Nitric oxide stimulates stress-activated protein kinases in glomerular endothelial and mesangial cells

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Abstract Exposure of rat glomerular mesangial cells and primary cultures of bovine glomerular endothelial cells to compounds releasing nitric oxide (NO), including MAHMA-NONOate, *S*-nitrosoglutathione, and spermine-NO, results in a time- and concentration-dependent activation of stress-activated protein kinases (SAPK) as measured by the phosphorylation of c-Jun in a solid phase kinase assay. Dibutyryl cGMP had no effect on SAPK activity. Pretreatment of the cells with the tyrosine kinase inhibitor genistein strongly attenuated NO-induced c-Jun phosphorylation. Furthermore, *N*-acetylcysteine markedly reduced the activation of SAPK in response to NO. These studies identify SAPK as a target for NO which may be critical for the NO-induced apoptosis of glomerular mesangial and endothelial cells.

Key words: Nitric oxide; Stress-activated protein kinase; Mesangial cell; Endothelial cell; Apoptosis

1. Introduction

Nitric oxide (NO) is a short-lived, free-radical gas synthesized by NO synthase and mediates cell-cell communication throughout the body. Produced in appropriate amounts by endothelial cells, neurons or macrophages, it contributes to blood pressure regulation, neuronal communication and immune defence. On the other hand, excessive and uncontrolled production of NO is associated with severe diseases like septic shock, stroke, neurodegeneration, diabetes mellitus, arthritis and other forms of acute and chronic inflammation [1–3]. Mesangial cells orchestrate inflammatory processes in the renal glomerulus by increased mediator production, increased matrix production, and increased mesangial cell proliferation [4,5]. An important and highly versatile member of this orchestra of inflammatory mediators produced by mesangial cells is NO [5,6]. Mesangial cells exposed to inflammatory cytokines like interleukin 1β or tumour necrosis factor α express the inducible NO synthase and produce high concentrations of NO [7–9] which may contribute to certain forms of glomerulonephritis [10,11]. We have recently reported that glomerular mesangial cells and endothelial cells are not only production sites of but are also themselves targets for NO and undergo apoptotic cell death upon exposure to high concentrations of NO. NO generated from different NO-releasing compounds as well as NO-saturated solution induced apoptosis in both cell types as demonstrated by intranucleosomal DNA fragmentation, enrichment of cytosolic DNA/histone complexes, an increasing number of fragmented DNA ends and typical nuclear chromatin condensation [12]. Apoptosis is a controlled biological strategy to remove unwanted cells from a given tissue and thus is involved in physiological and pathophysiological processes [13,14]. Recently, it has been reported that a cascade of the mitogen-activated protein kinase family, the stress-activated protein kinase (SAPK) module, is required for induction of apoptosis [15,16]. We therefore were interested to investigate whether NO is able to trigger this signalling pathway in glomerular mesangial cells and endothelial cells.

2. Materials and methods

2.1. Cell culture

Renal glomeruli from male Sprague-Dawley rats or from female Wistar rats (80–100 g body wt) were isolated under sterile conditions by a sieving technique, and glomerular cells were cultured as described previously [17]. In a second step, single cells were cloned by limited dilution in 96-microwell plates to obtain pure cultures of epithelial cells and mesangial cells [17]. Cells were identified as mesangial cells by (a) their typical stellate morphology; (b) positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells; (c) positive staining for Thy 1.1 antigen; (d) negative staining for factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contamination, respectively; and (e) generation of inositol trisphosphate upon activation of angiotensin II AT1 receptors was used as a functional criterion for characterizing the cloned cell line. For the experiments passages 8–15 of mesangial cells were used. Calf kidneys were obtained from a local slaughterhouse and were used to obtain glomerular endothelial cell cultures by a method previously described by Ballermann [18] and modified by Briner and Kern [19]. Individual clones of endothelial cells were characterized by positive staining for factor VIII-related antigen and uniform uptake of fluorescent acetylated low-density lipoproteins. Negative staining for smooth muscle actin and cytokeratin excluded mesangial cell and epithelial cell contaminations. Cells were utilized at passages 5–11.

2.2. c-Jun kinase assay

Quiescent mesangial cells in 60-mm diameter dishes were stimulated as indicated. To stop the reaction, the cells were washed with ice-cold PBS and scraped into 0.5 ml of TLB buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 25 mM β-glycerophosphate). Cells were then homogenized by passes through a 26 G needle, centrifuged for 5 min at 14000×*g* at 4°C and 200 μg of protein from the supernatant was taken for the Jun kinase assay. The solid-phase Jun kinase assay was performed as described [20] using a glutathione *S*-transferase (GST)-c-Jun (5–89) fusion protein coupled to glutathione-Sepharose beads as substrate. In brief, 10 μg of GST-c-Jun was coupled to 10 μl of glutathione-Sepharose in 0.5 ml of TLB buffer for 30 min at 4°C. The beads were then centrifuged for 20 s at 14000×*g*, washed twice with TLB buffer and incubated for 2 h at 4°C with cell extracts in TLB buffer containing 200 μg of protein. Thereafter, the complexes were washed twice with TLB buffer and once with 20 mM HEPES, pH 7.4, 20 mM $MgCl_2$ before the kinase reaction was started by addition of 30 μl of kinase buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 20 mM *p*-nitrophenyl-

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phosphate, 10 μM ATP and 2 μCi [γ-^{32}P]ATP) to the complexes and incubated for 20 min at 30°C. To stop the reaction, 30 μl of 2×Laemmli sample buffer was added and the samples were heated for 5 min at 95°C. Proteins were separated by SDS-PAGE (13% acrylamide gel) and phosphorylated GST-c-Jun was detected by autoradiography and quantitated by a phosphorimager (Molecular Dynamics).

2.3. Chemicals

(*Z*)-1-{*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)amino)}diazen-1-ium 1,2-diolate (MAHMA-NONOate), *S*-nitrosoglutathione (GSNO) and (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl] amino}diazen-1-ium 1,2-diolate (spermine-NO) were from Alexis Corp., Läufelfingen, Switzerland; [γ-^{32}P]ATP (spec. act. >5000 Ci/mmol), anti-rabbit horseradish peroxidase-linked IgG and hyperfilm were from Amersham; glutathione-Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden; GST-c-Jun (5–89) was produced by Ciba-Geigy Ltd., Basel, Switzerland; all cell culture nutrients were from Gibco-BRL, Basel, Switzerland; all other chemicals were from Fluka, Buchs, Switzerland.

3. Results

Western blot analysis of mesangial cell lysates with polyclonal antisera specific for SAPK isoforms demonstrated that SAPKα and -γ, but not SAPK-β, are expressed in mesangial cells (Huwiler et al., manuscript submitted). We have used a solid-phase kinase assay with c-Jun (5–89) coupled to glutathione *S*-transferase as a substrate that binds all SAPK isoforms and after precipitation with glutathione-Sepharose the N-terminal domain of c-Jun is phosphorylated by activated

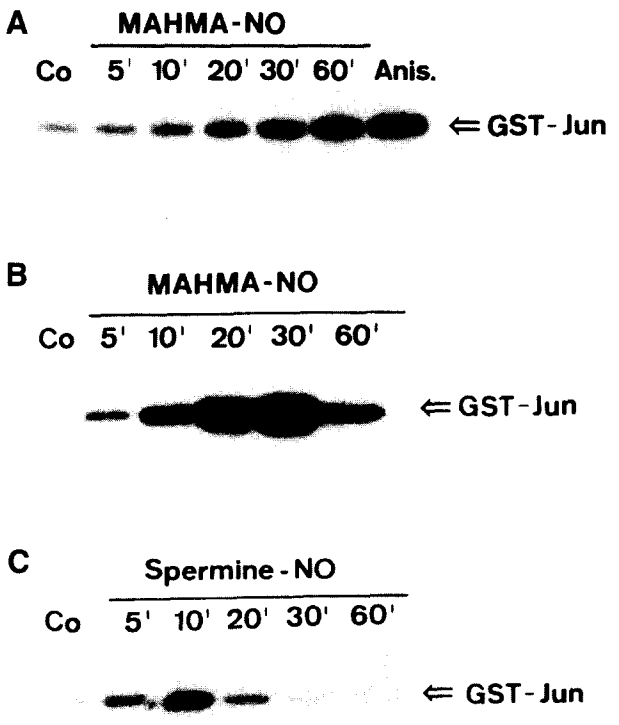

Fig. 1. Time course of MAHMA-NONOate- and spermine-NO-stimulated SAPK activity in glomerular endothelial cells (A) and mesangial cells (B,C). Quiescent endothelial cells (A) or mesangial cells (B,C) were treated for the indicated time periods with vehicle (Co), MAHMA-NONOate (1 mM) or spermine-NO (1 mM), as indicated. Thereafter, cells were harvested and SAPK activity was measured as described in Section 2. Similar results were obtained in three independent experiments.

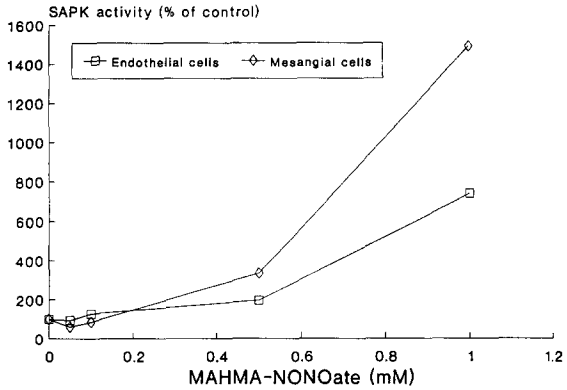

Fig. 2. Concentration dependency of MAHMA-NONOate-induced SAPK activity in glomerular endothelial cells and mesangial cells. Quiescent endothelial or mesangial cells were treated with the indicated concentrations of MAHMA-NONOate for 60 or 30 min, respectively. Cells were harvested and 200 μg of cell protein was taken for SAPK activity measurement as described in Section 2. Results are means of three independent experiments; the S.D. ranges from 12 to 80%.

SAPKs [20]. The data in Fig. 1A show that MAHMA-NONOate augments SAPK activity in glomerular endothelial cells within 10–20 min and further increases SAPK activity, with a maximal 10-fold stimulation at 60 min, the latest time point examined. Whereas the response to MAHMA-NONOate is sustained over at least 60 min in endothelial cells the response of mesangial cells is more transient as shown in Fig. 1B. Maximal SAPK activity in mesangial cells is observed 30 min after addition of MAHMA-NONOate and then declines again (Fig. 1B). MAHMA-NONOate is an NO donor that releases NO with a half-life of approx. 1–2 min under neutral pH conditions at 37°C [21]. This massive release of NO activates SAPK activity with a potency comparable to that of anisomycin (50 ng/ml) (Fig. 1A), a classical stress-activator of the SAPK pathway [22]. Spermine-NO is another NONOate that displays a much slower release of NO with a half-life of approx. 10–90 min [21]. When compared to MAHMA-NONOate, exposure of mesangial cells to spermine-NO results in weak stimulation of SAPK-activity which is maximal after 10 min and back to control levels after 30 min (Fig. 1B). Furthermore, a third NO donor, *S*-nitrosoglutathione (GSNO), induced c-Jun phosphorylation in glomerular endothelial and mesangial cells (data not shown) thus confirming that NO is able to the activate SAPK pathway in both cell types. Fig. 2 shows the concentration dependency of SAPK activation by MAHMA-NONOate in glomerular endothelial and mesangial cells. The addition of dibutyryl cGMP (0.5 mM) to the culture medium did not induce any SAPK activation (data not shown), thus excluding cGMP as a possible mediator of NO-induced SAPK stimulation.

In order to study the possible mechanisms controlling the activation of SAPKs by NO in mesangial and endothelial cells, we used a specific inhibitor of protein tyrosine kinases, genistein, which is an isoflavone compound from fermentation broth of *Pseudomonas* spp. [23]. As shown in Fig. 3, genistein markedly reduces MAHMA-NONOate-induced SAPK activation in endothelial and mesangial cells, thus suggesting that tyrosine phosphorylation is likely associated with the activation of the SAPK pathway by NO. In a subsequent step, we evaluated the effect of the free radical scavenger

and antioxidant *N*-acetylcysteine on NO-stimulated c-Jun phosphorylation. *N*-acetylcysteine has been reported to block SAPK and nuclear factor κB activation in response to UV light and other cellular stresses [24,25]. Coincubation of mesangial or endothelial cells with MAHMA-NONOate and *N*-acetylcysteine results in a strong inhibition of c-Jun phosphorylation (Fig. 3). *N*-acetylcysteine itself had no significant effect on SAPK activity (data not shown). These data emphasize the role of redox regulation in SAPK activation by NO.

It has recently been proposed that stimulation of the SAPK pathway contributes to cell death [15,16]. As shown in Fig. 4 spermine-NO and MAHMA-NONOate cause a marked increase in glomerular endothelial cell and mesangial cell apoptosis as measured by an ELISA based on the detection of cytosolic histone/DNA complexes. Coincubation with *N*-acetylcysteine and genistein inhibited NO-induced apoptosis in both cell types (Fig. 4).

4. Discussion

The function of the mitogen-activated protein kinase cascade, also known as the extracellular signal-regulated protein kinase pathway, is to convert extracellular signals to intracellular mediators that regulate metabolism, secretion, gene expression and cell growth [26].

Whereas the extracellular signal-regulated protein kinase module responds primarily to mitogenic agonists, two more recently characterized kinase cascades are responsive to cellular stresses such as interleukin 1, tumour necrosis factor α, heat shock, UV light, osmotic shock and metabolic poisons. These novel kinases were termed stress-activated protein kinase (SAPK, also termed c-Jun N -terminal kinase) and p38 kinase [22,26]. Activation of SAPKs and p38 kinase is a double-edged sword in that these kinases may trigger repair processes following cellular injury thus acting in a reparative manner or, alternatively, they may initiate the programm of cell death to remove unwanted or irreversibly damaged cells [22]. Recently it has been suggested that a dynamic equilibrium between the extracellular signal-regulated protein kinase pathway and the SAPK/p38 kinase pathway determines whether cells survive or undergo programmed cell death [15]. A shift in this dynamic balance by either activation of SAPK/p38 kinase activity or inhibition of the extracellular signal regulated protein kinase pathway may cause an increased rate of cell death. NO is also a double-edged sword. Synthesized in appropriate amounts it exerts important physiological functions. However, when produced in excessive amounts by the inducible NO synthase, it may contribute to the pathogenesis of a number of severe diseases, including circulatory shock and inflammation [11,27]. The exact mechanisms by which NO exerts its damaging effects are not yet fully understood. Inhibition of iron-sulfur proteins like aconitase, complexes I and II of the mitochondria respiratory chain, and ribonucleotide reductase or NAD(H)-dependent covalent modification of glyceraldehyde-3-phosphate dehydrogenase [28,29] and DNA damage as well as strand breaks [30–32] may contribute to the cytotoxic properties of NO. NO has recently been reported to initiate programmed cell death in a variety of cell types [33–40] including glomerular mesangial, endothelial and epithelial cells [12]. We now report that NO activates the SAPK pathway in glomerular mesangial and endothelial cells thus providing a link to the subsequent initiation of apoptosis in these cells (Fig. 4 and [12]). Our observations confirm and extend the very recent report of Lo et al. [41] who showed that reactive oxygen species mediate cytokine activation of SAPK in bovine chondrocytes. The exact mechanism by which NO causes SAPK activation has yet to be elucidated. Whether NO directly activates one of the at least 10 isoforms of SAPK known today [42] or interacts with the upstream regulators like SAPK kinase, p21-activated kinase (PAK) or the Ras-related small GTP-binding proteins (cdc 42, Rac 1) [43] are some pressing questions that immediately arise. Identification of direct targets of NO and signalling pathway triggered by NO will offer new approaches for therapeutic intervention in acute and chronic inflammatory diseases and tumour development.

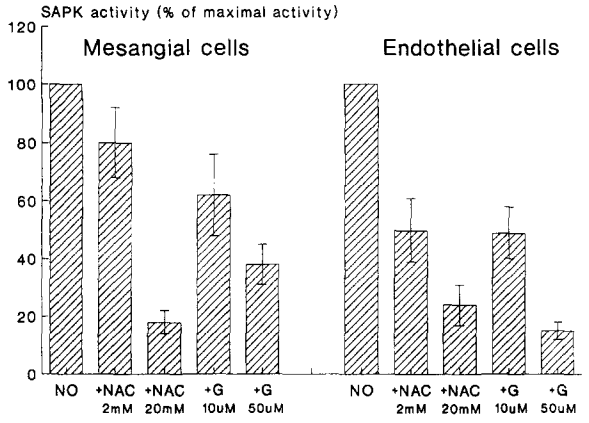

Fig. 3. Effects of *N*-acetylcysteine and genistein on SAPK activity in glomerular endothelial and mesangial cells. Quiescent endothelial or mesangial cells were treated with MAHMA-NONOate (1 mM, NO) in the absence or presence of the indicated concentrations of *N*-acetylcysteine (NAC) or genistein (G) for 60 min (endothelial cells) or 30 min (mesangial cells). Thereafter, cells were harvested and SAPK activity was measured as described in Section 2. Results are means ± S.D. of three independent 3 experiments.

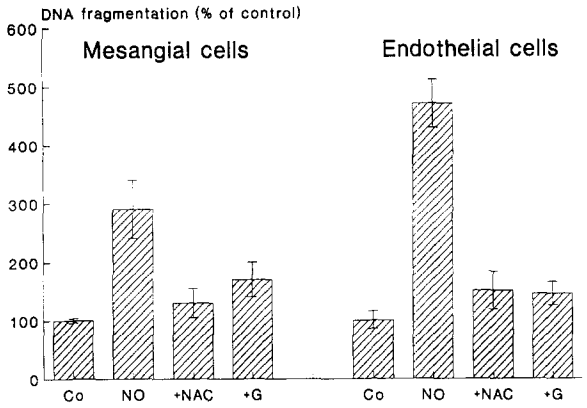

Fig. 4. MAHMA-NONOate induced apoptosis in gomerular endothelial and mesangial cells. Mesangial cells or endothelial cells were incubated with vehicle (Co) or MAHMA-NONOate (NO, 1 mM) in the absence or presence of *N*-acetylcyteine (NAC, 20 mM) or genistein (G, 50 μM) for 24 h. Cytosolic oligonucleosome-bound DNA was quantitated by an ELISA kit, DNA fragmentation is indicated as % of untreated control cells. Data are means ± S.D. of three independent experiments.

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