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Signalling pathways regulating inducible nitric oxide synthase expression in human kidney epithelial cells

Mirjana Poljakovic[a], Jens M. Nygren[a,b], Katarina Persson[a,c,*]

[a] Department of Clinical Pharmacology, Lund University Hospital, Lund, Sweden
[b] Department of Stem Cell Biology, Lund University Hospital, Lund, Sweden
[c] Department of Chemistry and Biomedical Sciences, University of Kalmar, Kalmar, Sweden

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Abstract

The purpose of this study was to elucidate the signalling pathways involved in the cytokine-activated inducible nitric oxide synthase (iNOS) response in a human kidney epithelial cell line, A498. Unstimulated cells did not express iNOS. Exposure of A498 cells to a cytokine mixture consisting of interferon gamma, interleukin-1β and tumor necrosis factor-α (TNF-α) increased nitrite production, iNOS mRNA and protein expression. Pharmacological inhibition of tyrosine kinases, including janus kinase (JAK2), and protein kinase C (PKC) inhibited cytokine-mediated nitrite production and iNOS protein expression. The involvement of mitogen-activated protein kinases (MAPKs) was investigated. Inhibition of p38 MAPK, but not of an upstream activator of extracellular signal-regulated kinase (ERK), caused a decrease in iNOS expression and nitrite production in response to cytokines. Electrophoretic mobility shift assay of nuclear extract from cytokine-stimulated cells demonstrated a pronounced binding to a nuclear factor κB (NF-κB) sequence present in the human iNOS promoter. Furthermore, the NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC) decreased cytokine-activated iNOS protein expression and nitrite production. The present study has demonstrated that cytokine-stimulated iNOS expression in human kidney epithelial cells involves activation of tyrosine kinases, including JAK2, PKC, p38 MAPK and NF-κB.

Keywords: Nitric oxide (NO); Urinary tract infection; MAP (mitogen-activated protein) kinase; NF-κB (nuclear factor-κB); Tyrosine kinase

1. Introduction

In urinary tract infection, uropathogenic bacteria attach to the uroepithelial cells and activate an inflammatory response. Uroepithelial cell activation triggers the secretion of cytokines, resulting in recruitment of inflammatory cells and clearance of the infection (Hedges et al., 1994). The expression of the inducible nitric oxide synthase (iNOS) gene is an important part of the host response to infection (Nussler and Billiar, 1993). iNOS expression has been demonstrated in uroepithelial cells in response to experimental urinary tract infection both in vivo (Poljakovic et al., 2001) and in vitro (Elgavish et al., 1996; Poljakovic et al., 2002). Studies on regulation of iNOS expression are of interest since inhibition of iNOS is expected to have therapeutic benefits in inflammatory diseases.

iNOS expression is known to be differentially regulated in different cell types and species (Rao, 2000). For iNOS induction, most human cells require a mixture of cytokines usually composed of interferon gamma, tumor necrosis factor-α (TNF-α) and interleukin-1β (Chu et al., 1998; Taylor et al., 1998). The cytokines synergize to obtain the maximal transcriptional activity of the iNOS promoter. The human iNOS gene is regulated through a complex promoter with consensus sequences for numerous regulatory elements including nuclear factor-κB (NF-κB) site, interferon gamma response element (Chartrain et al., 1994), activator protein-1 (AP-1) element (Chu et al., 1998) and TNF-responsive element (Chartrain et al., 1994). Studies have indicated that NF-κB is required for cytokine-mediated induction of the human iNOS gene and multiple NF-κB enhancer elements in the human iNOS promoter, which confer inducibility to TNF-α and interleukin-1β, have been identified in human liver and lung epithelial cells (Taylor et al., 1998).

* Corresponding author. Department of Clinical Pharmacology, Lund University Hospital, SE-221 85, Lund, Sweden. Tel.: +46-46-17-33-49; fax: +46-46-211-19-87.
E-mail address: Katarina.Persson@klinfarm.lu.se (K. Persson).

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Human iNOS expression has been demonstrated to depend on interferon gamma (Linn et al., 1997). We have previously shown that iNOS expression in human renal epithelial cells also depends on the presence of interferon gamma (Poljakovic et al., 2002). Stimulation of cells with interferon gamma results in activation of tyrosine kinases such as the janus kinases, JAK1 and JAK2, which phosphorylate and activate the signal transducer and activator of transcription (STAT) (Schindler and Darnell, 1995). After activation, STAT translocates into the nucleus and induces transcription of interferon gamma-regulated genes (Schindler and Darnell, 1995).

Signal transduction pathways employ protein phosphorylation and protein dephosphorylation as a mechanism to transmit signals within a cell (Whitmarsh and Davis, 1996). Protein kinases catylase the addition of phosphoryl groups while protein phosphatases catalyze their removal, causing positive or negative regulatory effects, depending on the target protein. Several kinases have been implicated in iNOS regulation. Kinases such as protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and protein tyrosine kinases have been found to be important in iNOS regulation in different cell types and species (Mullet et al., 1997). These kinases may in turn activate kinases that are downstream in the signalling cascade, like the mitogen-activated protein kinases (MAPKs). Three main subgroups of the MAPKs are known; extracellular signal-regulated kinase (ERK 1/2), p38 MAPK and Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (English et al., 1999). The ERK pathway is primarily activated by mitogenic factors (Whitmarsh and Davis, 1996), whereas the JNK/SAPK and p38 MAPK pathways are activated by stress-related stimuli such as inflammatory cytokines (Anderson, 1997). The major role of MAPKs is to transmit extracellular signals to the nucleus, where the transcription of specific genes is induced by phosphorylation and activation of transcription factors (Whitmarsh and Davis, 1996).

The cellular pathways for iNOS induction in human uroepithelial cells are not known but of interest as targets aimed to inhibit iNOS expression. In the present study, we investigated the pathways involved in cytokine-induced iNOS expression in human kidney epithelial cells using pharmacological modulation of intracellular signalling.

2. Materials and methods

2.1. Reagents

The following agents were used: NOS inhibitor NG-monomethyl-L-arginine (L-NMMA, Calbiochem, La Jolla, CA, USA), iNOS inhibitor aminoguanidine (Sigma, St Louis, MO, USA), NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC; Sigma), PKC activator phorbol 12-myristate 13-acetate (PMA; Sigma), MEK1/2 inhibitor U0126 (Promega, Madison, WI, USA), tyrosine kinase inhibitor genistein (Calbiochem), JAK2 tyrosine kinase inhibitor tyrphostin B42 (Calbiochem), p38 MAPK inhibitor SB203580 (Calbiochem), dimethyl sulfoxide (DMSO; Sigma), human interferon gamma (Sigma), recombinant human tumor necrosis factor-α (TNF-α, Sigma), recombinant human interleukin-1β (Sigma).

2.2. Human kidney epithelial cell line

The human kidney epithelial cell line, A498 (ATCC HTB-44), was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in phenol red-free Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma) at 37 °C in a 5% CO_2 atmosphere and subcultured when confluent.

2.3. Cell stimulation procedure

A cytokine mixture combined of interleukin-1β (1 ng/ml), TNF-α (25 ng/ml) and interferon gamma (400 U/ml) was used to induce iNOS expression in A498 cells. The concentrations were chosen based on the literature studies and pilot experiment. To analyze the effect of various inhibitors on iNOS induction, A498 cells were cultured in sterile 96-well plates. Cells were pretreated with the pharmacological agent; genistein (25–50 μM), tyrphostin B42 (50–100 μM), U0126 (1–50 μM), SB203580 (1–20 μM) or PDTC (1–50 μM) for 1 h and then stimulated with cytokines for 24 h. The different agents were included during the cytokine-stimulation period. In order to down-regulate PKC, the cells were first treated with PMA (0.01–0.1 μM) for 24 h and then stimulated with cytokines for an additional 24 h. In parallel, cells were treated with the pharmacological compound alone or the DMSO vehicle. All experiments were done in duplicate. Nitrite concentration in cell culture supernatants and cell viability were determined (see below).

For reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis and nuclear protein extraction, A498 cells were cultured in the 75-cm^2 flasks. When used for RT-PCR, the cells were stimulated with cytokines for 12 h. For Western blot analysis, the cells were pretreated with the different agents as previously described and then stimulated with cytokines for 24 h. When used for nuclear extraction, cells were serum-starved for 24 h prior to stimulation with cytokines for 0.5, 1 and 2 h.

2.4. Nitrite assay

Nitric oxide (NO) is rapidly converted into the stable end products nitrite and nitrate, which may be used as indirect measures of the amount of NO produced. Nitrite accumu-

lation in culture supernatants was analyzed in duplicate by the Griess assay. Briefly, 50 μl of the culture supernatants was mixed with 20 μl of water and 100 μl of Griess reagent (one part 0.1% *N*-(1-naphtyl) ethylene-diamine dihydrochloride in water and one part 1% sulfanilamide in 5% concentrated H_3PO_4 (both purchased from Sigma)). The mixture was incubated for 5 min at room temperature and the absorbance measured at 540 nm (Labsystems Multiscan PLUS; Labsystems, Lund, Sweden). The readings were compared to a standard curve of sodium nitrite with a lower detection limit of 1 μM nitrite. In order to confirm that the formed nitrite was derived from NOS, cells were incubated with the NOS inhibitor, L-NMMA (100 μM), or the iNOS inhibitor, aminoguanidine (100 μM), for 1 h prior to stimulation with cytokines.

2.5. RT-PCR

Total cellular RNA was prepared from A498 cells following the TRIzol® reagent RNA protocol (Life Technologies, Täby, Sweden). RT-PCR was performed according to the Perkin-Elmer PCR-kit (GeneAmp®RNA PCR kit; Perkin-Elmer, Foster City, CA), using 2 μg total RNA, MuLV reverse transcriptase and oligo-dT as the first strand primer according to the manufacturer's instructions. Primers for human iNOS and GAPDH were obtained from DNA Technology Aps, (Aarhus C, Denmark) and were as follows; iNOS sense, 5′-AGA CAT CAA CAA CAA TGT G-3′; and antisense, 5′-GAC CTG ATG TTG CCA TTG TTG-3′ (Adcock et al., 1994) amplifying a 658-bp product; GAPDH sense, 5′-ATT CCA TGG CAC CGT CAA GGC T-3′; and antisense 5′-TCA GGT CCA CCA CTG ACA CGT T-3′, amplifying a 571-bp product. PCR was performed in an automated thermal cycler (OmniGene, Hybaid, Middlesex, UK) with one initial step at 95 °C for 2 min, followed by 35 cycles at 95 °C for 60 s, at 58 °C for 60 s and at 72 °C for 60 s. Negative controls were performed in the absence of template or MuLV reverse transcriptase. PCR products were separated by 2% agarose gel electrophoresis and bands were visualized by ethidium bromide staining.

2.6. Western blot analysis

Cells were washed in sterile PBS (pH 7.2), lysed in Laemmli sample buffer and boiled. Protein concentrations were determined with Bio-Rad *DC* Protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (Pierce, Rockford, IL, USA) as standard. Equal amounts of protein (100 μg/lane) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories) and transferred to a polyvinylidene difluoride (PVDF)-Plus transfer membrane. Unspecific sites were blocked by incubating the membrane in 5% non-fat milk overnight at 4 °C. iNOS protein was detected using a rabbit polyclonal antibody raised to human iNOS (1/500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by donkey anti-rabbit immunoglobulin G (IgG) (1/5000) linked to horseradish peroxide (Santa Cruz Biotechnology). Blots were developed using enhanced chemiluminescence Western blotting detection reagent (ECL^+; Amersham Life Science, Arlington Heights, IL, USA) and exposed to X-ray film (Hyperfilm ECL; Amersham Life Science).

2.7. Extraction of nuclear proteins

Nuclear proteins were extracted by the rapid method previously described (Andrews and Faller, 1991). Cells were scraped into cold PBS and transferred to a cold microfuge tube. Cells were pelleted for 10 s at 14000 rpm and resuspended in a cold buffer (10 mM HEPES–KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM dithiotreitol, 0.2 mM phenylmethylsulfonylfluoride; all from Sigma). Cells were allowed to swell on ice, vortexed and centrifuged for 10 s at 14000 rpm. The pellet was resuspended in a cold buffer (20 mM HEPES–KOH pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1.0 mM Dithiotreitol, 0.2 mM PMSF; all from Sigma) and incubated on ice for 20 min for high-salt extraction. Cell debris was removed by centrifugation for 2 min at 14000 rpm at 4 °C and the supernatant fraction, which contains the DNA binding proteins, aliquoted in microfuge tubes and stored at −70 °C. Protein concentrations were determined by the Bradford assay using bovine serum albumin as standard.

2.8. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotide (1.75 pmol/μl) containing a binding sequence for NF-κB from the cloned human iNOS promotor (Taylor et al., 1998) was obtained from DNA Technology and was as follows: 5′-AGA GGG CTT TCC CAG AAC CA-3′. The oligonucleotide was 5′end labelled with γ-^{32}Phosphorus (^{32}P)-adenosine triphosphatate (Amersham Life Science) using T4 polynucleotide kinase (Promega) and purified by MicroSpin™ G-25 Column (Amersham Pharmacia). To saturate nonspecific binding sites, 5 μg of nuclear proteins was incubated with a binding buffer (1.0 mM EDTA, 50% glycerol, 12.5 mM dithiotreitol, 250 mM KCl, 50 mM HEPES (pH 7.9), 0.25 mg/ml poly(dI·dC)) and water for 10 min. ^{32}P-labelled NF-κB oligonucleotide was added to the nuclear protein mixture and incubated for 20 min. The resulting DNA–protein complexes were separated by electrophoresis in a 6% nondenaturating polyacrylamide gel in TBE buffer and visualized by autoradiography. For competition experiments, a 200-fold excess of unlabelled competitor NF-κB oligonucleotide was incubated in the mixture for 10 min prior to the addition of radiolabelled probe. In negative control experiments, the nuclear proteins were omitted.

2.9. Cell viability

To determine the possible cellular toxicity of the different agents, the viability of the cells was assayed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to formazan (Mosmann, 1983). The culture medium was removed and the cells were incubated with 20 μl of MTT solution (5 mg/ml) for 1 h at 37 °C. The MTT solution was removed and the cells were solubilized in 100 μl of DMSO (Sigma) with shaking for 5 min. The extent of MTT reduction to formazan was measured as the absorbance at 540 nm. Results are expressed as a ratio of stimulated compared to control cells. Pharmacological agents were considered toxic, and the experiment excluded, when the cell viability was lower in the presence than in the absence of the agent.

2.10. Analysis of data

Data are presented as means ± S.E.M. Student's unpaired *t*-test was used to compare two means and analysis of variance (ANOVA) followed by the Bonferroni–Dunn test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytokines induce iNOS mRNA expression in A498 cells

Unstimulated A498 cells did not show any iNOS mRNA as revealed by RT-PCR (Fig. 1). A498 cells stimulated with a cytokine mixture consisting of interleukin-1β, TNF-α and interferon gamma showed a pronounced iNOS mRNA expression.

3.2. Nitrite production reflects iNOS activity

Both the general NOS inhibitor L-NMMA (100 μM) and the specific iNOS inhibitor aminoguanidine (100 μM) caused a significant ($P < 0.001$) decrease in nitrite accumulation in cytokine-stimulated A498 cells (Fig. 2). These experiments demonstrated that the cytokine-stimulated increase in nitrite was a result of iNOS activity.

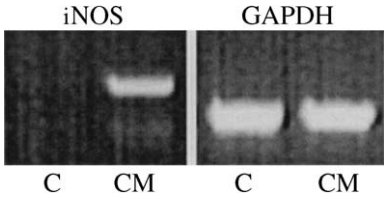

Fig. 1. RT-PCR analysis of iNOS and GAPDH mRNA expression in A498 cells stimulated for 12 h. C = control, CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma. Blots shown are representative of four similar experiments.

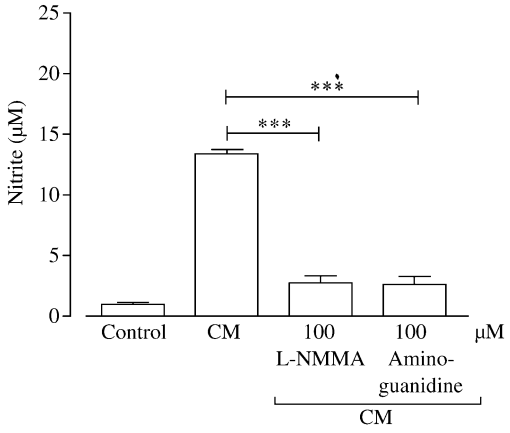

Fig. 2. The effect of L-NMMA, a general NOS inhibitor, and aminoguanidine, an iNOS inhibitor, on nitrite production in cytokine-stimulated A498 cells. Data are expressed as mean ± S.E.M. ($n = 4$). Statistical comparison, ***$P < 0.001$. CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma.

3.3. Inhibition of tyrosine kinase activity decreases nitrite production and iNOS protein expression in cytokine-stimulated cells

The general tyrosine kinase inhibitor, genistein, was used to investigate the involvement of tyrosine kinase activity. The highest concentration (50 μM) of genistein significantly ($P < 0.05$) decreased cytokine-induced nitrite production (3.9 ± 1.0 μM, $n = 5$) when compared to nitrite levels obtained in the absence of genistein (13 ± 2.8 μM, $n = 5$) (Fig. 3A). Genistein almost completely abolished cytokine-induced iNOS protein expression as judged by Western blot analysis (Fig. 3B).

3.4. Inhibition of JAK2 activity decreases nitrite production and iNOS protein expression in cytokine-stimulated cells

Tyrphostin B42 was used to inhibit JAK2 tyrosine kinase. The cytokine-induced nitrite production was significantly decreased after treatment with 75 μM (5.2 ± 1.1 μM, $n = 4$, $P < 0.05$) and 100 μM (4.2 ± 0.6 μM, $n = 5$, $P < 0.01$) tyrphostin B42 when compared to cytokine-stimulated cells in the absence of tyrphostin B42 (12 ± 3.0 μM, $n = 5$) (Fig. 4A). Tyrphostin B42 was also found to decrease, and at the highest concentration (100 μM) almost completely abolish, iNOS protein expression (Fig. 4B).

3.5. Inhibition of protein kinase C activity decreases nitrite production and iNOS protein expression in cytokine-stimulated cells

To determine the role of PKC in iNOS induction, A498 cells were pretreated with the PKC activator, PMA, or the vehicle (DMSO) for 24 h before challenged with the cytokines. Longtime exposure of cells to PMA is known to down-regulate the conventional and novel isoforms of

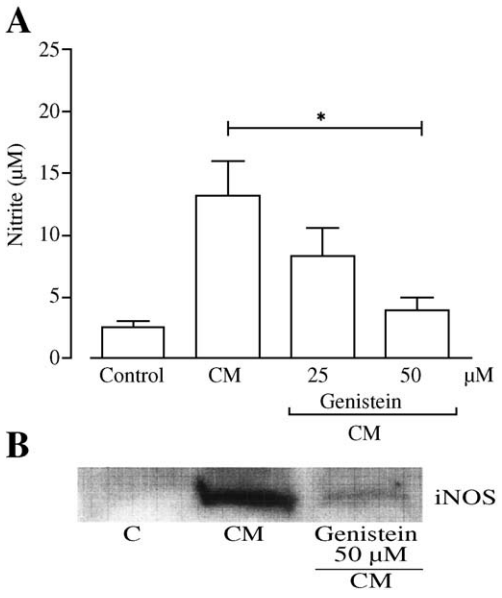

Fig. 3. The effect of genistein, a tyrosine kinase inhibitor, on nitrite production and iNOS protein expression in cytokine-stimulated A498 cells. (A) Cytokine-mediated nitrite production in the presence of genistein. Data are expressed as mean ± S.E.M. ($n = 5$). Statistical comparison, $^*P < 0.05$. (B) Western blot analysis of iNOS protein expression in cytokine-stimulated cells treated with genistein. C = control, CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma.

PKC. Pretreatment with PMA (0.01 μM and 0.1 μM) significantly decreased cytokine-induced nitrite production (2.8 ± 0.7 μM, $P < 0.05$ and 2.2 ± 0.4 μM, $P < 0.01$, respectively, $n = 3$) when compared to cells pretreated with corresponding concentrations of DMSO (12 ± 3.1 and 14 ± 4.1 μM, respectively, $n = 3$) (Fig. 5A). Western blot analysis demonstrated that PMA treatment caused a marked decrease in iNOS protein expression in cytokine-stimulated cells (Fig. 5B).

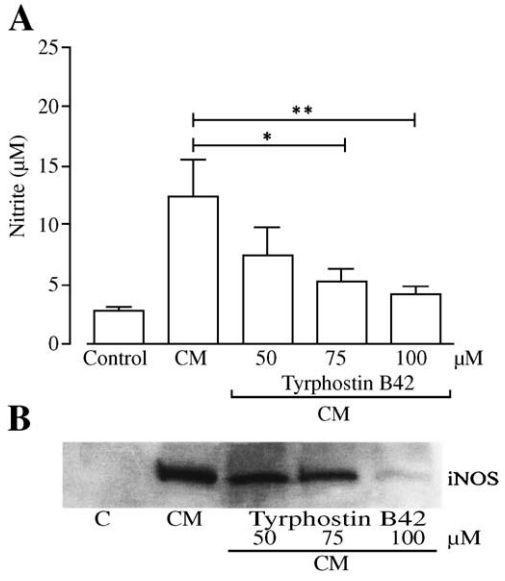

Fig. 4. The effect of tyrphostin B42, a JAK2 inhibitor, on nitrite production and iNOS protein expression in cytokine-stimulated A498 cells. (A) Cytokine-mediated nitrite production in the presence of tyrphostin B42. Data are expressed as mean ± S.E.M. ($n = 4–5$). Statistical comparison, $^*P < 0.05$, $^{**}P < 0.01$. (B) Western blot analysis of iNOS protein expression in cytokine-stimulated cells treated with tyrphostin B42. C = control, CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma.

3.6. The role of MAPKs activity in cytokine-induced nitrite production and iNOS protein expression

The role of ERK1/2 and p38 MAPK in cytokine-induced nitrite production was investigated. U0126 (1–20 μM), which inhibits an upstream activator of ERK1/2 (MEK1/2), did not decrease cytokine-induced nitrite production in A498 cells (data not shown). Higher concentrations of U0126 affected the cell viability as determined by MTT. SB203580 (1–20 μM), an inhibitor of p38 MAPK, significantly decreased cytokine-induced nitrite production at all concentrations used (Fig. 6A). A marked decrease in iNOS protein expression was revealed by Western blot analysis in A498 cells pretreated with SB203580 (Fig. 6B).

3.7. Inhibition of NF-κB decreases nitrite production and iNOS protein expression in cytokine-stimulated cells

The agent PDTC inhibits NF-κB by acting as an antioxidant and interfering with IκB degradation (Nakashima et al., 1999). PDTC caused a concentration-dependent decrease in nitrite accumulation in cytokine-stimulated cells, with a significant ($P < 0.01$) decrease in nitrite production at

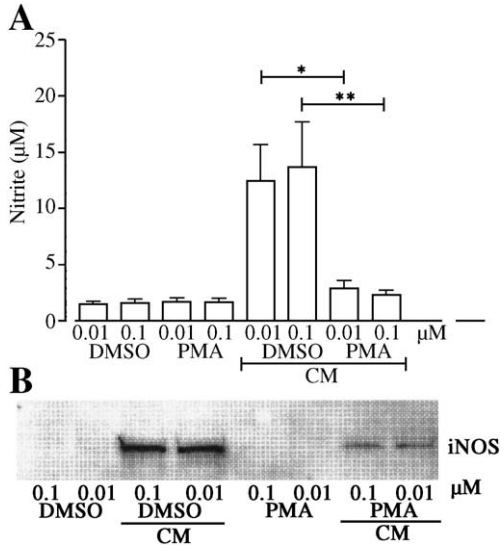

Fig. 5. The effect of PKC inhibition on nitrite production and iNOS protein expression in cytokine-stimulated A498 cells. (A) Cytokine-mediated nitrite production in PMA- and DMSO-treated cells. Prolonged treatment with PMA was used to down-regulate PKC. Cells exposed to corresponding concentrations of DMSO vehicle were used as control. Data are expressed as mean ± S.E.M. ($n = 3$). Statistical comparison, $^*P < 0.05$, $^{**}P < 0.01$. (B) Western blot analysis of iNOS protein expression in cytokine-stimulated A498 cells treated with PMA or DMSO vehicle. CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma.

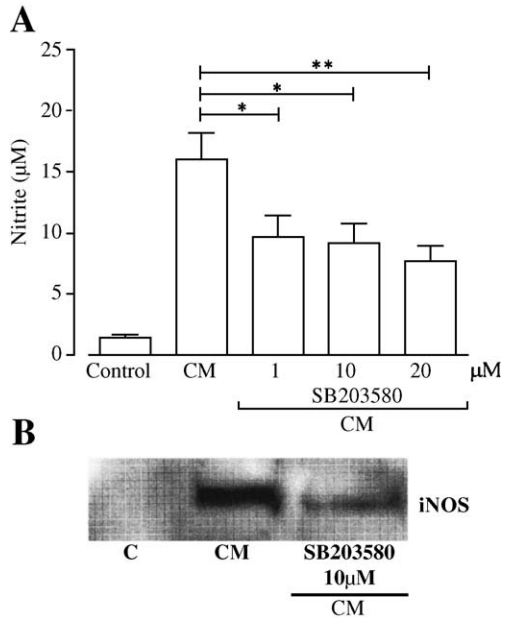

Fig. 6. The effect of SB203580, a p38 MAPK inhibitor, on nitrite production and iNOS protein expression in cytokine-stimulated A498 cells. (A) Cytokine-mediated nitrite production in the presence of SB203580. Data are expressed as mean ± S.E.M. ($n = 6$). Statistical comparison, $*P < 0.05$, $**P < 0.01$. (B) Western blot analysis of iNOS protein expression in cytokine-stimulated cells treated with SB203580. C = control, CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma.

the highest concentration used (50 μM) (Fig. 7A). PDTC treatment almost completely abolished iNOS protein expression in cytokine-stimulated A498 cells (Fig. 7B).

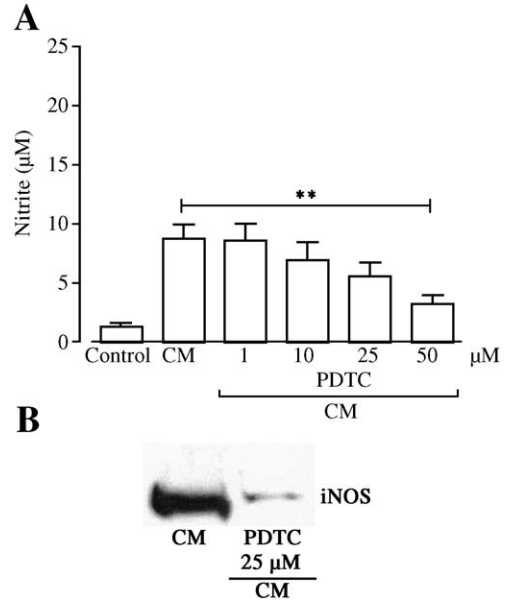

Fig. 7. The effect of PDTC, a NF-κB inhibitor, on nitrite production and iNOS protein expression in cytokine-stimulated A498 cells. (A) Cytokine-mediated nitrite production in the presence of PDTC. Data are expressed as mean ± S.E.M. ($n = 7$). Statistical comparison, $**P < 0.01$. (B) Western blot analysis of iNOS protein expression in cytokine-stimulated cells treated with PDTC. CM (cytokine mixture) = interleukin-1β/TNF-α/interferon gamma.

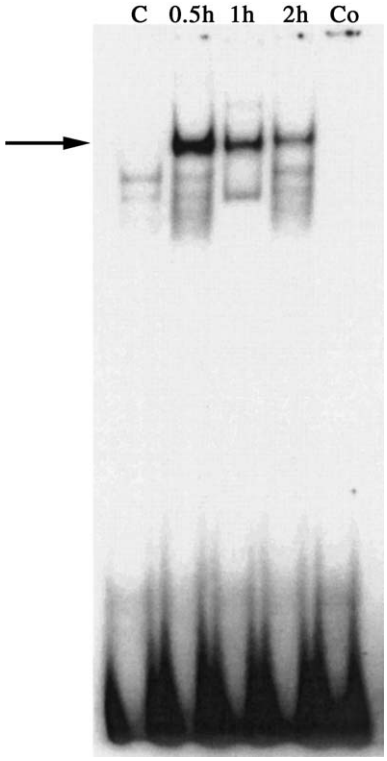

Fig. 8. Representative autoradiography of electrophoretic mobility shift assay of NF-κB binding to a ^{32}P labelled oligonucleotide probe in nuclear extracts from cytokine-stimulated A498 cells. Cells were stimulated for 0.5, 1 and 2 h with a cytokine mixture (interleukin-1β/TNF-α/interferon gamma.). The arrow shows the stimulation-induced binding complex. C = control, Co = 200-fold competition. The blot shown is representative of three similar experiments.

3.8. EMSA detection of NF-κB activation

EMSA was used to study binding of nuclear proteins from cells stimulated by cytokines to an iNOS specific NF-κB oligonucleotide. Nuclear extracts from unstimulated cells showed one NF-κB band (Fig. 8). Nuclear extracts from cytokine-stimulated cells produced a strong NF-κB band that was not seen in unstimulated cells at 0.5, 1 and 2 h after stimulation (Fig. 8). Competition studies were performed to determine the specificity of DNA–NF-κB interactions. Adding a 200-fold excess of unlabelled NF-κB oligonucleotide abolished binding of NF-κB in nuclear extracts from stimulated cells, indicating that the assay was specific for NF-κB (Fig. 8).

4. Discussion

Urinary tract infection is accompanied by a cytokine response in the infected host (Hedges et al., 1994). The bacteria stimulate the epithelial cells to produce cytokines and pro-inflammatory factors. Influx of neutrophils and other inflammatory cells occurs subsequently to the primary bacterial interaction with the mucosa. The cytokines

secreted by influxing cells may in turn stimulate the epithelial cells (Hedges et al., 1994). In the human kidney epithelial cell line A498, a cytokine mixture of interleukin-1β, TNF-α and interferon gamma was found to induce iNOS mRNA and protein as well as NO production. We have previously demonstrated that the combination interleukin-1β, TNF-α and interferon gamma gives the highest level of NO production in primary human renal tubular epithelial cells (HRTEC) (Poljakovic et al., 2002). The iNOS response in transformed A498 kidney cells was found to be faster than the response of primary HRTEC. Despite the time difference, A498 cells and HRTEC showed similar iNOS responses to cytokines, demonstrating that the A498 cell line is likely to be relevant for studies of cytokine-induced iNOS signalling pathways. None of the three cytokines alone were able to induce iNOS expression in A498 cells or HRTEC (Poljakovic et al., 2002), suggesting that activation of multiple signalling pathways is required for iNOS transcription in human kidney epithelial cells. In this study, we now describe some of the signalling pathways involved in the activation of cytokine-mediated iNOS expression in human kidney epithelial cells.

Activation of cytokine receptors often results in tyrosine phosphorylation of different proteins, including the receptor itself, with subsequent dimerization of the receptor and signal transduction (Dell'Albani et al., 2001). The investigated pathways in our study do not necessarily represent events downstream of all three cytokine receptors stimulated, and the described kinases are not necessarily parts of only one cascade. In the present study, the general tyrosine kinase inhibitor genistein significantly decreased cytokine-induced NO production and almost completely abolished iNOS protein expression in human kidney epithelial cells. To further elucidate the tyrosine kinases involved we used tyrphostin B42, a specific JAK2 tyrosine kinase inhibitor. Tyrphostin B42 also decreased cytokine-induced iNOS protein expression and NO production. These results demonstrate that tyrosine kinases, including JAK2, are involved in cytokine-mediated iNOS induction in human kidney epithelial cells. The JAK/STAT-signalling pathway has also been implicated for iNOS induction in other epithelial cells like human lung (Ganster et al., 2001) and colon epithelial cells (Kleinert et al., 1998b).

Down-regulation of PKC by prolonged PMA treatment was performed to elucidate the involvement of PKC in cytokine-mediated iNOS expression in A498 cells. Inhibition of PKC caused a marked decrease in iNOS protein expression and NO production, suggesting a critical role of PKC in iNOS induction in human kidney epithelial cells. PKC activation has previously been demonstrated to be important in activation of iNOS transcription and also to be a key component of iNOS mRNA stabilization in e.g., rat pancreatic cells (Carpenter et al., 2001). Cytokine-induced iNOS expression in human colon epithelial cells (Kleinert et al., 1998a) was, however, found not to involve PKC activity. It has been shown that the individual PKC isoforms regulate iNOS expression in both a positive and negative manner (Paul et al., 1997).

MAP kinases are involved in the expressional regulation of many genes that are induced by cytokines. The involvement of the ERK signalling pathway in cytokine-activated iNOS expression was investigated by using U0126, which inhibits MEK1/2, an upstream activator of ERK1/2. In the present study, U0126 did not inhibit cytokine-mediated NO production in A498 cells. In agreement with our data, the ERK pathway was not found to be involved in iNOS expression in cytokine-activated human colon epithelial cells (Kleinert et al., 1998a). JNK/SAPK and p38 MAPK are other members of the MAP kinase family that have been shown to be important in, e.g. interleukin-1β-induced iNOS expression in rat glomerular mesangial cells (Guan et al., 1999). SB203580, a p38 MAPK inhibitor, significantly decreased NO production and iNOS protein expression in cytokine-stimulated A498 cells. The involvement of the JNK/SAPK pathway was not specifically investigated in the present study. Taken together, our results demonstrate that activation of the p38 MAPK, but not ERK, signalling pathway is involved in cytokine-activated iNOS induction in human kidney epithelial cells.

The transcription factor NF-κB has an important role in regulating gene expression related to inflammation and injury (Liou and Baltimore, 1993). NF-κB remains dormant in the cytoplasm bound to its inhibitor, I kappa B (IκB). Stimulation with different agents such as interleukin-1, TNF-α and LPS leads to the dissociation of the inhibitor and translocation of the free NF-κB to the nucleus. The human iNOS gene promoter has been shown to contain multiple binding sites for NF-κB (Taylor et al., 1998). In our study, EMSA experiments demonstrated increased NF-κB binding to the human iNOS promoter in A498 cells treated with cytokines. NF-κB binding was also observed in nuclear extracts isolated from unstimulated A498 cells, indicating the presence of a constitutive NF-κB activity in these cells. The majority of agents that activate NF-κB also tend to trigger the formation of endogenous reactive oxygen species (Flohe et al., 1997). Antioxidants, such as PDTC, block NF-κB activation by preventing IκB phosphorylation (Nakashima et al., 1999). In our study, PDTC caused a decrease in NO production and iNOS protein expression in cytokine-stimulated A498 cells, further strengthening that NF-κB is involved in iNOS transcription in these cells.

The role of iNOS in epithelial cells for the host response to urinary tract infection is not clear but NO has been shown to interfere with uroepithelial cell proliferation, differentiation and shedding (Elgavish et al., 1996; Glynne et al., 2001). The present study showed that cytokine-stimulated iNOS expression in human kidney epithelial cells involves activation of tyrosine kinases, including JAK2, PKC, p38 MAPK, and NF-κB. Manipulation of iNOS expression in human kidney epithelial cells may be achieved by interaction with signalling pathways identified in this study.

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