NITRIC OXIDE STIMULATES THE STRESS-ACTIVATED PROTEIN KINASE p38 IN RAT RENAL MESANGIAL CELLS

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Summary

Nitric oxide (NO) has gained increased attention as a diffusible universal messenger that plays a crucial role in the pathogenesis of inflammatory and autoimmune diseases. Recently, we reported that exogenous NO is able to activate the stress-activated protein kinase (SAPK) cascade in mesangial cells. Here, we demonstrate that exposure of glomerular mesangial cells to compounds releasing NO, including spermine-NO and (Z)-1-{N-methyl-N-[6-(N-methylammoniohexyl)amino]diazen}-1-ium-1,2-diolate (MAHMA-NO), results in an activation of the stress-activated p38-mitogen-activated protein kinase (p38-MAPK) cascade as measured by the phosphorylation of the activator of transcription factor-2 (ATF2) in an immunocomplex kinase assay. Activation of the p38-MAPK cascade by a short stimulation (10 min) with the NO donor MAHMA-NO causes a large increase in ATF2 phosphorylation that is several times greater than that observed after stimulation with interleukin-1β, a well-known activator of the p38-MAPK pathway. Time course studies reveal that MAHMA-NO causes rapid and maximal activation of p38-MAPK after 10 min of stimulation and that activation declines to basal levels within 60 min. The longer-lived NO donor spermine-NO causes a comparable rapid activation of the p38-MAPK pathway; however, the increased activation state of p38-MAPK was maintained for several hours before control values were reattained after 24 h of stimulation. Furthermore, the NO donors also activated the classical extracellular signal-regulated kinase (ERK) p44-MAPK cascade as shown by phosphorylation of the specific substrate cytosolic phospholipase A2 in an immunocomplex kinase reaction. Both MAHMA-NO and spermine-NO cause a rapid activation of p44-MAPK after 10 min of stimulation. Interestingly, there is a second delayed peak of p44-MAPK activation after 4–24 h of stimulation with NO donors.

These results suggest that there is a differential activation pattern for stress-activated and mitogen-activated protein kinases by NO and that the integration of these signals may lead to specific cell responses.

Key words: nitric oxide, stress-activated protein kinase, p38-MAPK, mesangial cell, mitogen-activated protein kinase.

Introduction

In recent years, nitric oxide (NO), a gas previously considered to be a potentially toxic chemical, has become established as a diffusible universal messenger mediating cell–cell communication throughout the body. NO is a well-known mediator of blood vessel relaxation that helps to maintain blood pressure. In the central nervous system, NO acts as an unconventional type of neurotransmitter contributing to long-term potentiation. In addition, NO is responsible for some of the cytotoxic activity of macrophages that helps to kill microbes and tumour cells. Excessive and uncontrolled production of NO is associated with severe diseases such as septic shock, stroke, neurodegeneration, diabetes mellitus, arthritis and other forms of acute and chronic inflammation (Nathan, 1992; Knowles and Moncada, 1994; Krönke et al., 1995; Pfeilschifter, 1995). In mammals, the synthesis of NO is catalyzed by nitric oxide synthase, which exists in several isoforms. Nitric oxide synthase catalyzes the oxidation of the amino acid L-arginine to citrulline and NO.

Renal mesangial cells exposed to proinflammatory cytokines such as interleukin-1 or tumour necrosis factor-α express an inducible nitric oxide synthase (iNOS) and produce large amounts of NO (Pfeilschifter and Schwarzenbach, 1990; Pfeilschifter et al., 1992), which may contribute to certain forms of glomerulonephritis (Pfeilschifter, 1995; Cattell and Cook, 1995). Glomerular mesangial and endothelial cells not only are the sites of production of NO but are also themselves targets for NO and undergo apoptotic cell death upon exposure to high concentrations of NO (Pfeilschifter and Huwiler, 1996; Mühl et al., 1996). Apoptosis is a controlled biological strategy to remove unwanted cells from a given tissue and thus is involved in important physiological and pathophysiological processes (Cohen, 1993).

Recently, we and others have shown that NO triggers the
Materials and methods

**Chemicals**

[^32P-γ]ATP (specific activity >185 TBq mmol⁻¹) and Hyperfilm were purchased from Amersham International, Amersham, Bucks, UK; anti-rabbit alkaline-phosphatase-linked IgG was purchased from Bio-Rad, München, Germany; anti-p38-MAPK-specific antibodies were purchased from New England Biolabs, Schwalbach, Germany; synthetic peptides based on the C-terminal sequence of p44-MAPK (IFQETARFQPGAEP) and p42-MAPK (IFEETARFQPGYRS) were synthesized and coupled to keyhole limpet haemocyanin using glutaraldehyde and used to immunize rabbits. The detailed characterization of the antibodies is described elsewhere (Huwiler and Pfeilschifter, 1994a; Huwiler et al., 1995).

Protein A-sepharose 4B-CL was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; GST-ATF2 was produced and kindly provided by Novartis Pharma Inc., Basel, Switzerland; cytosolic phospholipase A₂ was kindly provided by Dr Ruth Kramer, Lilly Research Labs, Indianapolis, IN, USA. (Z)-1-{[N-methyl-L-[6-{(N-methylammonio-hexyl)-amino]}diazen-1-ium-1,2-diolate (MAHMA-NO) and (Z)-1-{[N-3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]-diazen-1-ium-1,2-diolate (spermine-NO) were purchased from Alexis Corporation, Läufelfingen, Switzerland; all cell culture nutrients were purchased from Gibco Life Technologies, Eggenstein, Germany.

**Cell culture**

Rat renal mesangial cells were cultured as described previously (Pfeilschifter, 1990a,b). Single cells were then cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells were grown in RPMI 1640 supplemented with 10 % (v/v) foetal calf serum, penicillin (100 units ml⁻¹), streptomycin (100 μg ml⁻¹) and bovine insulin (0.66 units ml⁻¹). Mesangial cells exhibited the typical stellate morphology. Moreover, these cells stained positively for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells, stained positively for Thy 1.1 antigen and stained negatively for factor-VIII-related antigen and cytokeratin, which excludes endothelial and epithelial contamination. The generation of inositol trisphosphate upon activation of the angiotensin II AT₁ receptor (Pfeilschifter, 1990c) was used as a functional criterion for characterizing the cloned cell line. For the experiments in this study, passages 8–15 of the cell line MZ B1 were used.

**p38- and p44-MAPK activity assay**

Confluent mesangial cells in 100 mm diameter dishes were incubated for 2 days in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1 mg ml⁻¹ fatty-acid-free bovine serum albumin (BSA) and then stimulated at 37 °C with the different NO donors. To stop the reaction, the medium was removed, and the cells were washed with ice-cold phosphate-buffered saline (PBS). The cells were then scraped directly into lysis buffer (50 mmol l⁻¹ Hepes, pH 7.4, 150 mmol l⁻¹ NaCl, 1.5 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ EDTA, 1 mmol l⁻¹ EGTA, 10 % glycerol, 1 % Triton X-100, 20 mmol l⁻¹ β-glycerophosphate, 50 mmol l⁻¹ sodium fluoride, 1 mmol l⁻¹ Na₃VO₄, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ aprotinin, 1 μmol l⁻¹ pepstatin A, 1 mmol l⁻¹ PMSF) and homogenized by ten passages through a 26 gauge needle fitted to a 1 ml syringe. The homogenate was centrifuged for 10 min at 14000 g, and the supernatant was retained for immunoprecipitation. Samples containing 500 μg of protein and 5 % foetal calf serum in a total volume of 100 μl were incubated with specific anti-phospho-p38-MAPK antibody (at a dilution of 1:100) or with a specific anti-p44-MAPK antibody (at a dilution of 1:1000) for 20 h at 4 °C. Then, 20 μl of a 50 % slurry of protein A-sepharose 4B-CL in PBS was added, and the mixture was incubated for 1 h on a rotation wheel. After centrifugation for 5 min at 3000 g, immunocomplexes were washed three times with a low-salt buffer (50 mmol l⁻¹ Tris-HCl, pH 7.4, 150 mmol l⁻¹ NaCl, 0.2 % Triton X-100, 2 mmol l⁻¹ EDTA, 1 mmol l⁻¹ EGTA, 0.1 % sodium dodecy sulphate, SDS) and three times with a high-salt buffer (50 mmol l⁻¹ Tris-HCl, pH 7.4, 500 mmol l⁻¹ NaCl, 0.2 % Triton X-100, 2 mmol l⁻¹ EDTA, 2 mmol l⁻¹ EGTA, 0.1 % SDS) and once with 20 mmol l⁻¹ Hepes, pH 7.4, 20 mmol l⁻¹ MgCl₂ before the kinase reaction was started by adding 30 μl of kinase buffer (for p38-MAPK: 25 mmol l⁻¹ Hepes, pH 7.4, 25 mmol l⁻¹ MgCl₂; 25 mmol l⁻¹ β-glycerophosphate, 0.1 mmol l⁻¹ Na₃VO₄, 2 mmol l⁻¹ diithiothreitol (DTT), 20 μmol l⁻¹ ATP, 74 kBq of [32P-γ]ATP, 1 μg of glutathione-S-transferase (GST)-ATF2 for 30 min at 37 °C; for p44-MAPK: 25 mmol l⁻¹ Tris-HCl, pH 7.5, 5 mmol l⁻¹ β-glycerophosphate, 2 mmol l⁻¹ DTT, 0.1 mmol l⁻¹ Na₃VO₄, 10 mmol l⁻¹ MgCl₂, 37 kBq of [32P-γ]ATP and 1 μg of recombinant cytosolic phospholipase A₂ (cPLA₂) for 15 min at 37 °C). SDS-Laemmli buffer was added to stop the reaction, and the samples were heated for 5 min and separated using SDS–PAGE. After fixing in 25 % (v/v) isopropanol, 10 % (v/v) acetic acid, the gels were dried and analyzed on a phosphoimager (Molecular Dynamics).

**Results**

Western blot analysis of mesangial cell lysates with a
Stimulation of p38-MAPK by nitric oxide

...polyclonal antiserum specific for p38-MAPK reveals a single band at 38–40 kDa, indicating that p38-MAPK is expressed in mesangial cells (Fig. 1). To measure p38-MAPK activity, we immunoprecipitated p38-MAPK from stimulated cell extracts and performed an in vitro kinase assay using GST-ATF2 as a substrate.

Since its discovery as a stress-activated protein kinase, it has generally been assumed that stress stimuli, such as high osmolarity, ultraviolet irradiation and cytokines, are potent activators of the p38-MAPK pathway (Cano and Mahadevan, 1995). As shown in Fig. 2, stimulation of mesangial cells with interleukin-1β indeed leads to a concentration-dependent activation of p38-MAPK consistent with the observation that stress stimuli are able to trigger p38-MAPK activation. Interestingly, the NO donor MAHMA-NO (at 2 mmol l⁻¹) causes a several-fold greater activation of p38-MAPK than does 10 nmol l⁻¹ interleukin-1β, a concentration of interleukin-1β sufficient to trigger maximal physiological responses in mesangial cells (Pfeilschifter et al., 1989; Pfeilschifter and Schwarzenbach, 1990), thus clearly suggesting that exogenously applied high concentrations of NO represent a severe stress for mesangial cells. Time course experiments reveal that MAHMA-NO, a NO donor with a very short half-life (t₁/₂=1 min), causes a very rapid and maximal increase in GST-ATF2 phosphorylation after 10 min of stimulation (Fig. 3). This activation of p38-MAPK returns to basal level after 60 min of stimulation.

Spermine-NO, a NO donor with a longer half-life (t₁/₂=40 min), induces a comparable rapid activation of p38-MAPK with a maximum at 20 min. However, the increased activation state of p38-MAPK was maintained for up to 8 h before control values were reattained after 24 h of stimulation (Fig. 4). Sustained activation of p38-MAPK may be important...
for the long-term functional responses of cells since it has been well documented for the other MAPK pathways (Meloche et al., 1992).

We then tested the effect of NO on the classical MAPK activity in mesangial cells. For this purpose, we immunoprecipitated p44-MAPK from stimulated cell extracts and performed in vitro kinase reactions using recombinant cytosolic phospholipase A₂ (cPLA₂) as a substrate. As shown in Fig. 5, both MAHMA-NO (Fig. 5A,C) and spermine-NO (Fig. 5B,C) cause a rapid activation of p44-MAPK after 10 min of stimulation. Interestingly, there is a second delayed peak of p44-MAPK activation occurring after 1–24 h of stimulation with both NO donors (Fig. 5). In contrast to p44-MAPK, no activation of p42-MAPK could be detected upon stimulation with NO, irrespective of whether cPLA₂ or myelin basic protein was used as a substrate (results not shown). This suggest a functional divergence of the MAPK pathway, with p42 and p44 having separate sets of activators but also separate effectors (Chuang and Ng, 1994).

Discussion

The mitogen-activated protein kinases are a family of enzymes that play an important role in converting extracellular signals to intracellular messengers that regulate metabolism, secretion, gene expression and cell growth (Cano and Mahadevan, 1995). In contrast to the classical MAPK or extracellular-signal-regulated protein kinase (ERK) module, which is primarily activated by mitogenic stimuli, the more recently discovered stress-activated protein kinases SAPK/JNK and p38-MAPK are preferentially activated by cellular stresses such as inflammatory cytokines (interleukin-1, tumour necrosis factor-α), heat shock, ultraviolet light, osmotic shock and metabolic poisons (Cano and Mahadevan, 1995; Woodgett et al., 1996).

Our present results demonstrate that high concentrations of exogenously applied NO represent a severe stress factor for mesangial cells and potently activate the stress kinase p38-MAPK within minutes. Moreover, there is a parallel activation of the classical ERK/MAPK module upon stimulation with NO, thus confirming previous observations (Callsen et al., 1998). We have also reported that the SAPK/JNK cascade is induced by NO in glomerular mesangial and endothelial cells (Pfeilschifter and Huwiler, 1996). Generally, activation of the classical MAPKs and the stress kinases SAPK/JNK and p38-MAPK is assumed to initiate opposing processes. On the one hand, they can trigger repair processes following cellular injury and thereby act in a reparative manner; on the other hand, they may initiate apoptosis to remove irreversibly damaged cells (Woodgett et al., 1996). An integration of signals will lead to cell-type-specific responses which depend critically on which individual components of the different signalling pathways are present in a particular cell.

NO is a multifunctional intra- and intercellular signalling molecule, which may exert cytoprotective as well as cytotoxic effects. However, the exact mechanism by which NO acts is...
still unclear. NO is thought to react with iron–sulphur centres and protein thiols in important cellular molecules (Kröncke et al., 1995; Stamler, 1994). Another important reaction partner of NO is superoxide, with the subsequent generation of peroxynitrite, which, in the presence of a metal catalyst (such as Fe3+), can be converted to the nitronium ion that readily nitrates tyrosine residues in proteins and thus may block critical phosphorylation reactions in signal transduction cascades (Beckman and Koppenol, 1996).

The number of newly discovered targets for NO is steadily increasing, and NO has been shown to affect crucial intracellular signalling pathways. It has been found to activate MAPK cascades, including the SAPKs, in Jurkat T cells (Lander et al., 1996), glomerular mesangial and endothelial cells (Pfeilschifter and Huwiler, 1996; Callsen et al., 1998), human embryonic kidney cells (HEK293) (Kim et al., 1997) and chondrocytes (Lo et al., 1996).

The direct mechanism by which NO activates the p38-MAPK cascade described in the present study has still to be elucidated. However, it is tempting to speculate that NO will directly affect a kinase or an opposing phosphatase by nitrosylation, thereby leading to activation/inactivation and a subsequent increased phosphorylation and activation state of the p38-MAPK. Possible and intriguing candidates are members of the MAPK phosphatase (MKP) family, which have been shown to critically regulate the classical MAPKs as well as SAPKs and p38-MAPK (Keyse, 1995; Groom et al., 1996). Caselli et al. (1994) reported that NO inactivated a low-molecular mass phosphorytrosine protein phosphatase from bovine liver in vitro by S-nitrosylation of two vicinal cysteine residues in the active site of the enzyme. Alternatively, NO may directly modulate an upstream kinase of the p38-MAPK pathway and cause activation of the enzyme and of the downstream cascade.

It is of special interest to elucidate whether the induction of iNOS and the production of endogenous NO in mesangial cells can also lead to activation of the above kinases. In this context, it is worth mentioning that interleukin 1β, one of the most potent inducers of iNOS expression in mesangial cells (Pfeilschifter and Schwarzenbach, 1990; Pfeilschifter et al., 1992), causes a biphasic induction of the classical ERK/MAPK cascade in mesangial cells (Huwiler and Pfeilschifter, 1996), causing a biphasic induction of the classical ERK/MAPK cascade in mesangial cells (Huwiler and Pfeilschifter, 1994b). The late and persistent ERK activation triggered by interleukin 1 was indeed attenuated by the NOS inhibitor nitroarginine methyl ester (Callsen et al., 1998), suggesting that the amounts of NO generated under pathophysiological conditions are sufficient to activate the ERK/MAPK cascade. In contrast, interleukin 1 causes a rapid and pronounced activation of the p38-MAPK cascade but does not evoke a late stimulation of this kinase cascade (A. Huwiler and J. Pfeilschifter, unpublished observations), indicating that counter-regulatory mechanisms may be active to suppress a delayed NO-mediated activation of p38-MAPK. Whether other inflammatory mediators can bypass this inhibition remains to be investigated.

The identification of the direct targets of NO and the consecutive changes in signal outflow triggered by NO will offer exciting new insights and eventually offer new approaches for therapeutic intervention in acute and chronic inflammatory diseases and tumour development.

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References


