iNOS N-terminal antibody.

2.7. Cloning of iNOS promoter region

A Lambda Dash library (Stratagene, Amsterdam, Netherlands) that contains genomic rat DNA fragments partially digested with *Sau*3A was screened for iNOS positive clones with a radiolabelled DNA-fragment that represents a 1.1-kb promoter-distal *Hin*cII/*Xba* fragment of the recently cloned iNOS-promoter [19,25]. One positive clone that contains about 16 kb rat genomic DNA further referred to as λiNOS2-1 was characterized by restriction analysis. Southern blot analysis showed identical restriction patterns of λiNOS2-1 and rat genomic DNA as long restriction sites represented in λiNOS2-1 were used indicating that λiNOS represents a continuous genomic DNA. A *Pst*I/*Pst*I fragment that contains the iNOS 5′-flanking region from −49 to about −6000 was cloned into the vector Bluescript KS^+ (Stratagene) further referred to as pBSPP3.

2.8. Construction of reporter gene fusions and luciferase reporter gene assay

pGL3/2 was obtained by cloning a 1846-bp *Hin*cII/*Hin*cII fragment [25] into the *Sma*I site of Bluescript KS^+. A fusion of the iNOS promoter to the luciferase gene was generated by cloning a *Hin*dIII/*Sst*I fragment to the respective sites in the polylinker of the vector pGL3 basic (Promega, Mannheim, Germany). pGL3/4 was constructed by exchanging a 1.8-kb *Kpn*I/*Pst*I fragment from pGL3/2 with a 4.5-kb *Kpn*I/*Pst*I fragment from pBSPP3. Correct cloning of all constructs used was verified by sequence analysis using the automated sequence analyzer A310 (Perkin Elmer Applied Biosystems, Weiterstadt, Germany). Transfection of MC with pGL3/2, pGL3/4 and plasmid pRL-CMV used as a control was performed with the superfect reagent (Qiagen, Hilden, Germany). Luciferase activity was measured using the dual reporter gene system (Promega).

2.9. Statistics

Statistical analysis was performed by Student's *t*-test. *P* values < 0.05 were considered as statistically significant.

3. Results

To evaluate effects of O_2^- on IL-1β-induced iNOS transcription in MC we used two O_2^- generating systems. One of them is xanthine oxidase in combination with its substrate hypoxanthine further referred to as HXXO [26]. More recently another O_2^- generating reagent, the redox cycler DMNQ has been used as a O_2^- donor for cell culture experiments [27].

HXXO caused a concentration (2–32 mU) dependent potentiation of NO_2^- levels in IL-1β-activated MC measured after 24 h coincubation with a maximum of 5.5-fold (IL-1β plus HXXO vs. IL-1β alone, Fig. 1A). In a similar manner but to a lesser extent (up to 2.3-fold), DMNQ increased NO_2^- levels in MC coincubated with IL-1β (Fig. 1B). This effect was significant at concentrations higher than 1 μM. Both O_2^- generators showed no significant effect on NO_2^- production when applied without IL-1β (data not shown). As shown by immunoblot analyses production of NO_2^- is paralleled by a corresponding amplification of iNOS protein levels in MC challenged with IL-1β and HXXO (Fig. 2A) or DMNQ (Fig. 2B).

The molecular basis for NO production in response to cytokines, cAMP or lipopolysaccharide is formed by a transcriptional upregulation of iNOS gene expression in MC. [16,17]. Therefore we examined the influence of the O_2^- donors HXXO and DMNQ on IL-1β-induced iNOS-mRNA steady-state levels. HXXO and DMNQ augmented IL-1β-induced

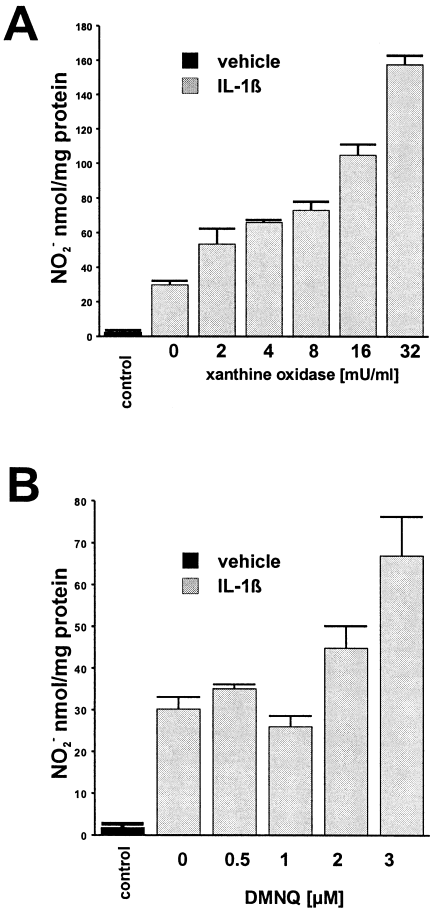

Fig. 1. Effects of HXXO and DMNQ on cytokine induced NO_2^- production. Quiescent MC were treated with vehicle (control), IL-1β (1 nM) or IL-1β plus the indicated concentrations of xanthine oxidase and 50 μM hypoxanthine, or DMNQ for 24 h. NO_2^- concentrations were determined in the supernatant by the Griess reaction. Values were corrected for total protein content per 100-mm dish. Data are means ± S.D. and were obtained from three independent experiments.

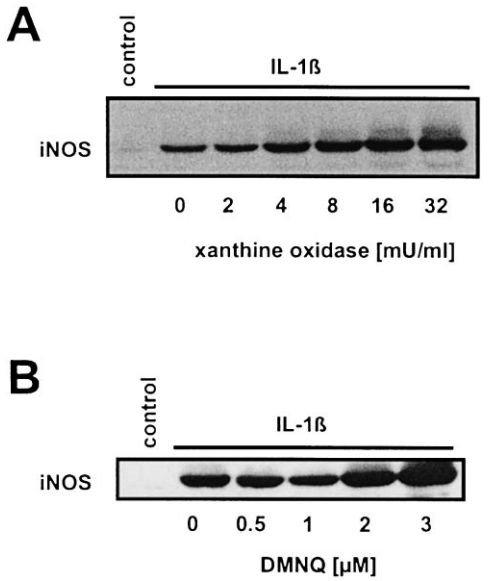

Fig. 2. Coinduction of IL-1β evoked iNOS protein expression by O_2^- generators. Quiescent MC were treated with vehicle (control), IL-1β (1 nM) or IL-1β plus the indicated concentrations of xanthine oxidase and 50 μM hypoxanthine (Fig. 2A), or DMNQ (Fig. 2B) for 24 h. Protein lysates (100 μg) were subjected to SDS-PAGE and immunoblotted using the anti-iNOS N-terminal antibody (see Section 2). The blots were developed with the ECL-System (Amersham-Buchler) and the intensity of the bands representing iNOS protein (130 kDa) evaluated by densitometry.

iNOS mRNA content in MC in a concentration dependent manner up to 23-fold and 3.1-fold for HXXO and DMNQ, respectively (Fig. 3). This indicates that coinduction of cytokine-driven iNOS expression by O_2^- occurs most likely at the transcriptional level.

We have recently cloned genomic DNA fragments that represent the promoter of the rat iNOS gene [19,25]. Functional analysis of the iNOS promoter showed that only 600 bp of the promoter region are required for cytokine- or cAMP-mediated induction of iNOS [16]. Nevertheless, so far there is no evidence that this promoter region is sufficient to mediate all modulatory effects of cytokines or cAMP-elevating agents on iNOS expression. Therefore we cloned an additional 14 kb of the 5′-flanking region of the iNOS gene. To evaluate whether the observed effects of ROS on IL-1β-mediated iNOS expression occur on a transcriptional level we transfected two promoter fragments of 1700 bp length, represented by pGL3/2, and 4500 bp length, represented by pGL3/4, that were fused to the luciferase gene in MC. Transiently evoked luciferase activity was then compared in cells treated with IL-1β and IL-1β plus HXXO for 24 h after transfection. HXXO (5 mU/ml) enhanced transient luciferase activity IL-1β treated MC transfected with either pGL3/2 or pGL3/4. However, whereas O_2^- caused a 10-fold amplification of luciferase activity in pGL3/4-transfected MC there was only a 3.3-fold increase in pGL3/2-transfected cells (Fig. 4) indicative for O_2^--sensitive regulatory elements in the far upstream region of the iNOS promoter.

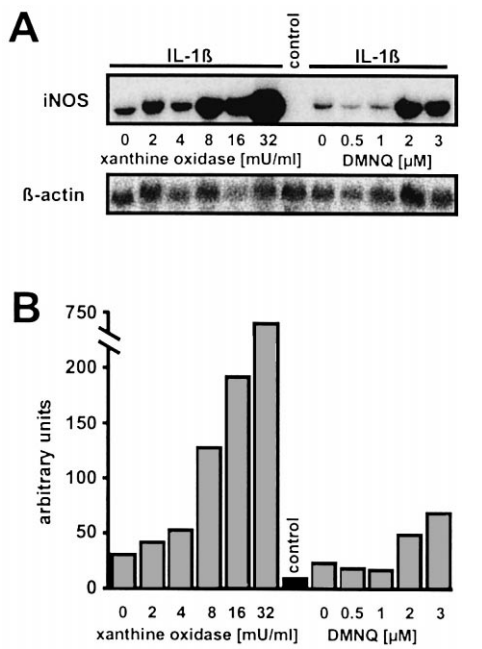

Fig. 3. Effects of HXXO and DMNQ on cytokine-induced iNOS-mRNA steady-state levels. Quiescent MC were treated with vehicle (control), IL-1β (1 nM) or IL-1β plus the indicated concentrations of xanthine oxidase and 50 μM hypoxanthine, or DMNQ. After 24 h cells were harvested for total RNA preparation. Membranes were hybridized to a radiolabelled cDNA fragment of pMac-NOS. Equivalent loading of the RNA probes was corrected after rehybridization of the filter with a probe for β-actin (pEX6). iNOS mRNA levels were detected, visualized (A) and evaluated (B) by use of the automated detector system BAS 1500 of Fujifilm (Raytest, Straubenhardt, Germany). mRNA levels for iNOS were corrected with mRNA levels for β-actin and expressed as arbitrary units (B).

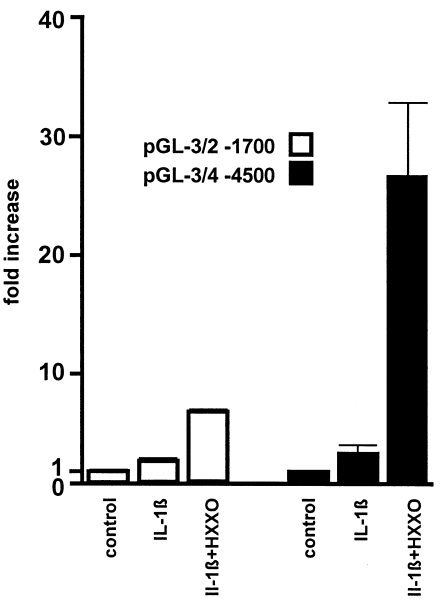

Fig. 4. Modulation of IL-1β-induced iNOS promotor activity by HXXO. MC grown in six-well plates were transfected with 2.5 μg pGL3/2 or pGL3/4 plasmid DNA and additionally with plasmid pRL_CMV that contains the gene for Renilla luciferase fused to a strong constitutive promoter of the Cytomegalus virus (Promega). After 24-h incubation with the indicated agents, dual luciferase assays (using two different substrates for the different luciferase enzymes) were performed with an automated chemiluminescence detector (Berthold, Bad Wildbad, Germany). Values for beetle luciferase were related to values for Renilla luciferase and the promoter activity expressed as fold induction vs. luciferase activity in vehicle treated MC. Data are means ± S.D., $n = 3$.

4. Discussion

The concept of ROS and NO forming the major effectors in immune-mediated glomerular diseases is based on an increasing number of in vitro and in vivo studies [9,10,28,29]. Brüne et al. [30] have established that a balanced and simultaneous generation of both radicals is non-destructive for MC whereas the unopposed formation of either O_2^- or NO induces apoptosis or even necrotic cell death [11,15,31]. Moreover, ROS and NO also exert their effects by regulating the expression of other inflammatory mediators involved in glomerular disease [20,32–35], possibly by influencing the activity of transcription factors [36,37]. In line with these reports we have observed a dramatic amplification of IL-1β-mediated activity of the iNOS promoter by O_2^-.

Recently, Jaimes et al. [38] reported that H_2O_2, the first ROS product generated in the O_2^- metabolizing pathway inhibits IL-1β-mediated NO production in MC via a posttranscriptional mechanism. At first glance this is in contrast with our observations, but Jaimes and colleagues also observed an increase of iNOS mRNA steady-state levels by H_2O_2 in MC challenged with IL-1β. In fact, the high transcriptional activity of the iNOS gene (Figs. 3 and 4) is in contrast with the relatively low values concerning the iNOS protein content and activity (Figs. 1 and 2). This suggests that mechanisms are operative that counteract at the posttranscriptional level the high transcriptional activity induced by O_2^- donors an assumption that is currently under investigation in our laboratory. The regulation of iNOS gene expression by O_2^- or more generally speaking by oxidants or the redox state of the cell, possibly involving redox-regulated transcription factors like NF-κB or AP-1 [14], is of prime importance and may provide new therapeutic approaches for the treatment of inflammatory diseases.

In summary, we conclude from our data that O_2^- generation in the inflamed glomerulus may augment severity of glomerular diseases at least in part by up-regulation of iNOS expression. The crosstalk between O_2^- and NO and their generating systems as well at its relevance for the in vivo situation has to be elucidated in further experiments.

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