Nitric Oxide Activates IL-6 Production and Expression in Human Renal Epithelial Cells

Isak Demirel a Ravi Vumma a Camilla Mohlin b Lovisa Svensson b, c Susanne Säve b Katarina Persson a, b

a School of Health and Medical Sciences, Örebro University, Örebro, b School of Natural Sciences, Linnaeus University, Kalmar, and c Department of Medical Sciences, Uppsala University, Uppsala, Sweden

Abstract

Background/Aims: Increased nitric oxide (NO) production or inducible form of NO synthase activity have been documented in patients suffering from urinary tract infection (UTI), but the role of NO in this infection is unclear. We investigated whether NO can affect the host response in human renal epithelial cells by modulating IL-6 production and mRNA expression.

Methods: The human renal epithelial cell line A498 was infected with a uropathogenic Escherichia coli (UPEC) strain and/or the NO donor DETA/NO. The IL-6 production and mRNA expression were evaluated by ELISA and real-time RT-PCR. IL-6 mRNA stability was evaluated by analyzing mRNA degradation by real-time RT-PCR.

Results: DETA/NO caused a significant (p < 0.05) increase in IL-6 production. Inhibitors of p38 MAPK and ERK1/2 signaling, but not JNK, were shown to significantly suppress DETA/NO-induced IL-6 production. UPEC-induced IL-6 production was further increased (by 73 ± 23%, p < 0.05) in the presence of DETA/NO. The IL-6 mRNA expression increased 2.1 ± 0.17-fold in response to DETA/NO, while the UPEC-evoked increase was pronounced (20 ± 4.5-fold). A synergistic effect of DETA/NO on UPEC-induced IL-6 expression was found (33 ± 7.2-fold increase). The IL-6 mRNA stability studies showed that DETA/NO partially attenuated UPEC-induced degradation of IL-6 mRNA.

Conclusions: NO was found to stimulate IL-6 in renal epithelial cells through p38 MAPK and ERK1/2 signaling pathways and also to increase IL-6 mRNA stability in UPEC-infected cells. This study proposes a new role for NO in the host response during UTI by modulating the transcription and production of the cytokine IL-6.

Key Words
Nitric oxide · Urinary tract infections · IL-6 · MAPK signaling · Renal epithelial cells

Introduction

The free radical nitric oxide (NO) is formed from a reaction between molecular oxygen and the substrate l-arginine by the enzyme NO synthase (NOS). In the kidney, NO plays an important role in various physiological processes, including salt and fluid reabsorption, renal hemodynamics, renin secretion and tubuloglomerular feedback [1]. The inducible form of NOS (iNOS) is absent under physiological conditions, but is expressed in many cells in response to microbes and inflammatory cytokines [2]. Increased NO levels or iNOS activity have been re-
ported in patients suffering from urinary tract infection (UTI) [3–5], with a cellular localization of iNOS described in neutrophils and renal and bladder epithelial cells [5–8]. Thus, NO is produced by host cells in UTI, but the functional role of NO in this infection remains unclear.

UTIs, including cystitis and pyelonephritis, are mainly caused by different strains of uropathogenic Escherichia coli (UPEC). Attachment of the bacteria to the urinary tract epithelium is the first step in the pathogenesis of UTI. When bacteria adhere to the epithelial cells, these are triggered to release proinflammatory cytokines and chemokines (e.g. IL-6 and IL-8) followed by recruitment and activation of inflammatory cells [9]. Release of proinflammatory mediators from renal epithelial cells in response to different UPEC strains and toll-like receptor-4 (TLR4) signaling has been extensively studied [10]. In some tissues, NO appears to be able to regulate the host production of chemokines and cytokines. Both stimulatory and suppressive effects of NO on cytokine expression and production have been reported, which suggests that NO may have a role during both induction and resolution of inflammation [11]. However, it is not known if NO regulates the release of proinflammatory cytokines such as IL-6 from renal epithelial cells. IL-6 is a multifunctional cytokine with proinflammatory and immunoregulatory functions, and is important for bacterial clearance. Elevated levels of IL-6 are found in patients with UTI, and several cell types within the urinary tract can produce IL-6 including fibroblasts, macrophages and epithelial cells [12].

This study was performed to determine whether NO could affect the host IL-6 responses in human renal epithelial cells by modulating IL-6 release and mRNA expression.

**Materials and Methods**

**Bacteria and Renal Epithelial Cells**

The UPEC strain IA2 was originally isolated from a patient with acute pyelonephritis. Bacteria were grown and maintained on tryptic soy agar (Becton Dickinson co, Le Pont Clai, France). The human renal epithelial cell line A498 (ATCC HTB-44) was obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA). Cells were grown in DMEM supplemented with 10% fetal bovine 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-Aldrich, Germany) at 37°C in a 5% CO2 atmosphere. Gentamicin (50 μg/ml; Sigma-Aldrich) was added to cells 24 h before infection, but was excluded during the experiments. The viability of the cells was >90% during the experiments as determined by trypan blue exclusion test.

**Measurement of IL-6 Production**

The NO-donor DETA/NO (Alexis Biochemical, Lausen, Switzerland) was prepared immediately before the start of the experiment. To exclude effects of the parent compound (a polyamine) on IL-6 secretion, some experiments were performed with deacetylated DETA/NO (incubation at 37°C for >96 h followed by ventilation in open air for 48 h). The effect of DETA/NO (0.1 mM and 1 mM) and UPEC (105 CFU/ml) on IL-6 production was assessed after 6 and 24 h. To study the effect of DETA/NO on an ongoing UPEC infection, UPEC was added 2 h prior to DETA/NO stimulation. In some experiments, the NOS inhibitor L-NAME (1 mM) was added 2 h prior to UPEC infection to study the involvement of endogenously produced NO in UPEC-evoked IL-6 production. To study the involvement of MAPK, the cells were preincubated with the JNK inhibitor SP600125 (20 or 40 μM; InSolution JNK Inhibitor II, Calbiochem, USA), the p38 MAPK inhibitor SB203580 (10 or 20 μM) or the ERK1/2 inhibitor PD98059 (10 or 20 μM) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 1 h prior to DETA/NO (1 mM) stimulation for 24 h. IL-6 production was analyzed by ELISA using the BD OptEIA™ human IL-6 ELISA kit II (BD Biosciences, San Diego, Calif., USA) according to the manufacturer’s instructions.

**Reverse Transcription-Polymerase Chain Reaction**

RNA extraction was performed using Qiagen RNeasy minikit (Qiagen, Hilden, Germany). The cDNA synthesis (1 μg of total RNA) was performed by using the Omniscript Reverse Transcriptase for first-strand cDNA synthesis (Qiagen) and oligo-dT primers (Applied Biosystems, Foster City, USA). RNA was controlled for genomic DNA contamination. The cDNA was amplified using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Little Chalfont, UK), together with PCR-specific primers (Invitrogen Life Technologies, Carlsbad, Calif., USA). The primers were IL-6 (forward) 5’-ATGACTCTTCTTCTCACAAGCGG-3’, IL-6 (reverse) 5’-GAAGAGCCCTCAAGGCTGACT-3’ (628 bp) and GAPDH (forward) 5’-ATTCATGGACCGTCAAGGC-3’, GAPDH (reverse) 5’-TCAGGTCCA CCACGTACAGT T-3’ (571 bp).

**Real-Time Reverse Transcription-Polymerase Chain Reaction and Stability Experiments**

The real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out in a total volume of 20 μl with 10 μl SoFast EvaGreen supermix (Bio-Rad Laboratories, Hercules, Calif., USA), 0.8 μl ROX passive reference dye (Bio-Rad Laboratories), 2 μl of each primer IL-6 (Qiagen, Hs_IL6_1_SG) and GAPDH (Qiagen, Hs_GAPDH_SG) and 2 μl cDNA and 5.2 μl water. The amplification was performed in an ABI 7900HT thermocycler (Applied Biosystems) with the following protocol: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s followed by a dissociation curve. All samples were run in duplicates. The Ct values were analyzed by the comparative Ct (ΔΔCt) method and normalized to the endogenous control GAPDH. Fold difference was calculated as 2−ΔΔCt.

The IL-6 mRNA degradation was evaluated by comparing the expression in samples treated with actinomycin D (5 μg/ml; Santa Cruz Biotechnology Inc.) with control samples without actinomycin D treatment.
Statistics

Data are given as mean ± standard error of the mean (SEM), and n indicates the number of independent experiments. Student’s unpaired t test was used to compare two treatments. For multiple comparisons, ANOVA followed by Bonferroni-Dunn test was used. Statistical significance was considered at p < 0.05.

Results

IL-6 Production in Response to DETA/NO and UPEC

Production of IL-6 was measured after exposing the cells to the NO donor DETA/NO (0.1 and 1 mM) in the absence or presence of the UPEC strain IA2. DETA/NO did not affect the basal IL-6 production after 6 h compared to non-stimulated cells (fig. 1a). However, after 24 h of stimulation, a 2-fold and 6-fold increase (p < 0.05) in basal IL-6 production was noted in response to DETA/NO 0.1 and 1 mM, respectively (fig. 1b). We next studied if DETA/NO could modify an ongoing UPEC-induced IL-6 secretion. UPEC-stimulated IL-6 production was not significantly affected by DETA/NO after 6 h of stimulation (fig. 1a). However, after 24 h of stimulation, DETA/NO (1 mM) increased UPEC-evoked IL-6 production by 73 ± 23% (p < 0.05; fig. 1b).

No significant increase in IL-6 production was found in experiments using DETA/NO (1 mM) that had been inactivated prior to application (fig. 2a). The NOS inhibitor L-NAME did not affect IL-6 production in UPEC-infected cells, verifying that UPEC-evoked IL-6 release does not involve endogenously produced NO (fig. 2b). Measurement of nitrite in cell supernatants after stimulation with UPEC for 24 h confirmed that UPEC are unable to activate NO production in A498 cells (data not shown).

IL-6 mRNA Expression in Response to DETA/NO and UPEC

RT-PCR analysis was performed to examine whether the DETA/NO-induced increase in IL-6 production involves increased gene expression. No expression of IL-6 mRNA was detected in non-stimulated cells, but an increased expression of IL-6 mRNA was noted 6–24 h after DETA/NO stimulation (fig. 3a). As expected, RT-PCR analysis demonstrated a strong expression of IL-6 mRNA in A498 cells stimulated with UPEC (fig. 3b). Real-time RT-PCR was used for quantitative studies of IL-6 expression in A498 cells. The IL-6 mRNA expression increased 2.1 ± 0.17-fold in the presence of DETA/NO, while the UPEC-induced increase was pronounced (20 ± 4.5-fold). DETA/NO and UPEC in combination caused a 33 ± 7.2-fold increase in IL-6 mRNA expression (fig. 4). Thus, a synergistic trend in IL-6 mRNA expression was observed for the combined treatment of DETA/NO and UPEC.
MAPK Signaling in Response to DETA/NO

Different inhibitors were used to evaluate if the DETA/NO-induced IL-6 production involves MAPK signaling pathways. The p38 MAPK (SB203580) and the ERK1/2 (PD98059) inhibitors markedly suppressed DETA/NO-induced IL-6 production in a dose-dependent manner (fig. 5). The DETA/NO-induced increase in IL-6 was reduced, at the highest concentration used, by 99.81% (p < 0.001) and 95.83.5% (p < 0.001) in response to SB203580 and PD98059, respectively. The JNK inhibitor (SP600125) did not inhibit the IL-6 production induced by DETA/NO (fig. 5). None of the inhibitors significantly suppressed the basal IL-6 production (data not shown).

IL-6 mRNA Stability Experiments

Actinomycin D, an inhibitor of transcription, was used to evaluate the effect of DETA/NO on IL-6 mRNA stability in A498 cells. Degradation of IL-6 mRNA was evaluated by comparing actinomycin D-treated samples stimulated with IA2 (10^8 CFU/ml), DETA/NO (1 mM) or with cell medium alone to a control sample without actinomycin D treatment and expressing the differences as fold change. The mRNA levels were relatively stable for up to 6 h in un-stimulated control cells (fig. 6). UPEC-stimulated cells showed a time-dependent IL-6 mRNA degradation after actinomycin D treatment (fig. 6). In cells treated with DETA/NO, the IL-6 mRNA levels were stable for up to 6 h and not different from control cells (fig. 6). Treatment of UPEC-infected cells with DETA/NO caused a decreased IL-6 mRNA degradation compared to cells treated with only UPEC. The effect of DETA/NO on mRNA stability in UPEC-infected cells was most pronounced 1–4 h after actinomycin D treatment (fig. 6).
Discussion

The results in this study showed that addition of the NO donor DETA/NO to human renal epithelial cells caused an augmentation of basal IL-6 production. The DETA/NO-induced increase in IL-6 production was associated with an increase in IL-6 mRNA verifying an effect of DETA/NO at the transcriptional level. Both IL-6 and NO are known to be produced during the host response in UTI [7, 13], supporting that an interaction between these mediators may take place in vivo. In a rat model of ascending pyelonephritis, tubular immunoreactivity for IL-6 as well as iNOS was seen in cortical and medullary areas, which also corresponded to sites of bacterial colonization [7]. Production of IL-6 from urinary tract epithelial cells after in vitro infection with UPEC strains increases within hours by a TLR4-dependent mechanism [10, 14]. This was also confirmed in our study where UPEC strain IA2 increased IL-6 secretion approximately 7-fold after 6 h of infection, i.e. a much faster response than noted for DETA/NO. Infection of A498 cells with UPEC strain IA2 did not evoke any endogenous NO production. This is in agreement with a previous study [15] demonstrating that activation of iNOS expression in A498 cells depends on cytokines such as IL-1β and IFN-γ while UPEC per se are weak inducers of iNOS in vitro.

Neutrophils and renal epithelial cells express the iNOS enzyme in different UTI-models, which suggests that these cell types are able to produce NO during UTI [4, 5, 7, 8]. The neutrophils upregulate iNOS early in the infection in response to the bacteria [5, 8] while induction of iNOS in human renal epithelial cells is delayed and dependent on secreted cytokines from infiltrating inflammatory cells [8, 15]. Additional work is required to define more accurately the relative roles of NO signaling from epithelial cells versus the neutrophils in regulation of the...
IL-6 response. NO is an antimicrobial molecule that reduces the growth of a wide variety of Gram-negative and Gram-positive bacteria species, including UPEC [2, 16]. However, to protect against the damage caused by nitrosative stress, bacteria possess multiple defense mechanisms. Thus, it is possible that host-derived NO may exert an antibacterial effect in UTI, at least until appropriate defense mechanisms have been activated. We have previously shown that iNOS-deficient and wild-type mice were equally successful to clear UPEC-induced bladder and kidney infections [17], suggesting that iNOS-derived NO production was not important for bacterial clearance in this experimental UTI model. Unfortunately, we did not measure urinary IL-6 levels in the iNOS-deficient mice as this might have provided valuable information for validation of the in vitro findings in the present study. Further studies are needed to explore the importance of NO-mediated regulation of proinflammatory cytokines using in vivo UTI models.

Whether NO regulates cytokines in a positive or negative manner appears to depend on the type of responder cell, the cytokine and the local levels and duration of the NO exposure [11]. In our study, two concentrations of DETA/NO were used, and only the highest concentration showed a stimulatory effect on IL-6 production. High concentrations of NO donors may have an inhibitory effect on IL-6 production [18, 19], and it is possible that a higher concentration of DETA/NO (＞1 mM) would have decreased IL-6 also in renal epithelial cells. However, due to the risk of cytotoxicity, experiments using higher concentration of DETA/NO were not performed. In agreement with our data, NO has been reported to stimulate IL-6 production in murine skeletal myocytes [18] and human blood mononuclear cells [19] by activating ERK1/2 and p38 MAPK-signaling pathways [18]. We also found that ERK1/2 and p38 MAPK signaling were crucial for the DETA/NO-mediated activation of IL-6 in renal epithelial cells. Mechanisms through which NO inactivates tyrosine phosphatases leading to prolonged activation of MAPK and S-nitrosylation of p21Kas, an upstream signal of the MAPK pathway, have been reported [20, 21].

The results in this study also showed that addition of DETA/NO to cells pre-infected with UPEC caused an augmentation of the IL-6 response, suggesting a potentially wide influence of NO as an inducer and modulator of inflammation. Quantitative RT-PCR studies demonstrated that the effect of DETA/NO in combination with UPEC was synergistic rather than additive, at least at the mRNA level. It is well known that TLR4 and associated ERK1/2 and p38 MAPK signaling pathways play a role in IL-6 production in UPEC-stimulated human renal epithelial cells [22, 23]. This was also confirmed in our study where the p38 and ERK 1/2 inhibitors markedly suppressed IA2-induced IL-6 secretion from A498 cells [unpubl. observation]. Our study suggests that NO activates IL-6 production in human renal epithelial cells by signaling pathways that, at least partly, overlap with pathways used by UPEC to induce IL-6. Thus, NO signaling seems to represent a non-TLR4 pathway that may function as an additional pathway for activation of IL-6 release during kidney infection.

To further explore the mechanisms for NO in IL-6 regulation, we performed analysis of IL-6 mRNA stability. It was found that DETA/NO per se did not alter the IL-6 mRNA stability compared to the control. UPEC-stimulated cells caused a gradual degradation of IL-6 mRNA, but treatment with DETA/NO was able to partially attenuate this degradation at least during the first 4 h. In other studies, NO has been reported to stabilize IL-8 mRNA by p38 MAPK signaling pathways, and stabilization of IL-6 mRNA in mouse embryonic fibroblasts was also promoted by p38 MAPK signaling [24, 25]. Thus, the mechanisms through which DETA/NO mediates increased IL-6 mRNA stability in UPEC-infected renal epithelial cells may involve p38 MAPK signaling as we found this pathway to be activated by DETA/NO. The results from the IL-6 mRNA stability experiments indicate that NO may stabilize UPEC-evoked IL-6 production in renal epithelial cells by posttranscriptional events such as regulation of mRNA degradation.

This study demonstrates that NO activates IL-6 production and expression in renal epithelial cells and also provides insight into mechanisms, including activation of p38 MAPK and ERK1/2 signaling pathways and effects on IL-6 mRNA stability. It is suggested that NO may participate in the host response during UTI by modulating the epithelial cytokine response.

Acknowledgements

The authors acknowledge support from the Swedish Medical Research Council (12601), the Swedish Council for Working Life and Social Research, and the Natural Sciences Faculty at the University of Kalmar.

Disclosure Statement

No conflicts of interest.