Role of Mitogen-Activated Protein Kinases in *S*-Nitrosoglutathione-Induced Macrophage Apoptosis†

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Received September 23, 1998; Revised Manuscript Received November 19, 1998

ABSTRACT: The inflammatory mediator nitric oxide (NO•) promotes apoptotic cell death based on morphological evidence, accumulation of the tumor suppressor p53, caspase-3 activation, and DNA fragmentation in RAW 264.7 macrophages. Since nitrosothiols may actually be the predominant form of biologically active NO• in vivo, we used *S*-nitrosoglutathione (GSNO) to study activation of extracellular signal-regulated protein kinases1/2 (ERK1/2), c-Jun N-terminal kinases/stress-activated protein kinases (JNK1/2), and p38 kinases. Moreover, we determined the role of mitogen-activated protein kinase signaling in the apoptotic transducing ability of GSNO. ERK1/2 became activated in response to GSNO after 4 h and remained active for the next 20 h. Blocking the ERK1/2 pathway by the mitogen-activated protein kinase kinase inhibitor PD 98059 enhanced GSNO-elicited apoptosis. p38 was activated as well, but inhibition of p38 with SB 203580 left apoptosis unaltered. Activation of JNK1/2 by GSNO showed maximal kinase activities between 2 and 8 h. Attenuating JNK1/2 by antisense-depletion eliminated the pro-apoptotic action of low GSNO concentrations (250 μM), whereas apoptosis proceeded independently of JNK1/2 at higher doses of the NO donor (500 μM). Decreased apoptosis by JNK1/2 depletion prevented p53 accumulation after the addition of GSNO, which positions JNK1/2 upstream of the p53 response at low agonist concentrations. In line, JNK1/2 activation proceeded unaltered in p53-antisense transfected macrophages. However, with higher GSNO concentrations apoptotic transducing pathways, including p53 accumulation, were JNK1/2 unrelated. The regulation of mitogen-activated protein kinases by GSNO may help to define cell protective and destructive actions of reactive nitrogen species.

Apoptosis, a synonym for programmed cell death, has been identified as a conserved and important determinant in controlling cellular homeostasis. Apoptotic mechanisms participate in the removal of genetically or toxicologically damaged cells, operate during host defense, occur during embryonic development, and modulate the progression of diseases such as cancer, autoimmune diseases, and viral infections (*1*−*4*). Initiation of pro-apoptotic pathways is achieved by a wide range of stimuli including physiological activators, damage-related inducers, therapy-associated agents, or toxins (*5*). Among the potential extrinsic and intrinsic apoptotic signals, nitric oxide (NO•)[1] recently came into focus (*6*).

NO•, which is produced along with L-citrulline by enzymatic oxidation of L-arginine, gained considerable attention because of its ubiquitous presence in biology (*7*, *8*). Investigations have demonstrated important roles of NO• in diverse physiological processes but also elucidated cytotoxic signaling properties. Reactivities encompass endothelium-dependent relaxation, neurotransmission, and cell-mediated immune responses (*9*, *10*). In general, there is a rough correspondence between homeostatic and toxic functions of NO• and its production in small versus large quantities. Reactive nitrogen species include not only nitric oxide but also those species resulting from oxidation, reduction, or adduction in physiological milieus. The expression of a wide variety of effects is achieved through multiple interactions with intracellular targets via redox and additive chemistry, i.e., the formation of *S*-nitrosothiols such as *S*-nitrosoglutathione (GSNO) resulting from the reaction of intracellular glutathione (GSH) with reactive nitrogen species (*11*).

NO•-elicited apoptosis is established for various cell systems, among others, for peritoneal macrophages and for different macrophage cell lines (*6*, *12*). In RAW macrophages NO• evoked typical apoptotic features such as cell shrinkage, chromatin condensation, and DNA fragmentation (*6*). Biochemically we noticed up-regulation of the tumor suppressor p53 and activation of caspases, i.e., caspase-3 (*6*). Pro-apoptotic signaling was attenuated by Bcl-2 overexpression or by maneuvers that resulted in high expression rates of cyclooxygenase-2 (*13*, *14*). However, the detailed pro-apoptotic mechanisms remained elusive.

Recent studies focused on the activation of mitogen-activated protein kinases (MAPK) in affecting apoptosis (*15*). The MAPK superfamily encompasses three distinctive subgroups, namely, the extracellular signal-regulated protein kinases1/2 (ERK1/2), the stress-activated protein kinases also

† We are grateful to the Deutsche Forschungsgemeinschaft and in part to the European Community for financial support.

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[1] Abbreviations: AMC, 7-amino-4-methylcoumarin; DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-aminomethylcoumarin; ERK, extracellular signal-regulated protein kinases; GSNO, *S*-nitrosoglutathione; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated protein kinase kinase; NO•, nitric oxide.

10.1021/bi982292a CCC: $18.00 © 1999 American Chemical Society
Published on Web 01/30/1999

known as c-Jun N-terminal kinases 1/2 (JNK1/2), and p38 kinases (*16*−*18*). MAPK are serine/threonine kinases that become activated by an upstream kinase cascade via dual phosphorylation at a tyrosine and threonine residue (*16*). Their coordinated activation and interaction allows cells to respond to various genotoxins as well as survival factors by affecting a steadily growing list of downstream targets (*16*). The impact of MAPK activation on the decision whether a cell survives or dies is controversial. However, several studies associate JNK1/2 or p38 activation with apoptosis (*19*−*21*), whereas activation of ERK1/2 seems to signal protection (*22*, *23*). Others propose that the balance between ERK1/2 activation relative to active JNK1/2 and/or p38 determines the fate of a cell (*24*).

In our study we determined activation of all three MAPK subfamilies in response to GSNO, the predominant form of biologically active $NO^{\bullet}$, and elucidated their contribution to initiation or inhibition of macrophage apoptotic cell death. By using MAPK inhibitors and antisense oligonucleotides to down regulate JNK1/2, we provide evidence for a pro-apoptotic function of JNK1/2 and an anti-apoptotic role of ERK1/2. Modulation of MAPK activity is correlated to the occurrence of GSNO-evoked apoptotic parameters such as morphological changes, p53 accumulation, caspase activation, and DNA fragmentation. We conclude that JNK1/2 activation is a major constituent of GSNO-mediated apoptotic cell death in macrophages.

MATERIALS AND METHODS

Materials. RPMI 1640 and medium supplements were ordered from Biochrom, Berlin, Germany. Fetal calf serum was purchased from Gibco, Berlin, Germany. Hoechst dye 33258, β-glycerophosphate, trichloroacetic acid, diphenylamine, sodium pyrophosphate, protein A-sepharose, and reduced glutathione came from Sigma, Deisenhofen, Germany. Sodium vanadate was bought from Boehringer Mannheim, Mannheim, Germany. Phenylmethylsulfonyl fluoride was ordered from Fluka, Deisenhofen, Germany. Anti-p44/42 MAPK rabbit polyclonal antibody for Western blot analysis was purchased from IC Chemikalien GmbH, Ismaning, Germany. Phosphospecific p44/42 (ERK1/2) and p38 MAPK antibodies came from New England Biolabs GmbH, Schwalbach/Taunus, Germany, while the secondary antibody was purchased from Promega/Serva, Heidelberg, Germany. JNK1/2 and p38 antibodies were bought from Santa Cruz Biotechnology, Inc., Heidelberg, Germany. ECL-detection reagents and γ[^{32}P]ATP were from Amersham, Braunschweig, Germany. *N*-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC), AMC, and CHAPS were purchased from Biomol, Hamburg, Germany. PD 98059 and SB 203580 came from Calbiochem, Bad Soden, Germany. The GST-cJun (1/166) expression plasmid was kindly provided by Prof. P. Angel, Heidelberg, Germany. All other chemicals were of the highest grade of purity and commercially available.

Cell Culture. The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (complete medium). All experiments were performed using complete RPMI. To study the activation of ERK1/2 cells were cultured with 2% heat-inactivated fetal calf serum (reduced medium). PD 98059 and SB 203580 were added from DMSO stock solutions. Appropriate solvent controls were performed. p53-antisense transfected cells were as previously described (*25*).

GSNO Synthesis. *S*-Nitrosoglutathione was synthesized and characterized as previously described (*26*).

Western Blots. Cell lysis was achieved with ice-cold lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate, pH 8.0) and sonication with a Branson sonifier (10 s, duty cycle 100%, ouput control 1). Following centrifugation (14000*g*, 15 min) protein content was measured. For equal protein loading 150 μg of protein were mixed with the same volume of 2 × SDS-sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose sheets. Molecular weight of corresponding proteins was determined in relation to molecular weight rainbow markers, equal loading was confirmed by Ponceau S staining. Transblots were washed twice with TBS (140 mM NaCl, 50 mM Tris/HCl, pH 7.2) containing 0.06% Tween-20 before blocking unspecific binding with TBS/5% skim milk. Filters were incubated overnight at 4 °C with the phosphospecific p44/42 MAPK antibody (1:1000), the phosphospecific p38 MAPK antibody (1:1000), or the p53 antiserum (hybridoma supernatant, clone PAb122, 1:5, kindly provided by Prof. H. Stahl, Homburg/Saar, Germany). Proteins were detected by a horseradish peroxidase (HRP)-conjugated polyclonal antibody (1:10000) using the ECL method. Afterward, blots were stripped (62.5 mM Tris, 2% SDS, 95 mM β-mercaptoethanol, pH 6.7) for 30 min at 55 °C and reblocked with TBS/5% skim milk. In turn, the anti-p44/42 or anti-p38 MAPK rabbit polyclonal antibodies (1:1000 each) were incubated for 1 h before ECL detection.

JNK1/2 Activity Assay. RAW 264.7 macrophages were incubated for the times indicated, scraped off, centrifuged (5 min, 1200*g*), and resuspended in lysis buffer (20 mM Tris/HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, and 1 mM sodium vanadate). Cells were kept on ice for 5 min, vortexed, and centrifuged (10000*g*, 20 min, 2 °C). Protein (300 μg of the supernatant), in an equalized sample volume, was used for immunprecipitation. Anti-JNK1/2 antibodies were added, followed by gentle rotation at 4 °C for 2 h. Following protein A-sepharose addition, immune complexes were further incubated for 60 min. JNK1/2-protein A-sepharose complexes were washed two times with lysis buffer and once with kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glyerophosphate, 25 mM $MgCl_2$, 1 mM DTT, and 0.1 mM sodium vanadate). Immune complexes were centrifuged (10000*g*, 2 min, 4 °C) and resuspended in 25 μL of kinase buffer, with the addition of 2 μg GST-cJun and 5 μCi γ[^{32}P]ATP. Phosphorylation was performed for 20 min at 37 °C. Reactions were stopped by adding 25 μL of 2 × SDS-sample buffer. Samples were heated for 5 min at 95 °C and proteins were separated on 10% SDS-polyacrylamide gels. Gels were fixed, dried, and subjected to autoradiography.

Down Regulation of JNK1/2 by Antisense Oligonucleotides. RAW 264.7 macrophages were incubated with 2.5 μM JNK1 antisense (JNK1asn, 5′-CTC-TCT-GTA-GGC-

CCG-CTT-GG-3′) and 2.5 μM JNK2 antisense (JNK2asn, 5′-GTC-CGG-GCC-AGG-CCA-AAG-TC-3′) oligonucleotides (*27*) for 16 h prior to cell stimulation. The 5′-terminal fluorescein-labeled oligonucleotides were stabilized by phosphothioate linkages (Eurogentec, Seraing, Belgium). Uptake of antisense oligonucleotides was determined by scoring cells by fluorescense-microscopy as previously described (*28*).

Fluorogenic Caspase-3 Assay. Following individual incubations, cells were recovered and centrifuged (1200*g*, 5 min). Pellets were resuspended in lysis buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM PMSF, and 10 mM DTT), left on ice for 30 min, sonified (Branson sonifier, 10 s, duty cycle 100%, output control 1), and centrifuged (10000*g*, 10 min, 4 °C). Cytosolic protein (30 μg) was incubated with 12 μM of DEVD-AMC at 30 °C. Substrate cleavage was followed fluorometrically with excitation at 360 nm and emission at 460 nm for 2 h. Substrate cleavage during the linear phase of the reaction was quantitated by internal AMC standards.

DNA Fragmentation. DNA fragmentation was quantitated by the diphenylamine assay as previously reported (*29*). Briefly, following incubation cells were resuspended in 250 μL of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer), and incubated with an additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4 °C. Centrifugation (10000*g*, 15 min) allowed for the recovery of intact chromatin (pellet) and DNA fragments (supernatant). Pellets were resuspended in 500 μL of TE buffer and again precipitated with 500 μL of 10% trichloroacetic acid at 4 °C. Following centrifugation (4000*g*, 10 min) the supernatant was removed. After the addition of 300 μL of 10% trichloroacetic acid samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent. The percentage of cleaved DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Hoechst 33258 Staining. Cells, grown and stimulated in 24-well plates, were recovered, washed once in phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde for 5 min onto glass slides, stained with the Hoechst 33258 (8 μg/mL) for 5 min, washed twice with PBS, and mounted in Kaiser's glycerol gelatin. Nuclei were visualized using a Zeiss fluorescence microscope (Axiovert 100), counting a minimum of 200 cells in each sample to score apoptosis. Apoptotic nuclei are expressed as a percentage of total nuclei.

Lactate Dehydrogenase (LDH) Release. The percentage of LDH release from cells is a determinant of cellular necrosis and expressed as the proportion of LDH released into medium compared to the total amount of LDH present in intact cells. Total LDH was determined following cell lysis with 0.1% Triton X-100. LDH activity was monitored by following the oxidation of NADH as the decrease in absorbance at 334 nm. Reactions were carried out in a triethanolamine buffer (50 mM triethanolamine), pH 7.6, containing 5 mM EDTA, 127 mM pyruvate, and 14 mM NADH.

Statistical Analysis. Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's *t*-test. Normal distribution of data is ensured. Otherwise representative data, of at least three similar examinations, are shown.

RESULTS

GSNO-Mediated MAPK Activation in RAW 264.7 Macrophages. Since it is known that NO$^\bullet$ causes apoptotic cell death in macrophages, i.e., RAW 264.7 cells and further taking into consideration that various members of the MAPK superfamily affect apoptotic signaling cascades, we examined activation of ERK1/2, JNK1/2, and p38 kinases in response to GSNO and elucidated their impact in modulating GSNO-elicited apoptosis.

In a first set of experiments we investigated activation of ERK1/2, JNK1/2, and p38 kinases in response to GSNO. Agonist treatment caused activation of all three kinases, however, with different time and concentration dependency.

To assess GSNO-mediated activation of ERK1/2, we reduced the basal background of kinase activity by culturing cells in reduced medium (for details see Materials and Methods) for 16 h prior to GSNO addition. In turn, activation of ERK1/2 was followed by Western blot analysis using a phosphospecific p44/42 MAPK antibody that only showed reactivity to the tyrosine-phosphorylated and thus activated form of ERK1/2. Both ERK1 (p44) and ERK2 (p42) were efficiently activated in response to GSNO. Activation became evident after 4 h and kinases remained in their phosphorylated form for at least 20 h (Figure 1A, top panel). Activity increases were unrelated to changes in protein amount because detection of ERK1/2 by Western blot analysis revealed no protein increase (Figure 1A, middle panel). We conclude that GSNO promotes efficient and long-lasting ERK1/2 activation. In further experiments we established a dose-dependent ERK1/2 activity increase. Activation was noticed by Western blot analysis using the phosphospecific p44/42 MAPK antibody at 500 μM GSNO without any further increase up to 1 mM GSNO (Figure 1A, bottom panel).

In some comparison to ERK1/2 activation, JNK1/2, and p38 kinases were activated by GSNO as well. For JNK1/2 we noticed a higher degree of activity between 2 and 8 h. Activation, determined in a direct kinase activity assay using GST-cJun as the substrate, was strongest at 4 and 8 h, while kinase activity was absent at 1 or 16 h, respectively (Figure 1B, top panel). GSNO-elicited JNK1/2 activation was dose-dependent. Activation started around 250 μM GSNO and leveled off at 1 mM (Figure 1B, bottom panel). In further experiments we established that another NO$^\bullet$-releasing agent, spermine-NO, promoted JNK1/2 activation with an identical time and concentration dependency (data not shown).

Activation of p38 was followed by employing phospho-specific antibodies directed against the phosphorylated kinase (Figure 1C, top panel). GSNO (1 mM) promoted a fast kinase activation. p38 was phosphorylated after 1 h, activation peaked at 4 h, and activity declined afterward, although it remained slightly elevated for the next 20 h. The amount of p38 protein, determined by Western blot analysis using a p38 kinase antibody, remained unaltered during the entire incubation period (Figure 1C, middle panel). GSNO-induced p38 kinase activation was dose-dependent. Activation was noticed at 750 μM GSNO with a more pronounced activation in response to 1 mM GSNO (Figure 1C, bottom panel).

Activation of ERK1/2-Enhanced Survival of GSNO-Stimulated Macrophages. To elucidate the role of ERK1/2 during GSNO-induced apoptosis, we interrupted ERK1/2

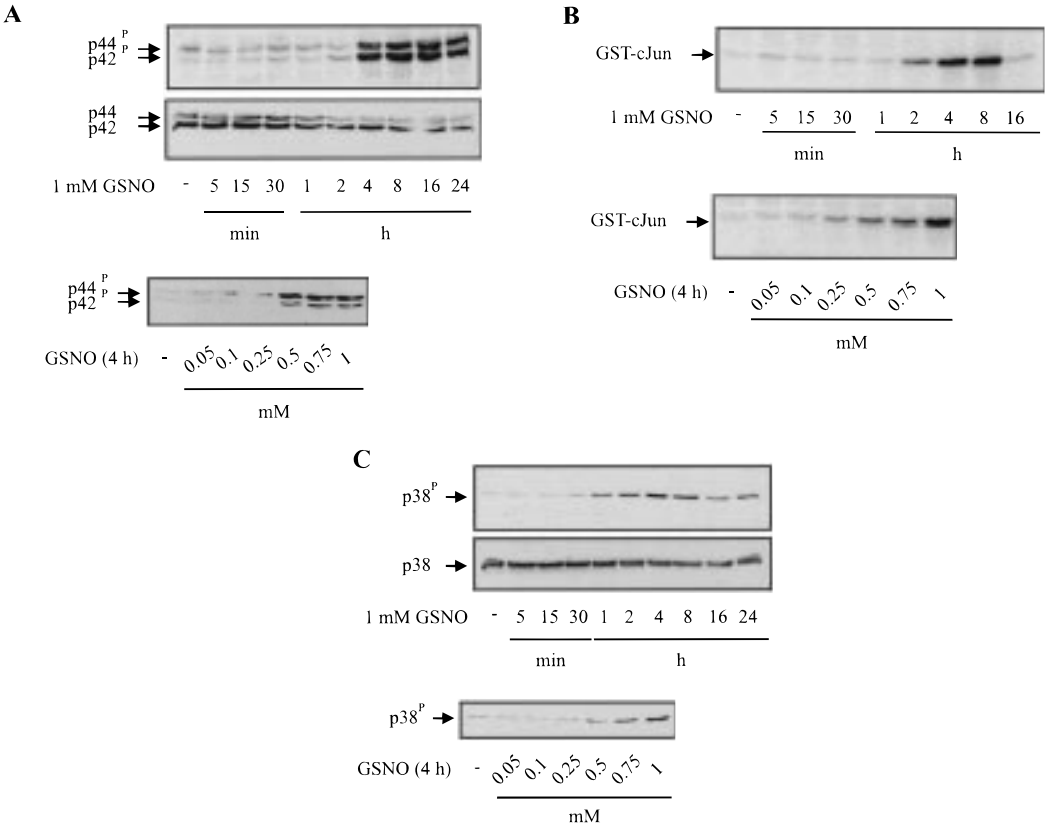

FIGURE 1: GSNO-induced MAPK activation. RAW 264.7 macrophages were time and concentration dependently exposed to GSNO. Phosphorylation/activation of ERK1/2 was determined in reduced medium as outlined under Materials and Methods by Western blot analysis with a phosphospecific p44/42 MAPK antibody ($p42^P/p44^P$; upper and bottom panel of A). Detection of JNK1/2 or p38 activity was performed in complete medium. JNK1/2 activation was determined by a radioactive kinase assay using GST-cJun as a substrate (B). p38 kinase activation was examined by Western blot analysis using a phosphospecific p38 MAPK antibody ($p38^P$; upper and bottom panel of C). Protein amounts of ERK1/2 and p38 were assessed by Western blots using an anti-p44/42 (p42/p44; middle panel of A) or a p38 MAPK (p38; middle panel of C) antibody. For Western blot analysis 150 μg of protein was loaded in each lane. Results are representative of three similar experiments.

signaling by employing PD 98059, a MEK-1 inhibitor (*30*). MEK-1 is the immediate upstream kinase that phosphorylates, i.e., activates ERK1/2. Inhibition of MEK-1 by PD 98059 blocked GSNO-induced ERK1/2 activation (Figure 2A) without affecting JNK1/2 and p38 kinase activities (data not shown). In this set of experiments cells were preincubated with PD 98059 (20 μM) for 30 min, followed by the coincubation of GSNO for 8 h. Apoptotic cell death was initiated by the addition of 0.25 or 0.5 mM GSNO and was analyzed on the basis of DNA fragmentation, caspase-3 activation, and p53 accumulation. Cell necrosis was assessed by determination of LDH-release.

In corroboration with earlier reports (*31*), we noticed dose-dependent DNA fragmentation in response to GSNO during a 8 h exposure period that was quantitated by the diphenylamine assay. The absence of LDH release underscored plasmamembrane integrity during GSNO exposure (data not shown). A concentration of 0.25 mM GSNO elicited 11−14% fragmentation, while 0.5 mM GSNO led to 20−22% DNA fragmentation. DNA cleavage of unstimulated controls was around 2−3% (Figure 2B).

Incubation of macrophages with 20 μM PD 98059 for 30 min followed by the subsequent addition of the NO donor (8 h) significantly enhanced GSNO-evoked DNA fragmentation. However, DNA fragmentation in unstimulated controls (Figure 2B) and the release of LDH were not affected by PD 98059 (data not shown).

Caspase-3 activation is considered a convenient marker of apoptosis and is regarded as the point of no return in the pro-apoptotic signaling cascade. GSNO elicited the cleavage of the fluorogenic caspase-3 substrate DEVD-AMC and promoted AMC accumulation. AMC production in response to 0.25 mM GSNO increased from 1.8 nM AMC/(mg min) to 2.6 nM AMC/(mg min) in the presence of 20 μM PD 98059, while the AMC production was elevated from 2.7 nM AMC/(mg min) to 3.5 nM AMC/(mg min) as a result of 0.5 mM GSNO addition and conincubation of PD 98059 (20 μM) and 0.5 mM GSNO, respectively (Figure 2C). Control incubations neglected any effect of PD 98059 on residual, unstimulated caspase-3 activity.

Western blot analysis of p53 revealed a similar, apoptotic potentiating role of PD 98059 (Figure 2D). Accumulation of p53 was absent in controls but occurred in response to 0.5 mM GSNO during a 4 h incubation period. PD 98059 (20 μM) by itself left p53 accumulation unaltered but enhanced the p53-promoting efficacy of 0.2 mM GSNO. Also, p53 accumulation in response to 0.5 mM GSNO was enhanced by the MEK-1 inhibitor.

Collectively, on the basis of DNA fragmentation, caspase activation, and p53 accumulation, our data imply an apoptosis enhancing capability of the MEK1-inhibitor PD 98059.

Attenuation of JNK1/2 Activity Decreased GSNO-Induced Apoptosis. To determine the participation of JNK1/2 activation during GSNO-induced apoptosis, we used 5′-terminal

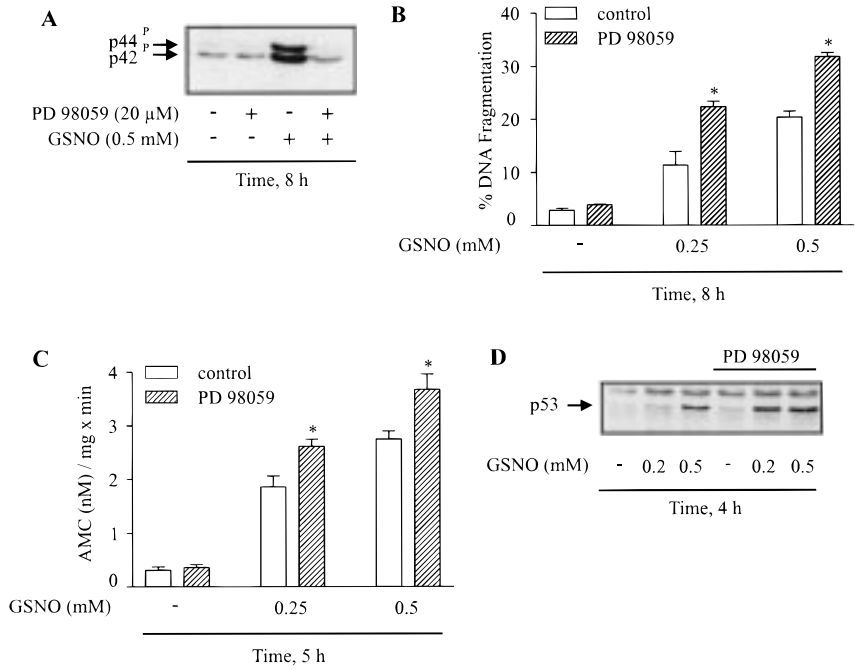

FIGURE 2: ERK1/2 inhibition enhanced GSNO-elicited apoptosis. Macrophages were pretreated with the MEK-1 inhibitor PD 98059 (20 μM) for 30 min prior to a subsequent conincubation period with GSNO for 8 h (A, B), 5 h (C), 4 h (D), or remained untreated (control). Phosphorylation/activation of ERK1/2 was determined by Western blot analysis with a phosphospecific p44/42 MAPK antibody ($p42^P$/$p44^P$) in response to 0.5 mM GSNO (A). DNA fragmentation was quantitated by the diphenylamine assay (B), caspase-3 activation was determined by the cleavage of DEVD-AMC (C), and p53 accumulation was analyzed by Western blot analysis (D) in response GSNO. For details see Material and Methods. Western blots were performed with 150 μg of protein loaded on each lane and are representative of four similar experiments. Results represent the mean ($\pm$ standard deviation) of four different experiments (* $P < 0.01$ versus GSNO-treated control macrophages).

fluorescein-labeled, phosphothioate-stabilized antisense oligonucleotides to down regulate JNK1 and JNK2, thereby blocking the JNK1/2 pathway (*27*). Incubation of RAW 264.7 macrophages with 2.5 μM JNK1/2 antisense oligonucleotides for 16−24 h showed a high percentage of positively affected cells (*28*) and subsequently a completely suppressed JNK1/2 activity (Figure 3A). As shown in Figure 3A, activation of JNK1/2 by GSNO up to 0.5 mM was attenuated by JNK1/2 antisense oligonucleotide pretreatment without affecting the activities of ERK1/2 or p38 (data not shown).

We then focused on the occurrence of apoptosis in macrophages in which JNK1/2 was down regulated by antisense oligonucleotide pretreatment. Low-dose GSNO (0.25 mM) -initiated apoptosis was largely attenuated as a result of suppressed JNK1/2 activity (Table 1). However, increasing GSNO concentrations to 0.5 mM revealed a different picture. Although JNK1/2 activity still was inhibited (Figure 3A), DNA fragmentation proceeded comparable to controls. These results point to a variable JNK1/2 requirement during GSNO-elicited apoptosis. Whereas low GSNO concentrations demand JNK1/2 activity, higher doses of GSNO do not.

The dichotomy became also apparent during caspase-3 activity determinations. Caspase-3 activation was attenuated in cells with down-regulated JNK1/2 when GSNO was used at a concentration of 0.25 mM (Figure 3B). However, with 0.5 mM GSNO caspase-3 activation in JNK1/2, antisense-treated cells and controls became indistinguishable. These results were further verified for p53 accumulation (Figure 3C). p53 accumulation was absent in unstimulated and control antisense-treated macrophages. The p53 response became significant following the exposure to 0.25 or 0.5 mM GSNO. In JNK1/2 antisense-treated macrophages p53 accumulation in response to 0.25 mM GSNO was suppressed but was detectable after the addition of 0.5 mM GSNO. We conclude that an attenuated JNK1/2 activity suppressed apoptosis in response to low agonist, i.e., GSNO concentrations, only.

JNK1/2 Activation in Response to GSNO Is Unaltered in p53-Deficient Macrophages. To confirm JNK1/2 activation as an upstream event of p53 accumulation, we investigated JNK1/2 activation in response to GSNO in stably p53-antisense-transfected RAW 264.7 macrophages (R-p53asn-11) (*25*) in comparison to control cells.

In p53-antisense-transfected macrophages p53 accumulation was absent after GSNO (0.5 mM, 4 h) treatment, whereas exposure of control macrophages evoked a significant p53 response (Figure 4A). Further experiments investigated GSNO-elicited JNK1/2 activation in p53-antisense-constructed macrophages. Exposure of R-p53asn-11 cells to 0.5 mM GSNO for 4 h revealed JNK1/2 activation that resembled the GSNO-response observed in parent cells (Figure 4B). Accumulation of p53 can be considered as a convenient and sensitive read-out system that indicates $NO^{\bullet}$ intoxication. GSNO exposure promotes efficient DNA binding of p53 as determined by gel-shift analysis (von Knethen and Brüne, unpublished observations), thus implying a transcriptional active tumor suppressor. However, presently it is unknown whether p53 initiates apoptosis via transcription-dependent or -independent pathways.

p38 Kinases Do Not Affect GSNO-Induced Apoptosis. In a last set of experiments, we determined the impact of p38 kinases on GSNO-mediated apoptotic cell death in mac-

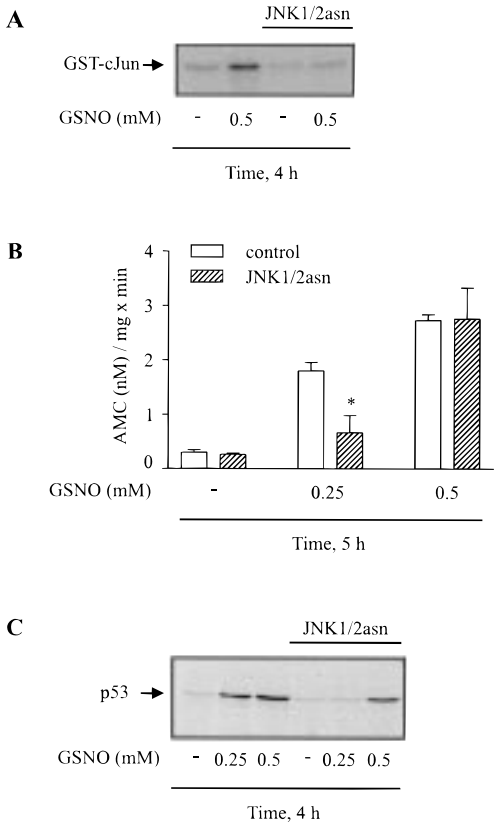

FIGURE 3: JNK1/2 antisense oligonucleotides attenuated GSNO-induced apoptosis. Macrophages were incubated 16 h prior to agonist treatment with 2.5 μM JNK1 and 2.5 μM JNK2 antisense oligonucleotides (JNK1/2asn), or remained as controls. Afterward, cells were exposed to 0.5 mM GSNO or vehicle for 4 h and JNK1/2 activity was determined in a radioactive kinase assay (A) as described under Material and Methods. In addition, caspase-3 activity (B) and p53 accumulation (C) were determined in controls and JNK1/2 antisense oligonucleotides (JNK1/2asn) treated cells. For details see Figure 2. Results represent the mean (± standard deviation) of four different experiments (* $P < 0.05$ versus GSNO-treated control macrophages). Western blots (150 μg of protein/lane) are representative of four similar experiments.

Table 1[a]

	apoptotic cells (%)			
	control	JNK1/2 asn	SB 203580	JNK1/2asn SB 203580
vehicle	1.1 ± 0.3	1.9 ± 1.3	1.7 ± 0.5	2.9 ± 2.5
0.25 mM GSNO	17.1 ± 2.0	6.4 ± 1.0[b]	18.4 ± 1.2	5.0 ± 1.3[b]
0.5 mM GSNO	30.8 ± 2.2	24.7 ± 3.2	26.5 ± 2.1	25.6 ± 1.3

[a] RAW 264.7 macrophages were incubated 16 h prior to agonist treatment with 2.5 μM JNK1 and JNK2 antisense oligonucleotides (JNK1/2asn) or remained as controls. Cells were then exposed to GSNO (0.25 and 0.5 mM) or vehicle for 8 h with or without pretreatment with 5 μM SB 203580 for 30 min. Apoptotic cell death was determined with the DNA-specific fluorochrome Hoechst dye 33258. For details see Materials and Methods. Mean values (± standard deviation) of four different experiments are given. [b] $P < 0.01$ versus GSNO (250 μM)-treated control cells.

rophages by employing the p38 kinase inhibitor SB 203580 (*32*). At a low concentration (5 μM) SB 203580 acted as a specific p38 kinase inhibitor without altering the activity of other MAPK family members in response to GSNO (0.5 mM, 4 h) (data not shown).

To define the role of p38, cells were pretreated with 5 μM SB 203580 for 30 min followed by a subsequent

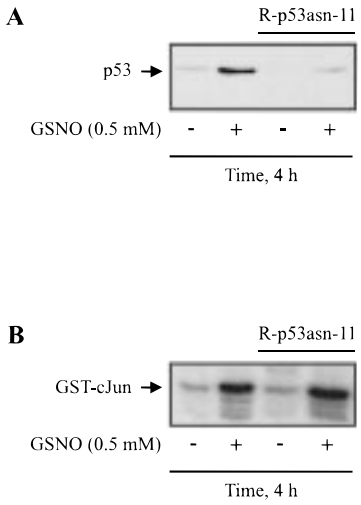

FIGURE 4: JNK1/2 activation proceeded unaltered in p53-deficient macrophages. RAW 264.7 and stably p53-antisense transfected RAW 264.7 macrophages (R-p53asn-11) were tested for p53 expression and JNK1/2 activity in response to GSNO (0.5 mM, 4 h) by Western blot analysis (150 μg of protein/lane) using a p53-antibody (A) and a radioactive kinase assay with the substrate GST-cJun (B). Western blot and kinase assay are representative for three similar experiments.

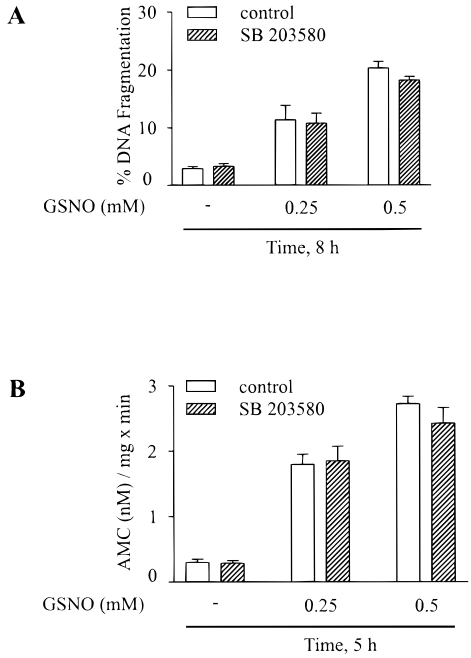

FIGURE 5: Inhibition of p38 kinase left GSNO-induced apoptosis unaltered. Macrophages were pretreated with 5 μM of the p38 MAPK inhibitor SB 203580 for 30 min or left as controls. SB 203580 was present throughout the incubations. Cells were stimulated with 0.25 or 0.5 mM GSNO for 8 h (A) or 5 h (B). DNA fragmentation was quantitated by the diphenylamine assay (A), whereas caspase-3 activity (B) was assayed by DEVD-AMC cleavage as outlined under Materials and Methods. Results represent the mean (± standard deviation) of four different experiments.

coincubation period with GSNO. DNA fragmentation in response to 0.25 or 0.5 mM GSNO remained unaffected by the addition of SB 203580 (Figure 5A). Moreover, SB 203580 did not influence caspase-3 activation following GSNO addition, neither in response to 0.25 mM nor with 0.5 mM (Figure 5B). In line, SB 203580 left p53 accumulation in GSNO-treated samples unaffected (data not shown). We also obtained evidence that a combination of SB 203580

and JNK1/2 antisense oligonucleotide treatment was equally effective in attenuating apoptosis as was JNK1/2 antisense oligonucleotide treatment alone (Table 1). As a result of this examination, it appeared that p38 kinases do not modulate GSNO-mediated apoptotic cell death in macrophages.

DISCUSSION

Our study provides evidence for MAPK activation by *S*-nitrosoglutathione (GSNO) and reveals a modulatory role of distinctive MAPK family members in affecting GSNO-mediated apoptotic cell death in macrophages. Whereas ERK1/2 activation contributed to protection, inhibition of p38 was without any interference. Low GSNO concentrations demanded activation of JNK1/2 to complete apoptosis, whereas the cell death program proceeded independently of JNK1/2 at higher GSNO concentrations. By analyzing p53 accumulation, caspase activation, and DNA fragmentation, we determined the impact of MAPK signaling relative to the occurrence of these apoptotic parameters.

$NO^{\bullet}$ emerged as an important biological messenger with diverse actions throughout physiology and pathology (*9*, *10*). Previous work appreciated the dual role of $NO^{\bullet}$ in either causing or preventing apoptotic cell death (*14*, *33*). Peritoneal macrophages and macrophage cell lines, among other systems, appear highly susceptible to a death promoting action of $NO^{\bullet}$ (*6*, *12*). The advanced knowledge of transducing components, especially the early accumulation of p53 prior to caspase-3 activation during pro-apoptotic signaling, makes macrophages a preferential model system to study $NO^{\bullet}$-mediated cell death, to elucidate MAPK activation by $NO^{\bullet}$, and to gain insights how MAPK family members modulate apoptosis. Since nitrosothiols may actually be the predominant form of biologically active $NO^{\bullet}$ in vivo, we studied modulation of MAPK family members by using GSNO.

In corroboration with other studies we realized the capacity of NO donors to cause tyrosine phosphorylation of various targets, among others, MAPK family members (*34*, *35*). Although not causatively related to apoptosis, $NO^{\bullet}$ has been reported to activate JNK1/2 in mesangial cells or chondrocytes (*36*−*38*) and has been examined as an activator of MAPK in Jurkat T cells (*39*). We present evidence for activation of all three members of MAPK, namely, ERK1/2, JNK1/2, and p38. However, activation of distinctive MAPK members was achieved with different time and concentration dependencies. In some analogy to the study performed in T cells (*39*), we noticed JNK1/2 to be highly sensitive, although macrophages responded with a much slower activation kinetic compared to T cells. This may point to different pathways of activation in response to NO donors. Rapid ERK1/2 activation in T cells occurred as a result of p21ras activation (*40*), whereas in mesangial cells $NO^{\bullet}$ elicited a fast ERK1/2 activation via the formation of cGMP and a delayed activity response that resulted from tyrosine phosphatase inhibition (*35*). Further examinations need to address molecular mechanisms underlying GSNO-mediated MAPK activation in RAW 264.7 macrophages. An activity increase may result from the combined action of GSNO on upstream kinases such as Ras-, Rac-, or Cdc42 (*18*, *40*) and/or may stem from inhibition of tyrosine phosphatases, i.e., MKP-1 or MKP-2, that function as deactivators of MAPK (*18*).

Treatment of macrophages with GSNO resulted in a time- and dose-dependent induction of apoptosis (*31*). Taking into consideration recent reports that link MAPK activation to inhibition or initiation of apoptosis, we wished to determine how attenuated MAPK affect GSNO-mediated macrophage apoptosis. By using specific inhibitors our results imply that ERK1/2 contributed to cell protection, p38 was without any interference, and JNK1/2 transmitted pro-apoptotic signals at low agonist concentrations.

JNK1/2, ERK1/2, and p38 kinases are regulatory proteins that convey extracellular signals such as growth factors, cytokines, or stress components into cellular responses (*16*). Kinase activation is implicated in a wide variety of responses ranging from proliferation to cell death. By eliminating JNK1/2 activity with antisense oligonucleotides (*27*), our experiments are in line with other reports that propose a pro-apoptotic role of JNK1/2 (*19*, *22*). Our examinations enabled us to put JNK1/2 activation upstream of the GSNO-evoked p53 response in macrophages by focusing on final apoptotic parameters such as DNA fragmentation and intermediate signaling components such as caspase-3 activation. Phosphorylation of p53 by JNK1/2 is known (*41*−*43*) and is described as one mechanism by which the protein half-life is prolonged (*41*), thus favoring a pro-apoptotic function of the tumor suppressor. Supporting evidence for the positioning of JNK1/2 upstream of p53 stems from analysis in p53-antisense constructed cells. Evidently, JNK1/2 activation proceeded unaltered irrespective to an attenuated p53 response. However, further analysis at higher agonist, i.e., GSNO concentrations revealed a different picture. Clearly, at higher concentrations GSNO is endowed with the ability to promote macrophage apoptosis independently of JNK1/2. The use of different pathways to promote apoptosis at various agonist concentrations is not surprising and may add an additional regulatory component that allows the modulation of pro-apoptotic pathways. Further experiments will address JNK1/2-independent mechanisms that stabilize p53 in response to GSNO and promote apoptosis.

In contrast to JNK1/2, activation of ERK1/2 attributes to cell survival. Inhibition of ERK1/2 by the MEK-1 inhibitor PD 98059 enhanced GSNO-induced apoptosis. Increased cell death became evident at the level of p53 accumulation, caspase-3 activation, and DNA fragmentation. Enhanced GSNO-mediated apoptosis by ERK1/2 inhibition is in good agreement with studies where PD 98059 increased H_2O_2-elicited apoptosis in HeLa cells (*22*). The relationship between ERK1/2 activation and apoptosis in response to GSNO seems consistent with that seen in other stress conditions that result in cell death, including UV-irradiation or growth factor withdrawal (*23*, *24*). Our results support the general hypothesis that the dynamic balance between ERK and JNK pathways modulates cell survival and apoptosis (*24*). In analogy to peroxide treatment, we noticed activation of all kinase members which is in contrast to the concept put forward for growth factor withdrawal and initiation of apoptosis, with the exclusive activation of distinctive MAPK members. Small shifts in the kinase balance achieved through manipulations of either the ERK or JNK1/2 pathway produces changes in the outcome of apoptosis. It is therefore likely that the differences in sensitivity of various cells to the apoptotic effects of $NO^{\bullet}$ reflect the relative activation of MAPK family members. Our

experiments imply that GSNO-evoked ERK activation may only respresent a minor component in protection from macrophage apoptosis, whereas the JNK pathway predominated at low agonist concentrations. We assume that the balance of kinase activation contributes, but not only regulates entry into apoptosis. In macrophages the proapoptotic efficacy of GSNO, with the activation of cell death initiating pathways, overrules the potency of GSNO-evoked antiapoptotic systems. This may be cell specific, may depend on the timing of MAPK activation, and may be related to the potency of the tumor suppressor p53 to signal apoptosis.

In conclusion, our study provides insights into the contribution of ERK1/2, JNK1/2, and p38 kinases in modulating GSNO-initiated macrophage apoptosis. We noticed a cell protective role of ERK1/2 and a death-promoting action of JNK1/2 that was restricted to low agonist concentrations. These conditions allowed to position JNK1/2 upstream of the p53 response. However, at higher concentrations of GSNO p53 accumulation and apoptosis were JNK1/2 unrelated. Studies are under way to identify MAPK upstream signaling components that may contribute to the decision of a cell to survive or to enter apoptosis in response to GSNO.

ACKNOWLEDGMENT

We thank Brigitte Rogge for excellent technical assistance.

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BI982292A