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Protection against Nitric Oxide-Induced Apoptosis in Rat Mesangial Cells Demands Mitogen-Activated Protein Kinases and Reduced Glutathione

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ABSTRACT

Inflammatory diseases such as proliferative glomerulonephritis are associated with the production of nitric oxide (NO), which can initiate apoptotic/necrotic cell death. We studied the role of the p42/44 mitogen-activated protein kinases (MAPKs) and c-Jun N-terminal kinases1/2 (JNK1/2) in NO-evoked cytotoxicity in rat mesangial cells (MC). The NO donor *S*-nitrosoglutathione time- and concentration-dependently promoted apoptotic cell death as detected by JNK1/2 and caspase-3 activation as well as DNA fragmentation. By using Ro 318220, a JNK1/2 activator, we established a correlation between apoptosis and JNK1/2 activation. Apoptosis is antagonized by the addition of fetal calf serum or the simultaneous generation of NO and superoxide (O_2^-), another biological inflammatory mediator. Fetal calf serum-induced protection required p42/44 MAPK activation as inhibition of the p42/44 MAPK pathway by the MAPK kinase-1 inhibitor PD 98059 attenuated MC protection. In contrast, cytoprotection by NO/O_2^- cogeneration demanded reduced glutathione but was p42/44 MAPK unrelated. Depletion of glutathione reversed NO/O_2^--evoked survival to cell destruction and reinstalled JNK1/2 activity. In conclusion, different signal transduction pathways facilitate protection against NO-induced JNK1/2 activation and apoptosis in rat MC.

Mammalian cells have developed a mechanism to diminish unwanted, damaged, or futile cells. This process, known as apoptosis, is initiated by diverse actions such as DNA damage, growth factor withdrawal, or radical generation or as a result of receptor occupancy. During the initiation of apoptosis, different proapoptotic and antiapoptotic proteins are up- or down-regulated, thus promoting the activation of caspases, chromatin condensation, and DNA fragmentation (Hale et al., 1996). A tight regulation of apoptosis, similar to proliferation, is indispensable to maintain a homeostatic cell number otherwise resulting in pathological conditions, exemplified by inflammatory kidney diseases. Under conditions of IgA nephropathy or anti-Thy1.1 nephritis, an early lysis of glomerular mesangial cells (MC) is followed by mesangial hypercellularity (Savill et al., 1995). Although hypercellularity is a well recognized risk factor for progression to irreversible scarring, increases in the number of MC are reversible. Removal of surplus MC mainly occurs by an apoptotic process (Baker et al., 1994; Shimizu et al., 1995). This phenomenon correlates with a significant production of nitric oxide (NO) and can be prevented by pretreatment with the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (Cattel et al., 1993). NO is synthesized by oxidation of L-arginine, catalyzed by a family of NO synthases. Once NO is produced, it elicits diverse physiological and pathophysiological functions such as endothelium-dependent relaxation, neurotransmission, cell-mediated immune responses, and induction of apoptosis (Nathan, 1992; Brüne et al., 1998). Cellular toxicity of NO may result from the interaction with oxygen, superoxide (O_2^-), or transition metals. In the case of O_2^-, the NO/O_2^- reaction product can be cytotoxic or protective depending on the cell type (Lin et al., 1995). In cultured rat MC, stimulation with NO or O_2^- induces apoptosis, whereas the simultaneous generation of NO and O_2^- in a balanced proportion is cell protective (Sandau et al., 1997a). However, underlying mechanisms are still unclear.

Besides NO, growth factors (GFs) play an important role in the pathogenesis of glomerular inflammation. Some of these mediators stimulate proliferation, whereas others drive excessive matrix accumulation (Johnson et al., 1992). In addition, GFs promote MC survival by attenuating apoptosis induced by serum withdrawal (Mooney et al., 1997). Studies

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ABBREVIATIONS: MC, mesangial cells; GSNO, *S*-nitrosoglutathione; DMNQ, 2,3-dimethoxy-1,4-naphtoquinone; IGF, insulin growth factor; DTT, dithiothreitol; NO, nitric oxide; O_2^-, superoxide; JNK1/2, c-Jun N-terminal kinases1/2; MAPK, mitogen-activated protein kinases; BSO, L-buthionine-sulfoxamine; LDH, lactate dehydrogenase; GSH, glutathione; MKP-1, mitogen-activated protein kinase phosphatase-1; GF, growth factor; MEK, mitogen-activated protein kinase kinase; DEVD, *N*-Acetyl-Asp-Glu-Val-Asp-7; AMC, amino-4-methylcoumarin.

in different cell types underscored the protective character of GFs, also against NO toxicity (Spear et al., 1997), but examinations in MC are elusive. Known targets for GFs or fetal calf serum (FCS) are the p42/44 mitogen-activated protein kinases (Ottlinger et al., 1993), which belong to the superfamily of serine/threonine kinases, named mitogen-activated protein kinases (MAPK). MAPK, which also encompass the stress-activated protein kinases c-Jun N-terminal kinases1/2 (JNK1/2), become activated by upstream kinase cascades via dual tyrosine and threonine phosphorylation (Guan, 1994; Seger and Krebs, 1995). Activated MAPK translocate in the nucleus, where they phosphorylate transcription factors such as ternary complex factor/Elk-1, c-Jun, or activating transcription factor-2 (Robinson and Cobb, 1997). The individual activation and cross-talk between MAPK family members allow cells to react on a specific stimulus with proliferation, differentiation, or death. The role of MAPK activation in promoting cell death or survival is controversially discussed. Some studies connect p42/44 MAPK activation with cell protection, whereas JNK1/2 activation is associated with apoptosis (Jarvis et al., 1997; Wang et al., 1998). It is also proposed that the balance between p42/44 MAPK and JNK1/2 activation determines cytoprotection or destruction (Xia et al., 1995; Sanchez-Perez et al., 1998).

The aim of our study was to analyze the modulatory role of p42/44 MAPK and JNK1/2 in NO-evoked apoptosis and for MC protection. We found a selective activation of JNK1/2 in response to NO and Ro 318220, a JNK1/2 activator, and a direct correlation of JNK1/2 activation and apoptosis. MC are rescued from apoptosis by the addition of FCS or the formation of O_2^- relative to NO. Cell protection by FCS is achieved by p42/44 MAPK activation, whereas cell survival as a result of NO/O_2^- interaction is solely based on the compensatory antioxidant effect of intracellular glutathione. We conclude that individual MAPK family members and the glutathione-redox system determine death and survival.

Experimental Procedures

Materials. Insulin, diphenylamine, triethanolamine, and L-buthionine-sulfoximine were purchased from Sigma (Deisenhofen, Germany). NADH and pyruvate were purchased from Boehringer Mannheim (Mannheim, Germany). RPMI 1640 and medium supplements were ordered form Biochrom (Berlin, Germany). FCS was purchased from Life Technologies (Berlin, Germany). Phosphospecific p42/44 antibody was obtained from New England Biolabs GmbH (Schwalbach/Taunus, Germany). The secondary antibody was purchased from Promega/Serva (Heidelberg, Germany). JNK1/2 antibody was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Enhanced chemiluminescence detection reagents and [γ-^{32}P]ATP were obtained from Amersham (Braunschweig, Germany). *N*-Acetyl-Asp-Glu-Val-Asp-7 (DEVD)-amino-4-methylcoumarin (AMC), AMC, and 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate were purchased from Biomol (Hamburg, Germany). PD 98059, 2,3-dimethoxy-1,4-naphtoquinone (DMNQ), and Ro 318220 came from Calbiochem (Bad Soden, Germany). The glutathione-*S*-transferase (GST)-c-Jun (fusion protein, 1/166) expression plasmid was kindly provided by Prof. P. Angel (Heidelberg, Germany). *S*-nitrosoglutathione (GSNO) was synthesized as described previously (Sandau et al., 1997a). All other chemicals were of the highest grade of purity and commercially available.

Culture of MC. Rat MC were cultured, cloned, and characterized as described previously (Pfeilschifter and Vosbeck, 1991). Cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml bovine insulin. One day before and during the experiments, cells were kept in medium without FCS. Passages 10 to 25 of MC and 2.5×10^5 cells/assay were used for DNA fragmentation, lactate dehydrogenase (LDH) release, or caspase-3 activity analysis, whereas 5×10^6 cells/assay were used for the kinase assay and the Western blot.

JNK1/2 Activity Assay. Confluent rat MC 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 (10,000*g*, 20 min, 2°C). Protein (300 μg) of the supernatant, in an equalized sample volume, was used for immunoprecipitation. Anti-JNK1/2 antibodies were added, followed by gentle rotation at 4°C for 2 h. After 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 dithiothreitol (DTT), and 0.1 mM sodium vanadate). Immune complexes were centrifuged (10,000*g*, 2 min, 4°C) and resuspended in 25 μl of kinase buffer with the addition of 2 μg of GST-c-Jun and 5 μCi of [γ-^{32}P]ATP. Phosphorylation was performed for 20 min at 37°C. Reactions were stopped by the addition of 25 μl of 2× SDS sample buffer. Samples were boiled for 5 min at 95°C, and proteins were separated on 10% SDS-polyacrylamide gels. Gels were fixed, dried, and subjected to autoradiography.

Western Blot Analysis. After individual incubations, medium was removed and cells were scraped into ice-cold PBS (supplemented with 1 mM sodium vanadate, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride), centrifuged (700*g*, 10 min, 4°C), and resuspended in 200 μl of lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 1 mM sodium fluoride, pH 8.0). Cell debris was sonicated with a Branson sonifier (10 s, duty cycle 100%, output control 1) and centrifuged (14,000*g*, 15 min), and protein content was measured. 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% 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 marker. Transblots were washed with TBS (140 mM NaCl, 50 mM Tris · HCl, pH 7.2) containing 0.06% Tween-20 before blocking with TBS/5% skim milk. Phosphospecific p42/44 MAPK antibodies, dissolved in TBS/5% skim milk at a final concentration of 0.1 μg/ml, were incubated overnight at 4°C. Blots were washed five times before the anti-rabbit peroxidase-labeled secondary polyclonal antibody was added (1 h, diluted 1:10,000 in TBS/0.5% milk). Blots were washed five times, followed by enhanced chemiluminescence detection.

Fluorogenic Caspase-3 Assay. After individual incubations, MC were recovered and centrifuged (1200*g*, 5 min). Pellets were resuspended in lysis buffer [100 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM DTT], left on ice for 30 min, sonified (Branson sonifier, 10 s, duty cycle 100%, output control 1), and centrifuged (10,000*g*, 10 min, 4°C). Cytosolic protein (30 μg) was incubated with 12 μM 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 quantified by internal AMC standards.

DNA Fragmentation. DNA fragmentation was quantified by use of the diphenylamine assay as previously reported (Sandau et al., 1997a). Briefly, after incubation, MC 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 (10,000*g*, 15 min) allowed recovery of intact chromatin (pellet) and DNA frag-

ments (supernatant). Pellets were resuspended in 500 μl of TE buffer and again precipitated overnight with 500 μl of 10% trichloroacetic acid at 4°C. After centrifugation (4000*g*, 10 min), the supernatant was removed. After the addition of 150 μl of 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantified 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.

LDH Release. The percentage of LDH release from cells is a determinant of cellular necrosis and expressed as the proportion of LDH released into the medium compared with the total amount of LDH present in intact cells. Total LDH was determined after 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. Statistical probability is expressed as $*p < .05$ and $**p < .01$. Normal distribution of data is ensured. Otherwise, representative data of at least three similar examinations are shown.

Results

JNK1/2 Activation by NO is Time- and Concentration-Dependent. Previous studies have shown that different NO donors, such as GSNO, spermine-NO, and *S*-nitroso-*N*-acetylpenicillamine, caused apoptosis in rat MC as characterized by chromatin condensation, DNA fragmentation, and accumulation of the tumor suppressor p53 (Sandau et al., 1997b). With the notion that activation of the JNK1/2 pathway may promote cell death (Jarvis et al., 1997; Sanchez-Perez et al., 1998), we examined JNK1/2 activation in response to the NO donor GSNO in rat MC.

GSNO (1 mM) time-dependently promoted JNK1/2 activation as determined in a direct kinase assay using the substrate GST-c-Jun. Activation became evident after 1 h and was strongest at 2 and 4 h. Although activity then declined, it remained slightly elevated for the next 12 h (Fig. 1A). In further experiments, we established a dose-dependent JNK1/2 activity increase during a 4-h incubation period. Low GSNO concentrations (250 μM, 4 h) activated JNK1/2, which became more pronounced at higher concentrations and was strongest at a 0.75 to 1 mM concentration of the NO donor (Fig. 1B). JNK1/2 activation in response to NO is in close correlation to GSNO-induced apoptosis as described in earlier studies (Sandau et al., 1997b). Therefore, we assume a participation of the JNK1/2 pathway in NO-mediated apoptosis. In addition, we examined the time dependence of GSNO-evoked p42/44 MAPK activation. Only high concentrations of the NO donor caused p42/44 MAPK activation (Callsen et al., 1998), whereas 250 μM GSNO left p42/44 MAPK activation unaltered (data not shown). By proposing activation of JNK1/2 in promoting apoptosis, it should be possible to achieve cell death through manipulation of activation/deactivation of JNK1/2.

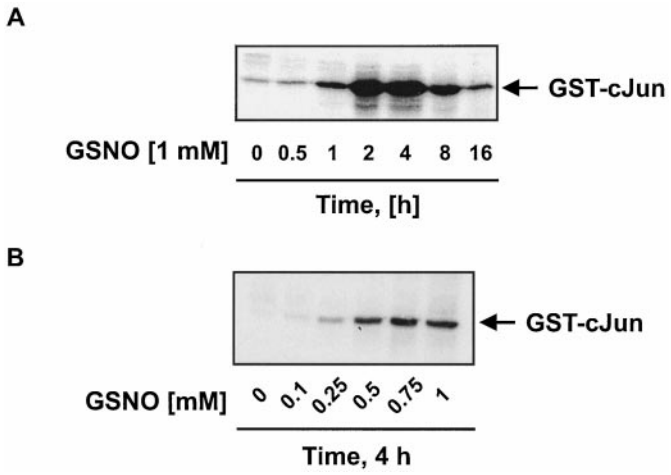

Fig. 1. Dose- and time-dependent JNK1/2 activation by GSNO. A, confluent and quiescent rat MC were stimulated with 1 mM GSNO and JNK1/2 activity was analyzed at times indicated. B, alternatively, MC were exposed to increasing concentrations of GSNO for 4 h or remained as controls. JNK1/2 activation was determined by a radioactive kinase assay using GST-c-Jun as a substrate as described in the text. Blots are representative of three similar experiments.

Ro 318220 Mediated JNK1/2 and Caspase-3 Activation. In the following experiments, we used the JNK1/2 activator Ro 318220 to determine whether sustained JNK1/2 activation evokes apoptosis in rat MC. Ro 318220, originally characterized as a specific protein kinase C inhibitor (Davis et al., 1992), was identified to activate JNK1/2 via inhibition of mitogen-activated protein kinase phosphatase-1 (MKP-1) expression (Beltman et al., 1996). As expected, we noticed a significant increase in JNK1/2 activity after the addition of Ro 318220 or after coincubation of Ro 318220 with GSNO.

For these experiments, rat MC were pretreated with 10 μM Ro 318220 for 30 min, followed by a 4-h lasting coincubation period with vehicle or 250 μM GSNO. GSNO as well as Ro 318220 activated JNK1/2, whereas coincubation of Ro 318220 with GSNO increased the effect (Fig. 2A). For control reasons, we ensured that p42/44 MAPK remained unaffected by Ro 3118220 treatment (data not shown).

Caspase-3 activation is considered a convenient marker of apoptosis and is regarded as the point of no return in the proapoptotic signaling cascade. GSNO induced the cleavage of the fluorogenic caspase-3 substrate DEVD-AMC and promoted AMC accumulation in apoptotic cells. AMC production increased in response to 250 μM GSNO after an incubation period of 8 h compared with unstimulated cells. A similar increase in caspase-3 activity occurred after the treatment with 10 μM Ro 318220 for 8 h. Coincubation of rat MC with 250 μM GSNO and 10 μM Ro 318220 significantly enhanced GSNO-evoked caspase-3 activation (Fig. 2B). To ensure that the effects of Ro 318220 are based on JNK1/2 activation and not on side effects due to protein kinase C inhibition, we repeated the experiments with staurosporine (40 nM), a well known protein kinase C inhibitor. In contrast to Ro 318220, staurosporine did not promote JNK1/2 activation, nor was the apoptotic rate affected (data not shown). In conclusion, JNK1/2 activation by Ro 318220 or GSNO is correlated to apoptotic cell death in rat MC.

FCS Promoted Cell Survival via p42/44 MAPK Pathway. MC elaborate diverse mechanisms to protect themselves against toxic insults. Protection is achieved via the simultaneous generation of O_2^- relative to NO production (Sandau et al., 1997a) and/or secretion of growth factors (Mooney et al., 1997). Although these protective factors are described, little is known about intracellular pathways that signal cell survival.

To establish a protective role of FCS against NO-mediated apoptosis, we assayed DNA fragmentation in response to 250

μM GSNO with or without the further addition of 10% FCS. Supplementation of FCS completely abrogated GSNO-induced DNA fragmentation (Fig. 3). To unravel FCS-evoked transducing pathways, we probed for p42/44 MAPK activation, which is considered a protective mechanism (Xia et al., 1995; Wang et al., 1998). Incubations of MC with 10% FCS resulted in rapid p42/44 MAPK activation (Fig. 3, inset) when determined by Western blot analysis using a phosphospecific p42/44 MAPK antibody that showed reactivity to the tyrosine-phosphorylated and thus activated forms of p42/44 MAPK only. To elucidate any contribution of p42/44 MAPK activation during FCS-induced cell survival, we interrupted p42/44 MAPK signaling by using 10 μM PD 98059, an MAPK kinase-1 (MEK-1) inhibitor (Alessi et al., 1995). MEK-1 is the immediate upstream kinase that phosphorylates and thereby activates p42/44 MAPK. The inhibitory effect of PD 98059 toward FCS-induced p42/44 MAPK phosphorylation was ensured by a Western blot against the phosphorylated form of the p42/44 MAPK (Fig. 3, inset). During DNA fragmentation analysis, PD 98059 (10 μM), incubated for 24 h, slightly increased apoptotic cell death by itself. Higher concentrations of PD 98059 were cytotoxic for MC (data not shown); therefore, any inhibition of p42/44 MAPK activity was compensated for by the onset of apoptosis, which restricted the use of PD 98059 for detailed dose-response studies. Importantly, PD 98059 partly reversed FCS-evoked protection toward GSNO-mediated apoptosis (Fig. 3). This is in correlation with the p42/44 MAPK activation pattern in which 10% FCS initiated p42/44 MAPK phosphorylation, even in the presence of 250 μM GSNO. GSNO itself had no effect on p42/44 MAPK activation (data not shown); however, p42/44 MAPK activation by FCS was suppressed by the addition of 10 μM PD 98059 (Fig. 3, inset). In conclusion, by using PD 98059, our data implicate a protective principle of FCS-activated p42/44 MAPK during NO-mediated apoptosis in rat MC.

Coincubation of NO/O_2^- Abolished Caspase-3 and JNK1/2 Activation. Besides FCS, the simultaneous generation of NO/O_2^- in a balanced ratio protects cultured rat MC against the toxicity of NO or O_2^- when applied individually (Sandau et al., 1997a). In corroboration with earlier reports, we observed NO- or O_2^--induced apoptosis in rat MC. This was verified in this study based on caspase-3 activation with the notion that coincubation of NO and O_2^- was cross-protective (Fig. 4A).

The addition of 250 μM GSNO for 24 h increased caspase-3 activity compared with unstimulated controls. O_2^-, generated by 1, 5, and 10 μM concentrations of the redox cycler DMNQ, which penetrates the plasma membrane and releases O_2^- inside the cell as measured by the cytochrome C assay (data not shown), dose-dependently initiated caspase-3 activation (Fig. 4A).

In extending experiments, we coincubated MC with GSNO (250 μM) and increasing DMNQ concentrations (1, 5, and 10 μM) for 24 h. Caspase-3 activity declined toward GSNO treatment when cells were costimulated with GSNO and DMNQ (Fig. 4A). Similar effects were seen by using other NO- and O_2^--generating systems such as spermine-NO and

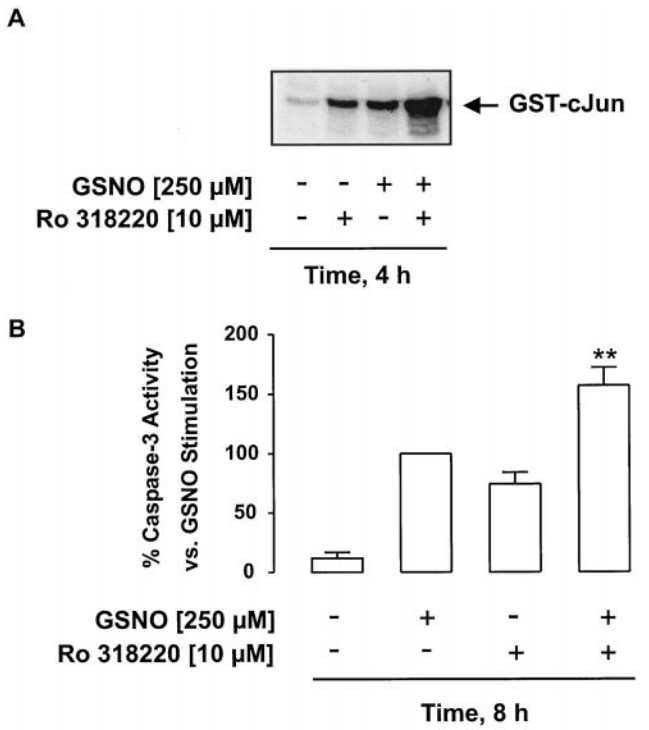

Fig. 2. Ro 318220 enhances GSNO-induced JNK1/2 and caspase activation. MC were exposed to GSNO (250 μM), the MKP-1 inhibitor Ro 318220 (10 μM), or remained as controls. Ro 318220 was preincubated for 30 min. A, JNK1/2 activity was analyzed in a radioactive kinase assay after a 4-h incubation period. B, caspase-3 activity was determined by the cleavage of DEVD-AMC after 8 h. For details, see *Experimental Procedures*. GSNO-induced caspase-3 activation was set 100%, and other values were calculated proportionally to GSNO. Caspase-3 activity values are the mean ± S.D. of four different experiments (**significant versus GSNO-treated cells), whereas the kinase assay is representative of four similar experiments.

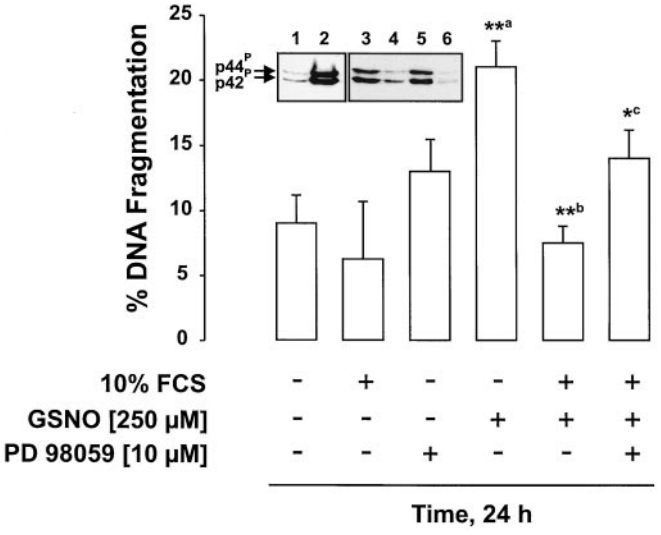

Fig. 3. GSNO-induced apoptosis is attenuated by FCS via p42/44 MAPK activation. Inset, MC were exposed to vehicle (lane 1), 10% FCS (lanes 2 and 3), 10% FCS in combination with 10 μM PD 98059 (lane 4), 250 μM GSNO (lane 5), or both agents (lane 6). Activation/phosphorylation of p42/44 MAPK was detected as outlined in *Experimental Procedures* by Western blot analysis with a phosphospecific p44/42 MAPK antibody ($p42^P/p44^P$). p42/44 MAPK activation in lanes 1 and 2 was detected after 5 min, whereas the inhibitory effect of PD 98059 was analyzed after 24 h (lanes 3–6). For DNA fragmentation, MC were cultured for 24 h with or without 10% FCS, the MEK-1 inhibitor PD 98059 (10 μM), and/or GSNO (250 μM) as indicated. DNA fragmentation was quantified using the diphenylamine assay described in the text. Data are mean ± S.D. values of at least five separate experiments (**, [a], significant versus control, **, [b], significant versus GSNO, *, [c], significant versus GSNO/FCS).

the hypoxanthine/xanthine oxidase system. After the assumption that JNK1/2 activation is associated with apoptosis, we determined phosphorylation of GST-c-Jun under conditions of NO/O_2^- cogeneration. Interestingly, GSNO (250 μM, 4 h)-induced JNK1/2 activation was reduced with increasing DMNQ concentrations. Activation vanished completely with the combination of GSNO (250 μM) and 10 μM DMNQ (Fig. 4B). Intriguingly, GSNO/DMNQ coincubation did not activate p42/44 MAPK (data not shown). In line, PD 98059 left NO/O_2^--mediated protection unaltered as determined by DNA fragmentation and caspase-3 activation analysis (data not shown). These examinations propose that protection evoked by NO/O_2^- coformation is p42/44 MAPK independent.

NO/O_2^- Protection Demands Intracellular GSH. The intracellular redox agent GSH plays a crucial role in intercepting oxidative and/or nitrosative stress and therefore is an important determinant in controlling stress-evoked apoptosis. To assess the role of intracellular GSH in NO/O_2^--mediated protection in rat MC, we depleted the GSH pool by 90% with a 24-h lasting addition of 500 μM L-buthionine-sulfoxamine (BSO; data not shown), a specific γ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of GSH synthesis (Richman et al., 1973).

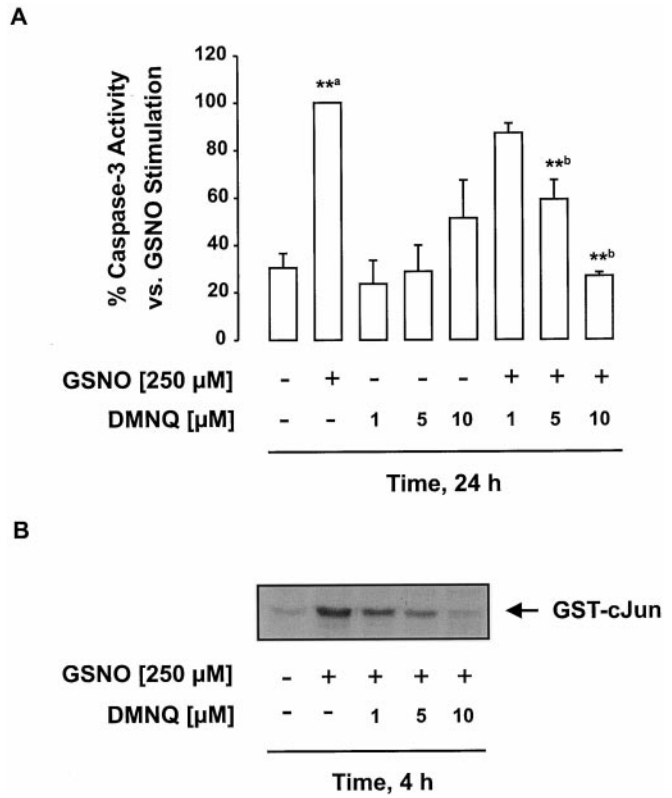

Fig. 4. GSNO-induced caspase-3 and JNK1/2 activation is abrogated by DMNQ. Rat MC were cultured in the absence or presence of 250 μM GSNO and/or increasing concentrations of DMNQ (1, 5, and 10 μM). A, caspase-3 activation was determined by the cleavage of DEVD-AMC after 24 h as described in *Experimental Procedures*. GSNO-induced caspase-3 activation was set at 100%, and other values were calculated proportionally to GSNO. Data are mean ± S.D. values of five separate experiments (**, [a], significant versus control; **, [b], significant versus GSNO). B, JNK1/2 activation was measured after 4 h by a direct kinase assay using GST-c-Jun as a substrate. The blot is representative for three similar experiments.

In a first set of experiments, we analyzed DNA fragmentation and LDH release as a result of 250 μM GSNO or 10 μM DMNQ addition (Table 1). The simultaneous addition of GSNO and DMNQ significantly lowered DNA fragmentation and LDH release compared with GSNO as shown in Table 1. Reduced DNA fragmentation achieved by NO/O_2^- coincubation did not result in a shift toward necrosis as confirmed by LDH measurements. However, GSH depletion by BSO enhanced GSNO-evoked cytotoxicity, shifting apoptosis to necrosis. Lowering GSH also completely abolished NO/O_2^--mediated protection, resulting in enhanced apoptosis and necrosis (Table 1) and thus suggesting GSH is a critical parameter for NO/O_2^--mediated cell protection in rat MC.

We further verified these results for JNK1/2 activation (Fig. 5). Kinase assays revealed that BSO (500 μM, 24 h) treatment did not activate JNK1/2. However, GSNO (250 μM, 4 h)-evoked JNK1/2 activation was dramatically enhanced by BSO prestimulation (Fig. 5A). In addition, GSH-depleted cells showed massive JNK1/2 activation after a 4-h lasting exposure period with GSNO (250 μM) and DMNQ (10 μM) that otherwise was abrogated in GSH-containing cells (Fig. 5B). GSH depletion obviously eliminates NO/O_2^--mediated protection and reestablishes JNK1/2 activation.

Ro 318220 Abolished NO/O_2^--Mediated Protection. To position JNK1/2 as a downstream event during NO/O_2^- protection, we investigated apoptotic cell death under conditions of NO/O_2^- cogeneration in response to Ro 318220. MC were treated with 10 μM Ro 318220 in the presence or absence of 250 μM GSNO and 10 μM DMNQ for 4 h. Activation of JNK1/2 was evident in response to Ro 318220 but was absent in GSNO/DMNQ-treated cells. Coincubation of Ro 318220, GSNO, and DMNQ resulted in JNK1/2 activation (Fig. 6A). These data were further confirmed during DNA fragmentation analysis.

Exposure of MC for 24 h with 250 μM GSNO resulted in 24% DNA fragmentation, whereas coincubation with 10 μM DMNQ significantly reduced DNA fragmentation. In line with caspase-3 activation (Fig. 2B), Ro 318220 also promoted massive DNA fragmentation. Coincubation of Ro 318220 with GSNO/DMNQ for 24 h completely abrogated the GSNO/DMNQ-induced protective principle and allowed 40% DNA fragmentation (Fig. 6B). With respect to initiation of apoptosis, we suggest that JNK1/2 activation can be positioned

TABLE 1
GSNO/DMNQ-evoked protection was attenuated by glutathione depletion
MC were preincubated for 24 h with or without BSO (500 μM). Then, vehicle (control), GSNO (0.25 mM), DMNQ (10 μM), or the combination of GSNO and DMNQ was added for 24 h. DNA fragmentation was quantified by the diphenylamine assay, and necrosis was measured by LDH release. For details, see *Experimental Procedures*. Data are mean values (±S.D.) of more than four separate experiments.

	DNA Fragmentation		LDH Release	
	−BSO	+BSO (500 μM)	−BSO	+BSO (500 μM)
	%			
Control	9 ± 2	14 ± 3	6 ± 2	8 ± 2
GSNO (0.25 mM)	24 ± 5	26 ± 3	16 ± 3	40 ± 4[c]
DMNQ (10 μM)	13 ± 2	20 ± 2	5 ± 1	13 ± 4
GSNO (0.25 mM) + DMNQ (10 μM)	17 ± 2[a]	25 ± 2	7 ± 0[d]	45 ± 3[b]

[a] Significant versus GSNO without BSO (DNA fragmentation).
[b] Significant versus GSNO/DMNQ without BSO (LDH release).
[c] Significant versus GSNO without BSO (LDH release).
[d] Significant versus GSNO without BSO (LDH release).

downstream of the point at which NO/O_2^--mediated protection interferes because JNK1/2 activation as a result of MKP-1 inhibition circumvents NO/O_2^--evoked cell survival.

Discussion

Apoptosis is an indispensable and continuously occurring process that eliminates obsolete, damaged, or futile cells, whereas uncontrolled apoptosis culminates in various diseases. The induction of apoptosis in response to various agonists is dependent on the signal strength, cellular self-defense mechanisms, and promoting or antagonizing signaling pathways. NO represents a stimulus with diverse properties. When produced at low concentrations, NO elicits cGMP-dependent, physiological functions (Schmidt and Walter, 1994). In contrast, under inflammatory settings, large amounts of NO are generated, which lead to cell destruction as described for mesangiolysis during proliferative glomerulonephritis (Cattell et al., 1993). NO-induced apoptosis has been shown for macrophages, neurons, and thymocytes among others (Brüne et al., 1998). In MC, NO initiates typical apoptotic features such as DNA fragmentation, chromatin condensation, and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling-positive reactions, which are preceded by accumulation of the tumor suppressor p53 and Bax (Sandau et al., 1997b) and down-regulation of the antiapoptotic protein Bcl-2 (K.B.S. and B.B., unpublished data). Altered protein expression occurs between 2 and 4 h after NO stimulation, but earlier signaling pathways remain unknown. Therefore, we concentrated on the JNK1/2 pathway, which is proposed as an early signal transduction mechanism associated with cell death. In line with an earlier observation (Pfeilschifter and Huwiler, 1996), we noticed a concentration- and time-dependent JNK1/2 activation by NO (Fig. 1, A and B). To establish a correlation between JNK1/2 activation and mesangial cell death, we manipulated JNK1/2 activation/deactivation by using the MKP-1 phosphatase inhibitor Ro 318220. MKP-1 was shown to dephosphorylate/inactivate JNK1/2, and therefore, treatment with Ro 318220 evoked JNK1/2 activation (Beltman et al., 1996). JNK1/2 activation as a result of Ro 318220 treatment was established (Fig. 2A, lane 2) in a JNK1/2 kinase assay with GST-c-Jun as the substrate, with the notion that coincubation of Ro 318220 and GSNO even enhanced JNK1/2 activity. By measuring caspase-3 activation (Fig. 2B), we demonstrated a direct correlation of JNK1/2 activation and the rate of apoptosis. Conclusively, the JNK1/2 pathway is an important and early signal transducing mechanism in NO-induced apoptosis in rat MC. For RAW macrophages, we have shown earlier in antisense experiments that JNK1/2 is located upstream of p53 accumulation in the NO-initiated apoptotic pathway (Callsen and Brüne, 1999), whereas others have described that JNK1/2 can phosphorylate p53 and thus prolong its half-life (Fuchs et al., 1997, 1998). These interactions may explain how JNK1/2 activation promotes apoptosis.

As discussed earlier, the apoptosis-inducing abilities are affected by other mediators such as oxidants or cytokines; in

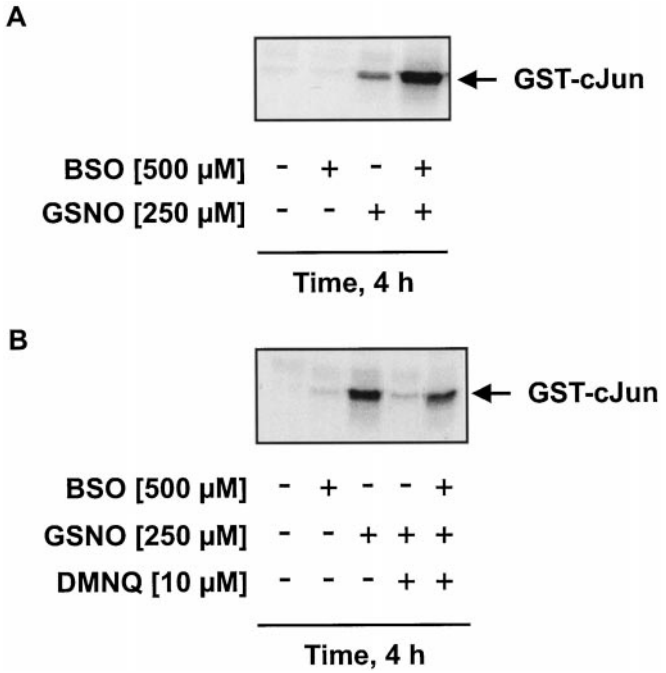

Fig. 5. Depletion of GSH enhanced JNK1/2 activation. Rat MC were exposed to 250 μM GSNO (A) or the combination of GSNO/10 μM DMNQ (B) or were kept as controls for 4 h. BSO (500 μM) was preincubated for 24 h. JNK1/2 activity was measured in a direct kinase assay as described in the text. Results are representative of five separate experiments.

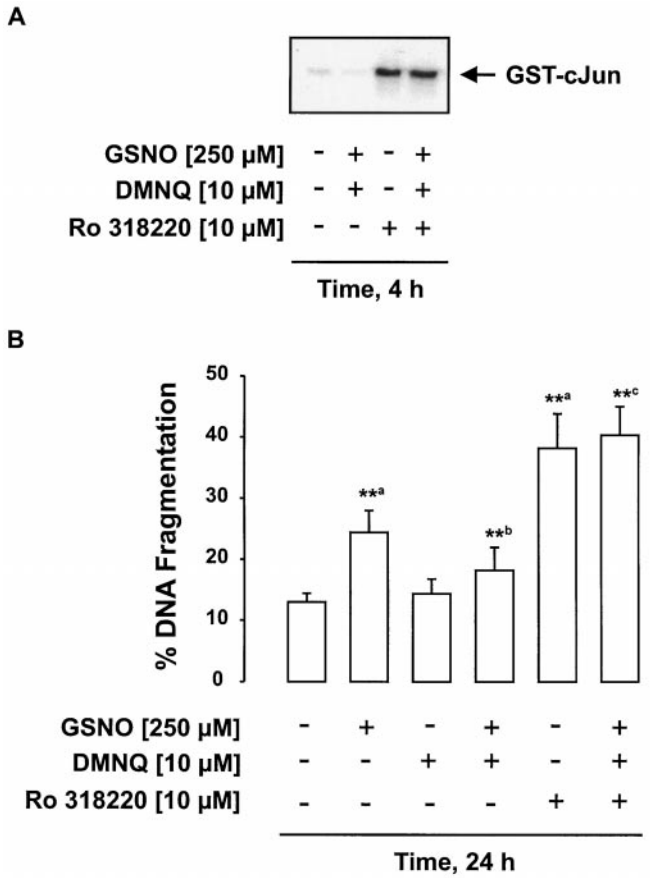

Fig. 6. Ro 318220 abolished GSNO/DMNQ-elicited protection. Rat MC were stimulated with GSNO (250 μM) and/or DMNQ (10 μM) with or without 10 μM Ro 318220. A, JNK1/2 activity was measured after 4 h in a direct kinase assay. B, DNA fragmentation was determined after 24 h using the diphenylamine assay as outlined in *Experimental Procedures*. Results are mean ± S.D. values of five separate experiments (**, [a], significant versus control; **, [b], significant versus GSNO; **, [c], significant versus GSNO/DMNQ). The blot is representative for three similar experiments.

addition, they are controlled by cellular self-defense mechanisms. This is exemplified for the NO system. Although NO generally induces apoptosis in MC, endogenously produced NO is not toxic. Detoxification of endogenously produced NO is achieved via the simultaneous generation of O_2^- (Sandau et al., 1997a). Experimentally, this is reproduced by the addition of a NO donor in combination with an O_2^-generating system. Here, we used GSNO and the redox cycler DMNQ, but combinations of spermine-NO with DMNQ or the O_2^--generating hypoxanthine/xanthine oxidase system work equally well (Sandau et al., 1997a). Based on caspase-3 activity determinations as an apoptotic marker, we proved GSNO and DMNQ coincubation in a balanced ratio was cell protective compared with the agonist GSNO (Fig. 4A). The coincidence of NO and O_2^- is not restricted to cells that specifically generate NO and O_2^- but may also occur under inflammatory conditions as a result of infiltration of activated immune cells. Once NO and O_2^- are produced, they react with each other in a diffusion-controlled reaction, resulting in the formation of peroxynitrite (Stamler, 1994). Peroxynitrite is a potential cytotoxic agent due to its oxidizing abilities. In cerebrocortical cultures or PC12 cells, peroxynitrite leaves the hallmarks of toxicity (Bonfoco et al., 1995; Spear et al., 1997), but the interaction is also established as a cellular protective mechanism during ischemia-reperfusion or myocardial injury (Lefer et al., 1997; Nossuli et al., 1997). However, detailed mechanisms remain elusive. In MC, NO/O_2^- coincubation abrogated JNK1/2 activation (Fig. 4B) and reduced caspase-3 activation (Fig. 4A). Previously, we noticed that NO/O_2^- cogeneration attenuated p53 and Bax accumulation (Sandau et al., 1997b), elicited Bcl-2 up-regulation (K.B.S. and B.B., unpublished data), and blocked DNA fragmentation (Sandau et al., 1997a). Because some studies connect p42/44 MAPK phosphorylation with cell protection (Xia et al., 1995; Wang et al., 1998), we analyzed whether an attenuated JNK1/2 activity was shifted toward p42/44 MAPK activation. This was ruled out by not observing p42/44 MAPK phosphorylation and by the lack of the MEK inhibitor PD 98059 interference (data not shown). We conclude that NO/O_2^--evoked cytoprotection is p42/44 MAPK independent. Wink et al. (1997) showed that generation of O_2^- quenched NO-mediated nitrosative reactions, thus resembling a shift from nitrosative to oxidative stress. Consequently, nitrosative stress decreased, whereas oxidative stress increased. The GSH system is established to be a first-line defense mechanism against oxidants. Under our conditions of NO/O_2^- coexistence, we measured a significant and long-lasting accumulation of oxidized GSH compared with the oxidative capacity initiated by NO or O_2^- itself (K.B.S. and B.B., unpublished data). This is in line with in vitro experiments in which oxidized GSH is formed in the presence of a NO donor, the xanthine oxidase system, and GSH (Wink et al., 1997). This encouraged us to analyze the role of GSH by using BSO to deplete intracellular GSH. GSH depletion sensitized MC toward apoptosis and necrosis (Table 1) and abrogated the protective effect of NO/O_2^- cogeneration. Notably, increased apoptosis again was accompanied by JNK1/2 activation (Fig. 5). It seems that GSH-mediated quenching of the oxidative stress that occurs as a result of the NO/O_2^- interaction is primarily responsible for protection. Attenuation of apoptosis by NO/O_2^- formation interferes upstream of JNK1/2 as stimulation with Ro 318220 reestablished JNK1/2 activation and apoptosis (Fig. 6, A and B).

In addition to the NO/O_2^- radical-radical interaction, alternative protective mechanisms against NO-induced toxicity may operate. In RAW 264.7 macrophages, cyclooxygenase-2 expression protects against NO-evoked apoptosis (von Knethen and Brüne, 1997); in hepatocytes, HSP 70 expression confers resistance (Kim et al., 1997); and activation of phosphatidylinositol 3-kinase mediates protection against peroxynitrite in PC12 cells (Spear et al., 1997). Often, cytokines or growth factors circumvent cell death under various settings. For MC, Mooney et al. (1997) reported that insulin-like growth factor (IGF)-I and -II and basic fibroblast growth factor suppressed apoptosis induced by serum deprivation, whereas transforming growth factor-β1, epidermal growth factor, and platelet-derived growth factor had no effect. IGF-I and -II also provided protection from etoposide- or cycloheximide-induced apoptosis (Mooney et al., 1997), although responsible mechanisms remained largely unknown. Therefore, we intended to corroborate protection by using FCS and specifically to address the likely involvement of MAPK. Our results (Fig. 3) show that 10% FCS protected MC against NO-induced DNA fragmentation by p42/44 MAPK phosphorylation. A cause-effect relation between MAPK activation and protection was established by the addition of the MEK inhibitor PD 98059. PD 98059 significantly abrogated FCS-evoked protection. These results support the concept that p42/44 MAPK activation conveys protection in MC. A similar mechanism has been described for primary rat cerebellar granule neurons in which the pituitary adenylate cyclase-activating polypeptide 38 induced protection against potassium deprivation via p42/44 MAPK activation through a cAMP-dependent pathway (Villalba et al., 1997).

In conclusion, the inflammatory mediator NO can induce apoptosis in rat MC through JNK1/2 activation. At the same time, cells elaborated different mechanisms to protect themselves. The simultaneous generation of NO and O_2^- allows avoidance of radical-induced toxicity as long as reduced GSH is present, whereas FCS conveys protection by causing p42/44 MAPK activation.

The involvement of different signaling pathways emphasizes how agonists such as IgA or anti-Thy1.1 antibodies can trigger a complex phenomenon such as glomerulonephritis that encompasses mesangial cell proliferation and cell death.

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