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Rapid and Delayed p42/p44 Mitogen-Activated Protein Kinase Activation by Nitric Oxide: The Role of Cyclic GMP and Tyrosine Phosphatase Inhibition

Dagmar Callsen,* Josef Pfeilschifter, † and Bernhard Brüne2*"  

The exposure of rat mesangial cells to cytokines promoted activation of the p42/p44 mitogen-activated protein kinase (MAPK). We identified a rapid and delayed phase of MAPK activation with distinctive activity increases at 5 to 15 min and 15 to 24 h. Rapid and late MAPK activation were attenuated by the redox-modulating agent N-acetylcyesteine. Specifically, late-phase activation coincided with endogenous nitric oxide (NO) generation and in turn was suppressed by the NO synthase-blocking compounds dihydrodiodionium or nitroarginine methyl ester. By using NO-liberating agents such as S-nitrosoglutathione and 3-morpholino-sydnonimine, we investigated intermediary signaling elements of NO in promoting MAPK activation. Early and transient activation at 5 min was suppressed by the soluble guanylyl cyclase-blocking agent "H-(1,2,4)-oxdiazolo-(4,3-α)-6-bromoquinazazin-1-one (NS 2028) and, moreover, was mimicked by the lipophilic cyclic GMP (cGMP) analogue 8-bromo-cGMP. In contrast, NO-mediated activation achieved within hours was unrelated to cGMP signaling. Late and persistent MAPK activation, induced by NO donors or endogenously generated NO, was found in association with inhibition of phosphatase activity. In vitro dephosphorylation of activated and immunoprecipitated p42/p44 by cytosolic phosphatases was sensitive to the readdition of NO and was found to be inhibited in cytosol of S-nitrosoglutathione-stimulated cells. Also, cells that had been exposed to cytokines for 24 h revealed a blocked phosphatase activity, which was successfully attenuated by the NO synthase inhibitor nitroarginine methyl ester and, therefore, was NO mediated. Conclusively, NO affects p42/p44 MAPK in rat mesangial cells twofold: rapid activation is cGMP mediated, whereas late activation is transmitted via inhibition of tyrosine dephosphorylation. The Journal of Immunology, 1998, 161: 4852–4858.

Mesangial cells (MC) are specialized smooth muscle cells located in the glomeruli of the kidney (1). MC take part in the pathogenesis of severe inflammatory conditions, i.e., glomerulonephritis (2, 3). Cellular proliferation of MC is thought to be pivotal for the development of crescent formation and progression of the proliferative glomerulonephritis (4). Following cell activation, in part as a result of cytokine release by infiltrating macrophages, MC respond with proinflammatory mediator secretion and inducible nitric oxide synthase (iNOS) up-regulation (5, 6). Nitric oxide (NO) is recognized for its participation in diverse biologic processes, ranging from physiologic to pathophysiologic/pathologic biologic processes, including inflammatory and proliferative/antiproliferative responses (2, 3, 7). The role of NO in promoting cell proliferation is controversial. NO was characterized as an antiproliferative agent in some systems (8, 9), but emerged as an upstream signal of cell proliferation in endothelial cells, keratinocytes, and fibroblasts (10–12).

Cell proliferation during glomerulonephritis may be augmented by endogenously produced NO by activating p42/p44 mitogen-activated protein kinases (MAPK), which are established proliferative signal components (4, 13). p42 MAPK (also known as extracellular-regulated kinase-2 or ERK-2) and the p44 MAPK (ERK-1) are activated as a result of a cascade of different upstream kinases (14, 15). Mitogen-activated serine/threonine kinases are unusual in requiring phosphorylation on both tyrosine and threonine residues within the signature sequence T-E-Y (threonine-183 and tyrosine-185) (16). Phosphorylation of the T-E-Y motif in MAPK is catalyzed by a dual-specificity kinase termed MAPK kinase (MAPKK, also known as MEK). MEK is itself activated by phosphorylation on either of two serine residues by a MEK kinase (MEKK) (17, 18).

Activation of MAPK is transient and highly regulated by the antagonizing or cooperating action of upstream kinases and phosphatases (19, 20). MAPK phosphatases are classified into two subfamilies according to their substrate specificity. There are serine/threonine phosphatases and tyrosine phosphatases, which are further subgrouped into V_{11}-like dual-specificity phosphatases and tyrosine-specific phosphatases (20, 21). Dephosphorylation of p42/p44 MAPK in vitro and in vivo is achieved by the cytosolic serine/threonine phosphatase PP2A, various inducible dual-specific phosphatases such as MKP-1/2 or PAC, and thus far uncharacterized tyrosine phosphatases (21–24). Common to all dual-specific and tyrosine-specific phosphatases is an active site motif consisting of a cysteine and an arginine separated by five residues (commonly known as the CxR motif) (25). Mutation of the catalytically active cysteine abrogated all phosphatase activity, which
can be rationalized by the requirement of a phosphoestern intermediate during catalysis (26, 27).

With the notion that histidine-activated thiols are preferentially targeted by NO redox species (28), we proposed that tyrosine phosphatases, which are involved in the regulation of the p42/p44 MAPK cascade, are NO sensitive. Besides up-regulation of cytokine-inducible iNOS, which generates NO by converting l-arginine to NO and citrulline (29, 30), a structurally diverse class of NO-releasing compounds such as S-nitrosothioglutathione (GSNO) or 3-morpholinosydnonimine (SIN-1) (31) was used to study NO-signaling pathways in rat MC.

In the present study, we demonstrate that the inflammatory mediator NO provoked rapid and delayed p42/p44 MAPK activation. Whereas rapid activation was cyclic GMP (cGMP)-mediated, delayed activation resulted from NO-evoked inhibition of tyrosine phosphatases.

Materials and Methods

Materials

RPM 1640 and medium supplements were ordered from Biochrom (Berlin, Germany). FCS was purchased from Life Technologies (Karlruhe, Germany). Insulin, β-glycerophosphate, 1,1,2-trichlorotrifluoroethane, tri-N- octylamine, trichloroacetic acid, sodium pyrophosphate, sulfanilamide, myelin basic protein (MBP), protein A-Sepharose, N-naphthylethenedia mine, reduced glutathione, and LPS came from Sigma (Deisenhofen, Germany). IL-1β, IFN-γ, sodium vanadate, and sodium fluoride were bought from Boehringer Mannheim (Mannheim, Germany). PMSF was ordered from Fluka (Deisenhofen, Germany). Enhanced chemiluminescence detection reagents and phosphospecific p44/p42 MAPK Ab was graciously supplied by Prof. Dr. R. Busse (University of Frankfurt, Frankfurt, Germany). All other chemicals were of the highest purity and commercially available.

MC culture

Rat MC were cultured, cloned, and characterized as described previously (32). Cells were grown in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and bovine insulin (5 μg/ml). One day before and during the experiments, cells were kept in medium without FCS. For experiments, passages 10 to 25 of MC were used.

GSNO synthesis

GSNO was synthesized and characterized as described previously (33).

Nitrite determination

Nitrite, a stable NO oxidation product, was determined by the Griess reaction. Cell-free culture supernatants were collected (200 μl) and mixed with 20 μl of sulfanilamide (dissolved in 1.2 M HCl)/20 μl of N- naphthylethenediamine dihydrochloride. After 5 min at room temperature, the absorbance was measured at 560 nm with a reference wavelength at 690 nm. Nitrite concentrations were calculated using a NaNO2 standard. For details, see Ref. 34.

Western blot analysis

Confluent and quiescent cells were incubated for the times indicated. After removing the medium, cells were washed once with ice-cold PBS (supplemented with 1 mM sodium vanadate) and rinsed into 400 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% NP-40, 1 mM PMSF, 1 mM sodium fluoride, and 1 mM sodium pyrophosphate) and with 1 mM PMSF), sonicated, and centrifuged (10,000 × g, 10 min, 4°C). The supernatant was neutralized with 750 μl of 0.5 M Tris-HCl, pH 8.0). Cell debris was sonicated with a Branson sonifier (10 s, duty cycle 100%, output control 1), centrifuged (14,000 × g, 15 min), and measured for protein content. For equal protein loading, 150 μg of protein was mixed with the same volume of 2× SDS-sample buffer (125 mM Tris-HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose sheets. The m.w. of corresponding proteins was determined in relation to m.w. markers. Transblots were washed twice with TBS (140 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing 0.06% Tween 20 before blocking nonspecific binding with TBS/5% skim milk. Phosphospecific p42/p44 MAPK Abs, dissolved in PBS, diluted 1:1,000 in TBS/5% milk, were incubated overnight at 4°C. Blots were washed five times before the anti-rabbit peroxidase-labeled secondary polyclonal Ab was added (1 h, diluted 1:10,000 in TBS/0.5% milk). Blots were washed five times and followed by enhanced chemiluminescence detection. Afterwards, blots were stripped (62.5 mM Tris, 2% SDS, 10% glycerol, 0.3 M mercaptoethanol, 100 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium pyrophosphate, and 1 mM sodium vanadate). Cells were lysed for 5 min on ice, vortexed, and centrifuged (10,000 × g, 20 min, 4°C). Protein content of the supernatant was measured and 1 mg of protein in an equalized sample volume was used for immunoprecipitation. Anti-p42/p44 MAPK Ab (35) were added to the lysate, followed by gentle rotation at 4°C. After 2 h, protein A-Sepharose was added, and the immunocomplexes were incubated for another 60 min. p42/p44 MAPK/protein A-Sepharose complexes were washed twice with lysis buffer and once with kinase buffer (25 mM HEPEs, pH 7.4, 25 mM β-glycerophosphate, 25 mM sodium pyrophosphate, and 1 mM sodium vanadate). Cells were lysed for 5 min on ice, vortexed, and centrifuged (10,000 × g, 20 min, 4°C). Protein content of the supernatant was measured and 1 mg of protein in an equalized sample volume was used for immunoprecipitation. Anti-p42/p44 MAPK Ab (35) were added to the lysate, followed by gentle rotation at 4°C. After 2 h, protein A-Sepharose was added, and the immunocomplexes were washed twice with 10 mM β-glycerophosphate and 5 μCi [γ-32P]ATP. Phosphorylation was performed for 20 min at 37°C. The reaction was stopped by adding 25 μl 2× SDS-sample buffer. Samples were heated for 5 min at 95°C and separated on 15% SDS-polyacrylamide gels. Gels were fixed, dried, and subjected to autoradiography.

Dephosphorylation assay

Dishes (10 cm) of confluent, quiescent cells were incubated with 5% FCS for 5 min. After removal of the medium, cells were washed with ice-cold PBS (supplemented with 1 mM sodium vanadate and 1 mM sodium fluoride, and 1 mM PMSF), scraped into lysis buffer (as outlined for the MAPK assay), kept for 10 min on ice, vortexed, and centrifuged (10,000 × g, 20 min, 4°C). The supernatant was used for immunoprecipitation of p42/p44 MAPK. Pelleted p42/p44 MAPK immunocomplexes were washed twice with lysis buffer and twice with hypotonic buffer (20 mM Tris, 10 mM NaCl, 1 mM PMSF, pH 7.5). Immunocomplexes were centrifuged (10,000 × g, 2 min, 4°C) and resuspended in hypotonic buffer. For individual dephosphorylation experiments, the resuspended p42/p44 MAPK immunocomplexes (immunoprecipitate (IP)) were divided into equal aliquots, which then were incubated with 500 μg of cytosolic cell extracts of untreated cells (controls) with or without the further addition of 1 mM GSNO and/or 10 μM PD 98059 during an incubation period of 30 min at 37°C. Alternatively, equal aliquots of IP were incubated with 500 μg of cytosolic protein derived from untreated, GSNO (1 mM, 8 h), or cytokine mix (CM) (24-h exposure period with 25 U/ml IL-1β, 100 U/ml IFN-γ, 1 ng/ml TNF-α, and 25 ng/ml LPS, in the presence or absence of 1 mM NaF)-exposed cells. For the preparation of cytosol, cells were scraped into ice-cold PBS (supplemented with 1 mM PMSF), centrifuged (10,000 × g, 10 min, 4°C), resuspended in hypotonic buffer (supplemented with 1 mM PMSF), sonicated, and centrifuged (10,000 × g, 15 min, 4°C). Dephosphorylation was terminated by centrifugation (1000 × g, 5 min, 4°C). IP were washed once with hypotonic buffer (supplemented with 5 mM sodium vanadate, 2 mM sodium fluoride, and 1 mM PMSF) and with kinase buffer. MAPK activation was determined as described for the MAPK assay.

Determination of cGMP

Confluent, quiescent cells were incubated for the times indicated. After removal of the medium, cells were lysed by the addition of 1 ml ice-cold 10% TCA. Lysates were kept for 10 min on 4°C and centrifuged (10,000 × g, 15 min, 4°C). The supernatant was neutralized with 750 μl of a 1:1 (v/v) mixture of tri-N-octylamine and 1,2,3-trichlorotrifluoroethane while vortexing. Centrifugation for 1 min at 10,000 × g generated three separate
measuring nitrite accumulation in the cell supernatant. Nitrite, a cytokine is known and was corroborated in these experiments by iNOS activation in rat MC. Up-regulation of iNOS in response to remained constant (Fig. 1). Further, the second but not the first phase (data not shown for the first phase) of MAPK activation was compromised by NAME treatment. Conclusively, delayed activation of MAPK in MC following CM addition coincided in time with endogenous NO formation and was compromised by NO synthase inhibitors. The redox-modulating agent NAC reduced rapid as well as delayed MAPK activation. Albeit modulation of MAPK activity, the amount of MAPK remained invariable (Fig. 2).

Results

Rapid and delayed p42/p44 MAPK activation by cytokine stimulation

Incubating rat MC with CM (25 U/ml IL-1β, 100 U/ml IFN-γ, 10 ng/ml TNF-α, and 25 ng/ml LPS) resulted in p42/p44 MAPK activation as determined by Western blot analysis. MAPK activation was detected by visualizing the tyrosine phosphorylated form of p42/p44 MAPK (Fig. 1A). The phosphospecific Ab mainly detected p42 and to a lesser extent p44. Cytokines elicited a biphasic response, showing rapid and delayed kinase activation. Immediate MAPK phosphorylation occurred within 5 to 15 min and vanished at 30 to 60 min following CM addition. Delayed kinase activation was observed after 15 to 24 h. Active MAPK was absent in controls and kinase activation was blocked by the MEK-1-specific inhibitor PD 98059 (data not shown) (37).

To rule out variations in the amount of MAPK to account for activity alterations, we probed for the protein amount of p42/p44 following CM addition. Within the time frame of the experiments and especially at longer exposure periods, the amount of MAPK remained constant (Fig. 1A). Further, we correlated MAPK and iNOS activation in rat MC. Up-regulation of iNOS in response to cytokines is known and was corroborated in these experiments by measuring nitrite accumulation in the cell supernatant. Nitrite, a stable NO oxidation product, significantly accumulated after 15 to 24 h following CM addition (Fig. 1, A and B). In extending experiments, we reestablished the concurrence between MAPK phosphorylation and its kinase activity. Activity determination is based on phosphate incorporation from radiolabeled [γ-32P]ATP into MBP. MC were time-dependently exposed to the CM followed by radioactive activity determination. A first and transient activation appeared 5 to 15 min after CM application. A second activation phase was noticed after 24 h in accordance with iNOS up-regulation as measured by nitrite accumulation in the cell supernatant (Fig. 1B). As outlined in the manufacturer description, we observed MBP to be separated on SDS-gels into two distinct m.w. fractions.

Delayed MAPK activation, seen at 24 h after CM exposure, was attenuated by the addition of 10 μM diphenyliodonium (DPI), an inhibitor of O2·−-producing NAD(P)H-like oxidases (38), or by including N-acetylcysteine (NAC) for the last 30 min of CM exposure (Fig. 2).

Further, the second but not the first phase (data not shown for the first phase) of MAPK activation was compromised by NAME, a specific NO synthase inhibitor. As expected, nitrite formation was additionally attenuated by NAME treatment. Conclusively, delayed activation of MAPK in MC following CM addition coincided in time with endogenous NO formation and was compromised by NO synthase inhibitors. The redox-modulating agent NAC reduced rapid as well as delayed MAPK activation. Albeit modulation of MAPK activity, the amount of MAPK remained invariable (Fig. 2).

Rapid and delayed activation of p42/p44 MAPK by NO donors

NO-releasing compounds such as GSNO or SIN-1 were employed to study MAPK activation in rat MC. GSNO elicited a biphasic MAPK activation (Fig. 3A). Following GSNO addition, we observed a rapid and transient MAPK phosphorylation, with a maximum at 5 min, that was suppressed by the addition of the specific inhibitor PD 98059 (data not shown). After 5 min, phosphorylation disappeared to basal values and finally reappeared after 4 h. Following that time point, MAPK stayed in its activated, i.e., phosphorylated, form for up to 15 h.

Direct kinase activity of p42/p44 MAPK as determined by MBP phosphorylation delivered identical results in response to GSNO (Fig. 3A) and assured the interchange of data obtained with either
of the assay systems. Further controls revealed that NAME left GSNO-mediated MAPK activation unaltered (data not shown).

To establish MAPK activation by NO donors irrespective of the chemical structure of NO-delivering agents, we tested kinase activation by SIN-1 (Fig. 3B). SIN-1 shared with GSNO the ability to evoke a biphasic MAPK activity modulation. Rapid activation was established between 5 to 15 min, whereas delayed activation occurred between 4 and 24 h. Intermediate time points revealed no kinase activity. For control reasons, we again probed for variations in the amount of MAPK during NO exposure (Fig. 3B). Evidently, neither rapid nor delayed MAPK activation was associated with fluctuations in the amount of protein. Importantly, increased enzyme activity at 4 to 24 h was unrelated to de novo kinase protein synthesis.

Some additional experiments again addressed the relation between p42/p44 phosphorylation and redox alterations. As a result, it became clear that the rapid and delayed GSNO-mediated MAPK activation was largely abrogated by NAC (data not shown). NAC by itself showed no kinase activation. Basal MAPK activation, which sometimes appeared, was also NAC sensitive.

Transducing pathways during NO-mediated MAPK activation

Experiments were designed to identify NO-associated signaling pathways that transmitted rapid and delayed MAPK activation. Based on the knowledge that soluble guanylyl cyclase is a primer target for NO, we intended to mimic MAPK activation by exposing cells to lipophilic cGMP analogues. Exposing rat MC for variable times to 1 mM 8-bromo-cGMP (Br-cGMP) revealed MAPK activation within 5 to 15 min. This was assured by Western blot analysis and the radioactive MAPK assay (Fig. 4A). With prolonged incubation, MAPK activation declined to basal values with
no indication of a second activation phase. Rapid MAPK phosphorylation, initiated by lipophilic cGMP derivatives within 5 min, was largely compromised by preincubating cells with NAC (Fig. 4B). We noticed a strongly decreased Br-cGMP-mediated MAPK phosphorylation response when cells were incubated beforehand for 30 min with 30 mM NAC.

In extending experiments, we employed NS 2028, which is a recognized soluble guanylyl cyclase inhibitor (39). NS 2028 by itself did not affect MAPK, but it largely attenuated rapid MAPK activation in response to GSNO (Fig. 4C). In contrast, late GSNO-mediated MAPK activation observed after 8 h (Fig. 4C) was unaffected by NS 2028. Our results imply that rapid and delayed NO-mediated MAPK activation are differently regulated. Whereas the first activation period occurred in close association with soluble guanylyl cyclase activation and cGMP formation, the second activation phase seemed unrelated to cGMP signaling.

To further support a role of cGMP during the rapid phase of NO-mediated MAPK activation, we determined cGMP levels by RIA in MC in response to GSNO. Stimulation with 1 mM GSNO for 2.5 min promoted a cGMP increase from a basal intracellular cGMP level of 10 ± 3 pmol/ml to stimulated values of 1047 ± 148 pmol/ml. At a 5-min incubation period with 1 mM GSNO, cGMP further increased to 1588 ± 32 pmol/ml. Neither basal nor stimulated cGMP responses were affected by the redox-modulating agent NAC (data not shown).

Further analysis investigated the mechanism of the CM-mediated rapid MAPK activation. Compared with the rapid NO-induced MAPK activation, CM-stimulated MAPK activation was not affected by the soluble guanylyl cyclase inhibitor NS 2028 (Fig. 4D) and so is unrelated to cGMP signaling pathways. NO-induced as well as CM-mediated MAPK activation was abolished by the MEK-1 inhibitor PD 98059 (Fig. 4E).

To gain mechanistic insights into the second delayed MAPK activation period, we performed in vitro MAPK dephosphorylation assays. For these experiments, phosphorylation of p42/p44 MAPK was initiated in serum-starved rat MC by a 5-min readdition of serum, which is known to activate p42/p44 MAPK (40). Phosphorylated, i.e., activated, p42 and p44 MAPK was immunoprecipitated (p42/p44 IP) and served as the substrate in the following dephosphorylation assays (Fig. 5). Following IP dephosphorylation, the remaining MAPK activity was then measured by its ability to cause MBP phosphorylation. Dephosphorylation of the substrate, i.e., phosphorylated p42/p44, was achieved during incubations with a cytosolic fraction of untreated, GSNO-, CM-, and/or NAME-exposed cells that contained active or blocked tyrosine phosphatases.

Cytosol of unstimulated cells promoted a rapid and near complete dephosphorylation (deactivation) of the p42/p44 MAPK substrate (Fig. 5A). Readdition of the NO donor GSNO to the cytosol prevented dephosphorylation of the substrate, thus pointing to inhibition of tyrosine phosphatases by NO. MAPK dephosphorylation and/or inhibition of dephosphorylation was unaffected by the MEK-1 inhibitor PD 98059. This ruled out the involvement of MAPK phosphorylation under the specified experimental conditions. Cytosol derived from cells that were pretreated with 1 mM GSNO for 8 h or cytosol from cells that had been stimulated with CM for 24 h revealed no dephosphorylation activity (Fig. 5B). However, phosphatase activity was apparent under conditions of costimulation with CM and the iNOS-blocking agent NAME for 24 h. MAPK dephosphorylation and/or inhibition of dephosphorylation in this assay was unaffected by the MEK-1 inhibitor PD 98059 (data not shown). Evidently, exogenously generated NO in rat MC caused phosphatase inhibition, thus promoting p42/p44 activation.

**Discussion**

Severe inflammatory conditions in the kidney mesangium, i.e., glomerulonephritis, are often associated with NO formation and increases in number of MC (2, 3). Consequently, a major focus underlying the pathogenesis of glomerulonephritis has been to define mechanisms of MC proliferation. Presently, the role of NO in modulating cell proliferation is discussed controversially. Although NO acts as an antiproliferative agent under some conditions (8, 9), NO has been established as a downstream signal in vascular endothelial growth factor-induced endothelial cell proliferation (10). The ability of NO to activate the p42/p44 MAPK pathway in cytokine-activated rat MC may serve as an explanation for cell proliferation during glomerulonephritis and is achieved through distinct signaling pathways.

**Cytokine-mediated MAPK activation**

The present study attributed special attention to cytokine-mediated NO generation and its signal-conveying properties leading to alterations in MAPK activity. Although primer effects of cytokines such as IL-1β, IFN-γ, and TNF-α are receptor-mediated (41, 42), cellular responses integrate activating and antagonistic signals and combine rapid as well as delayed alterations in the formation of diffusible second messengers and/or modulations in gene expression (6, 43). A major signal component in rat MC that is produced in response to cytokines is NO, which is found in association with iNOS up-regulation (3).
In corroboration with earlier reports (44, 45), we noticed a fast MAPK activation following cytokine addition, which successfully was attenuated by NAC addition. Transient MAPK activation is compatible with the notion that protein tyrosine phosphatases become active shortly following kinase activation (46). The resultant domination of dephosphorylation abrogated MAPK activity.

Surprisingly, a second phase of MAPK activation occurred 15 to 24 h after cytokine stimulation. Delayed and pronounced MAPK activation coincided with iNOS activation. A cause/effect relationship between NO generation and MAPK activation was established by the successful use of iNOS inhibitors such as NAME and DPI, which attenuated kinase activation. As a result of these experiments, we conclude that endogenously derived NO, stemming from an active iNOS, caused late-phase MAPK activation.

**NO-derived signaling pathways that promote rapid MAPK activation**

With the use of NO donors, we underscored an active role of NO in promoting rapid and delayed MAPK activation. Activation of MAPK by authentic NO and NO donors is in accordance with reports stating that activation of p21WAF1 promoted rapid MAPK activation in Jurkat cells (47, 48). However, delayed MAPK activation by NO donors and more importantly by endogenous NO generation is unknown.

In MC, rapid MAPK activation is compatible with NO-mediated soluble guanylyl cyclase activation and cGMP formation. Experimental evidence supporting this assumption is threefold. First, MAPK activation is mimicked by membrane-permeable cGMP analogues. Second, inhibition of soluble guanylyl cyclase by NS 2028, a recognized enzyme-blocking agent, abrogated immediate MAPK activation in response to NO donors (39). Third, cGMP formation in response to GSNO coincided with MAPK activation. As a further working hypothesis, we assume that cGMP-mediated phosphorylation initiates upstream signaling in the cascade leading to MAPK activation because the MEK-1 inhibitor PD 98059 hindered the cGMP response. Our results are in line with a recent study showing that NO mediates vascular endothelial growth factor-dependent p42/p44 MAPK activation via a cGMP-elicted response (40). The cGMP response may be in some analogy to the action of cAMP, which is known to activate p42/p44 MAPK through a B-Raf-dependent pathway (49). Transient activation is explained on the basis that cGMP-elevating agents, such as atrial natriuretic peptide or NO donors, contribute to MAPK inactivation through the induction of dual-protein tyrosine/threonine-specific phosphatases that show some selectivity toward MAPK (50).

Control experiments in the presence of NS 2028 rule out a cGMP action in cytokine-stimulated MC. However, cytokine and cGMP signals that led to a rapid MAPK activation were both PD 98059 sensitive. The integration of NO formation and MAPK activation may represent a so far underestimated target system that contributes to cGMP-evoked signal transduction.

**NO-derived signaling pathways that promote delayed MAPK activation**

Strikingly, NO evoked a second, delayed phase of activation, irrespective of whether NO was endogenously produced or delivered by NO donors. Delayed MAPK activation was not mimicked by lipophilic cGMP derivatives, was insensitive to the soluble guanylyl cyclase blocking agent NS 2028, and thus is unrelated to cGMP signaling.

Physiologic inactivation of MAPK is achieved by at least three distinctive MAPK phosphatase subfamilies with more than 100 isoenzymes that differ in structure and mode of function (25). Following the assumption that blocking thiol-sensitive protein tyrosine phosphatases may result in increased phosphorylation, we addressed the possibility that NO interfered with the MAPK phosphorylation/dephosphorylation equilibrium. By using FCS-activated p42/p44, we established dephosphorylation of MAPK by cytosolic tyrosine phosphatases. Dephosphorylation was sensitive to the readdition of NO and further was down-regulated in cytosol of NO- or cytokine-stimulated cells. An active role of NO in blocking phosphatase activity was proven in cells that were treated with cytokines in the presence of iNOS inhibitors. Inhibition of phosphatase activity by NO is rationalized when we consider the active and catalytically necessary thiol group in the Cys-R motif of tyrosine-specific phosphatases (25).

Taking into account that the active cysteine residue of tyrosine phosphatases exists as a thiolate anion (27, 51), inhibition by S-nitrosation or oxidation is predictable. Knowing that As3+ abrogated tyrosine phosphatase activity, and thereby promoted tumor growth, and binds with high affinity to vicinal thiols (52), and in view of the circumstances that some tyrosine phosphatases contain a second cysteine five residues away from the invariant thiol common to all tyrosine phosphatases, modifications and inhibition via intramolecular disulfide formation may attenuate protein activity.

Although inhibition of tyrosine phosphatases by NO donors has been performed, in part by measuring hydrolysis of p-nitrophenyl phosphate (53–55), cellular investigations with NO donors, and more importantly by iNOS induction in relation to MAPK activation, are elusive. Conclusively, late-phase activation of MAPK by NO resulted from inhibition of tyrosine phosphatases rather than activation of tyrosine kinases. NAC-evoked redox modulation of tyrosine phosphorylation may be in line with a regulatory role of the phosphatase reaction as well. Significantly, the use of NAC does not automatically imply activation of MAPK through an oxidation-dependent mechanism; rather, it may point to the activation of oxidation-sensitive phosphatases, thereby shifting the phosphorylation equilibrium toward dephosphorylation, i.e., MAPK deactivation.

NO-mediated rapid and delayed MAPK activation was separated on the basis of cGMP signaling vs inhibition of protein tyrosine phosphatases and may contribute to MC hypercellularity under inflammatory conditions. Our results emphasize the importance of NO as a balancing element in the molecular events affecting diverse cell responses such as cell proliferation and death.

**References**


