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Nitric Oxide Down-regulates MKP-3 mRNA Levels

INVOLVEMENT IN ENDOTHELIAL CELL PROTECTION FROM APOPTOSIS*

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MAP kinase-dependent phosphorylation processes have been shown to interfere with the degradation of the antiapoptotic protein Bcl-2. The cytosolic MAP kinase phosphatase MAP kinase phosphatase-3 (MKP-3) induces apoptosis of endothelial cells in response to tumor necrosis factor α (TNFα) via dephosphorylation of the MAP kinase ERK1/2, leading to Bcl-2 proteolysis. Here we report that the endothelial cell survival factor nitric oxide (NO) down-regulated MKP-3 by destabilization of MKP-3 mRNA. This effect of NO was paralleled by a decrease in MKP-3 protein levels. Moreover, ERK1/2 was found to be protected against TNFα-induced dephosphorylation by coincubation of endothelial cells with the NO donor. Subsequently, both the decrease in Bcl-2 protein levels and the mitochondrial release of cytochrome *c* in response to TNFα were largely prevented by exogenous NO. In cells overexpressing MKP-3, no differences in phosphatase activity in the presence or absence of NO were found, excluding potential post-translational modifications of MKP-3 protein by NO. These data demonstrate that upstream of the *S*-nitrosylation of caspase-3, NO exerts additional antiapoptotic effects in endothelial cells, which rely on the down-regulation of MKP-3 mRNA.

Apoptosis is the enzymatically controlled form of cell death induced by stimulation of distinct cellular signal transduction pathways, as opposed to the lethal cell damage that is known as necrosis (1). In the past few years, several signaling systems have been identified that control apoptotic cell death (2–4). Whereas the caspase cascade executes the apoptotic pathway, MAP kinases[1] are involved in modulating various regulatory pathways of the cell death machinery (5). Whereas the c-Jun N-terminal MAP kinase (stress-activated protein kinase) promotes apoptosis in various cell types (6), the MAP kinase ERK1/2 exerts prosurvival functions (7). Recently, ERK1/2 was shown to regulate protein levels of the antiapoptotic Bcl-2, thus linking ERK1/2 with the apoptotic signaling complex (8). In detail, by maintaining Bcl-2 in its phosphorylated status, ERK1/2 prevents Bcl-2 from ubiquitination, thereby inhibiting its degradation via the proteasome complex (8, 9). Bcl-2 in turn prevents the mitochondrial release of cytochrome *c* (10), an event that leads to formation of the apoptosome complex ultimately culminating in the activation of the executioner caspase-3 (11).

Besides the well established pro-apoptotic effect elicited by high concentrations of nitric oxide (NO), NO also exerts potent antiapoptotic effects in a variety of cells (12, 13). Several interactions of NO with the apoptotic signaling machinery have been postulated to explain the apoptosis inhibitory effects of NO. NO was shown to nitrosate not only the apoptosis executing enzyme caspase-3, where different apoptotic pathways converge (13, 14), but also caspase-6, -7, and -8 (15, 16). Furthermore, NO has been implicated to inhibit caspase-dependent Bcl-2 cleavage and, consequently, the release of mitochondrial cytochrome *c* in MCF-7 hepatocytes and endothelial cells (17–19). Importantly, NO was also reported to interact with $p21^{ras}$ and MAP kinase signaling (20). In Jurkat cells, NO was shown to activate the MAP kinases c-Jun N-terminal kinase and, though to a lesser extent, p38 and ERK1/2 by *S*-nitrosation of $p21^{ras}$ (21).

Here we address the effects of NO to interfere with the dephosphorylation of ERK1/2 as a potential target of the antiapoptotic capacity of NO in endothelial cells. We demonstrate that the down-regulation of the cytosolic MAP kinase phosphatase-3 (MKP-3) (22), which is known to dephosphorylate ERK1/2 (23), maintains ERK1/2 active and, thus, inhibits the execution of apoptosis by preventing Bcl-2 degradation and mitochondrial release of cytochrome *c*.

EXPERIMENTAL PROCEDURES

Materials—SNP, TNFα, and actinomycin D were obtained from Sigma; NG-monomethyl-L-arginine monoacetate and NOC-15 were from Alexis (Läufeling, Switzerland); and SNAP and 2′-amino-3′-methoxyflavone were from Biomol, Hamburg, Germany.

Cell Culture—Human umbilical vein endothelial cells (HUVEC; Cell Systems/Clonetics, Solingen, Germany; passage 2–4) were cultured in endothelial basal medium (Cell Cystems/Clonetics) supplemented with hydrocortisone (1 μg/ml), bovine brain extract (3 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 μg/ml), epidermal growth factor (10 μg/ml), and 10% fetal calf serum (Life Technologies, Inc.) until the third passage. After detachment with trypsin, cells were grown in culture dishes for 18 h before experiments were performed. HUVEC were exposed to constant laminar fluid flow by means of a cone and plate apparatus as described previously (24). COS-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with glutamine (2 mM), penicillin-streptomycin, and 10% fetal calf serum.

Plasmids and Transfection—MKP-3 was amplified by polymerase chain reaction with oligonucleotides that were synthesized to contain

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[1] The abbreviations used are: MAP kinase, mitogen-activated kinase; ERK, extracellular signal-regulated kinase; NO, nitric oxide; MKP-3, MAP kinase phosphatase-3; SNP, sodium nitroprusside; TNFα, tumor necrosis factor α; SNAP, *S*-nitroso-N-acetylpenicillamine; HUVEC, human umbilical vein endothelial cells; pNPP, *p*-nitrophenyl phosphate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ANOVA, analysis of variance; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; NOC-15, (Z)-1-[*N*-(3-ammoniopropyl)-*N*-(n-propyl)amino]diazen-1-ium-1,2-diolate).

*Bam*HI and *Eco*RV restriction sites and subsequently cloned into the respective sites of the pcDNA3.1-MycHis vector (InVitrogen, the Netherlands). Transient transfection of HUVEC was performed by incubation of 3.0×10^5 cells/6-cm well with 3 μg of plasmid as described previously (25). To transiently transfect COS-7 cells, 7 μg of pcDNA3.1 plasmid containing the respective insert were employed using Superfect™ (Qiagen, Hilden, Germany).

Western Blot Analysis—To determine ERK1/2 phosphorylation, HUVEC were lysed in buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2, 5 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM Na_3VO_4, and 1 μM/ml leupeptin, pH 7, 4) for 15 min at 4 °C followed by centrifugation (20,000 × *g*, 15 min). Then, samples were run on a 11% SDS-polyacrylamide gel and blotted onto polyvinylidene fluoride membranes, and finally protein was probed using a phosphospecific antibody against p42/p44 (New England Biolabs). Western blot analysis of MKP-3 and Bcl-2 protein levels was performed by using an antibody directed against MKP-3 (kindly provided by Dr. Steve Arkinstall, Serono) and against Bcl-2 (Roche Molecular Biochemicals), respectively. To determine cytosolic cytochrome *c* levels, the mitochondrial *versus* the cytosolic fraction was separated as described previously (26). Western blot membranes were blocked with 5% milk powder, 1% fetal calf serum at room temperature for 1 h and probed with anti-cytochrome *c* antibodies (PharMingen, San Diego, CA, 1:333 dilution).

Phosphatase Activity—HUVEC or COS-7 cells transfected with the respective plasmid were lysed in 300 μl of buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM dithiothreitol, 10 mM Tris/HCl (pH 8)) for 15 min at 4 °C. After centrifugation (20,000 × *g*, 15 min), phosphatase activity of the supernatant was detected by colorimetric measurements of the hydrolysis of the phosphatase substrate pNPP (Sigma) at 405 nm. Total protein content was analyzed, and enzyme activity was calculated as $\Delta OD \times mg\ protein^{-1} \times s^{-1}$.

MKP-3-induced Cell Death—Following the cotransfection of HUVEC with pcDNA3.1-lacZ (1 μg) and either pcDNA3.1-MKP-3 (2 μg) or the pcDNA3.1 control vector (2 μg), the transfected cells were identified by β-galactosidase staining. Viable *versus* dead stained cells were counted by two blinded investigators, and the results were expressed as dead/viable cells ×100. In addition, potential differences in cell death rate because of necrosis were excluded by measuring lactate dehydrogenase release.

Determination of MKP-3 Messenger RNA—RNA was prepared according to Batt *et al.* (27), and 10 μg was loaded on 0.8% formamide-agarose gels. RNA was blotted on nylon membranes, and the blots were hybridized with a radioactively labeled full-length human MKP-3 probe and incubated for 24 h. Then the blots were washed (0.1% SDS, 0.2× SSC) and exposed to x-ray films.

Measurement of MKP-3 RNA Transcription Rate—For preparation of nuclei, cells were detached with trypsin and lysed with Nonidet P-40. Nuclei (2×10^6) were separated by a 20.5% sucrose gradient and incubated in the presence of ATP, GTP, CTP, and [^{32}P]UTP for 30 min at 30 °C to allow for the transcription of ^{32}P-labeled mRNA. Then, RNA was extracted essentially as stated above (27). To prepare hybridization membranes, human full-length MKP-3 cDNA (100 μg) or glyceraldehyde-3-phosphate dehydrogenase cDNA (50 μg) were blotted onto nylon membranes using a dot blot device (Scotlab, Coatbridge, UK). Blots were cross-linked, hybridized with the radioactively labeled transcripts for 24 h at 65 °C, washed (0.1% SDS, 2× SSC), and exposed to x-ray films.

Statistical Analysis—Data are expressed as mean ± S.D. or mean ± S.E. as indicated from at least three independent experiments. Statistical analysis was performed by one-way ANOVA (variance: least significant difference test).

RESULTS

NO Prevents TNFα-induced ERK1/2 Dephosphorylation, Bcl-2 Degradation, and Mitochondrial Cytochrome c Release—To characterize a potential interference of NO with apoptotic signal transduction involving the MAP kinase p42/p44 (ERK1/2), HUVEC were stimulated with TNFα in the presence or absence of the exogenous NO donor SNP. Then, phosphorylation of ERK1/2 was determined by Western blot analysis using a phosphospecific antibody. As shown in Fig. 1*A*, stimulation of endothelial cells with the proapoptotic cytokine TNFα resulted in a time-dependent dephosphorylation of ERK1/2, as described previously (8). In contrast, the exogenous NO donor SNP abrogated ERK1/2 dephosphorylation by TNFα at all time points examined (Fig. 1*A*). Thus, exogenous NO interferes with ERK1/2 dephosphorylation in response to TNFα.

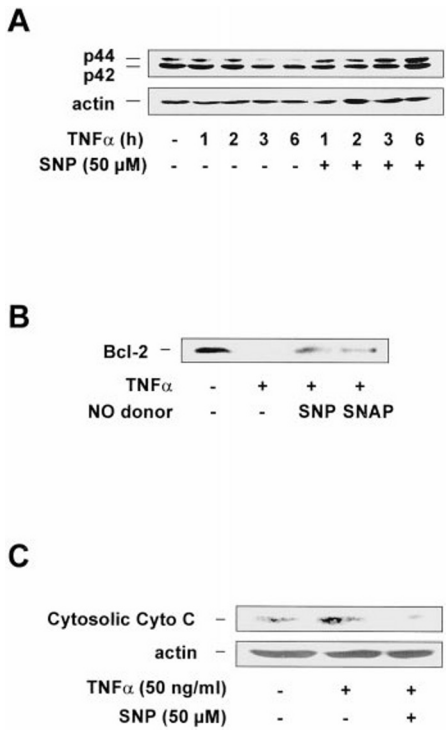

FIG. 1. **NO protection of TNFα-stimulated HUVEC from ERK dephosphorylation, Bcl-2 degradation, and mitochondrial cytochrome *c* release.** *A*, HUVEC were incubated with TNFα (50 ng/ml) for the indicated times in the absence or presence of SNP (50 μM), cells were lysed, and the amount of phosphorylated ERK1/2 was assayed by Western blot analysis employing a phosphospecific antibody. Equal loading of the gel was confirmed by an actin reprobe of the membrane. *B*, the intracellular Bcl-2 content of HUVEC following stimulation with TNFα (50 ng/ml) for 18 h in the absence or presence of SNP (50 μM) or SNAP (50 μM) was determined by Western blot. A representative blot of four different experiments is shown. *C*, HUVEC were stimulated with TNFα (50 ng/ml) in the absence or presence of SNP (50 μM) for 18 h. After cell lysis, the cytosolic fraction was separated, and the amount of cytochrome *c* was determined by Western blot analysis, followed by an actin reprobe of the membrane. A representative result out of three individual experiments is shown.

As ERK1/2 dephosphorylation is known to be a prerequisite for degradation of the antiapoptotic protein Bcl-2, the influence of exogenous NO on Bcl-2 protein degradation was investigated. For this purpose, Bcl-2 levels following exposure to TNFα in the presence or absence of the NO donors SNP or SNAP were determined by Western blotting. Fig. 1*B* illustrates that the degradation of Bcl-2 protein following stimulation with TNFα is largely prevented by coincubation with SNP or SNAP. To further confirm the functional significance of the observed protective effect of NO on ERK1/2 phosphorylation status and Bcl-2 protein levels, the release of cytochrome *c* from mitochondria in TNFα-stimulated cells was measured under the influence of NO. Subcellular protein fractions were isolated from endothelial cells stimulated with TNFα in the presence or absence of SNP to separate the cytosolic fraction from the mitochondrial. As depicted in Fig. 1*C*, the release of cytochrome *c* from mitochondria in response to TNFα is suppressed by coincubation with SNP. These data suggest an inhibitory role of NO in TNFα-induced apoptosis signaling upstream of the mitochondria by maintaining ERK1/2 phosphorylation. The inhibition of ERK1/2 dephosphorylation renders Bcl-2 resistant against degradation and, subsequently, inhibits the mitochondrial release of cytochrome *c*.

NO is known to functionally regulate proteins by *S*-nitrosation of essential cysteine residues (13, 28). The MAP kinase

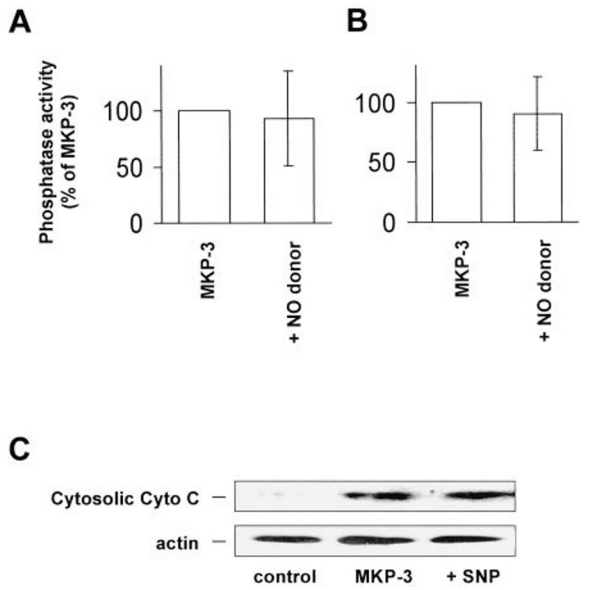

FIG. 2. **Lack of a posttranslational inhibitory effect of NO on MKP-3 activity in MKP-3-transfected cells.** *A*, HUVEC were transfected with the pcDNA3.1 vector that contained an insert encoding for MKP-3. Protein expression was performed by an 18-h incubation period in the absence or presence of SNP (50 μM). Subsequently, cells were lysed, and the whole cell lysate was assessed for phosphatase activity by measuring pNPP hydrolysis (mean ± SD, $n = 3$). *B*, COS-7 cells were transfected with the MKP-3 plasmid, and upon protein expression, cells were lysed and the whole cell lysate was incubated in the absence or presence of NOC-15 (500 μM for 30 min). Finally, pNPP hydrolyzing activity of the protein was assessed as above (mean ± S.D., $n = 3$). *C*, HUVEC were transfected with a plasmid that contained an insert encoding for MKP-3 or with the pcDNA3.1 vector alone. Then the cells were kept in the absence or presence of SNP (50 μM) for 18 h. Following cell lysis, the cytosolic fraction was separated, and the amount of cytochrome *c* was determined by Western blot. *Lower panel*, actin reprobe of the Western blot ($n = 3$).

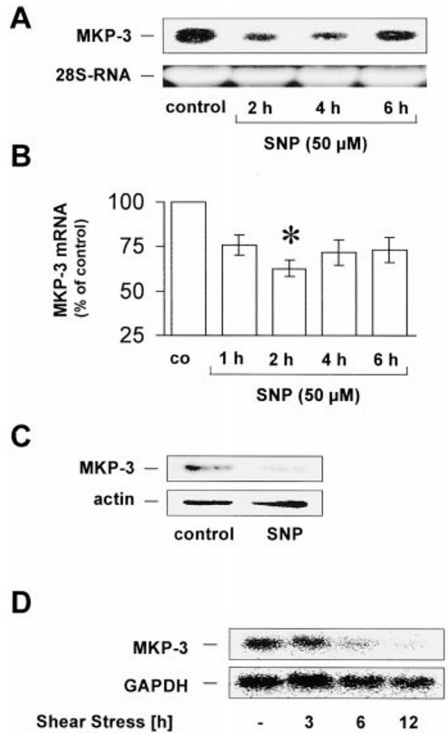

FIG. 3. **Down-regulation of MKP-3 mRNA and protein levels in NO- and shear stress-treated HUVEC.** *A*, Northern blot analysis of MKP-3 mRNA levels in HUVEC following the incubation with SNP (50 μM) for the indicated times. *Bottom*, 28 S RNA content to demonstrate that equal amounts of RNA were loaded into the gel ($n = 3$). *B*, densitometric analysis of MKP-3 mRNA levels normalized for the 28 S RNA content of the respective lanes, calculated as the percent of control in the absence of SNP. Mean values ± S.E. of three to five experiments are shown, *, $p < 0.01$ (ANOVA). *C*, the amount of MKP-3 protein in HUVEC following incubation of the cells in the absence or presence of SNP (50 μM) for 6 h was measured. The *upper panel* shows a Western blot of whole cell lysates probed with a polyclonal antibody against MKP-3, below the actin reprobe of the membrane ($n = 3$). *D*, MKP-3 mRNA expression in HUVEC exposed to constant laminar shears stress for the indicated periods of time applied by a cone and plate apparatus. At *bottom*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) reprobe of the Northern blot membrane to confirm equal loading of total RNA ($n = 3$).

phosphatase MKP-3, which dephosphorylates ERK1/2, contains an essential cysteine residue located at position 293 within its catalytic domain, the mutation of which was shown to inactivate MKP-3 phosphatase activity (23). Therefore, we investigated whether NO exerts an inhibitory effect on the phosphatase activity of MKP-3 on a posttranslational level, possibly by *S*-nitrosation of the enzyme. Phosphatase activity of HUVEC and COS-7 cell lysates overexpressing MKP-3 was analyzed in the presence and absence of NO donors by use of a pNPP hydrolysis assay. The phosphatase activity in HUVEC overexpressing MKP-3 was not influenced by an 18-h coincubation with SNP *in vivo* (93% of the enzymatic activity determined in MKP-3-transfected control cells with no SNP added, Fig. 2*A*) nor by a 30-min *in vitro* incubation with the NO donor NOC-15 of COS-7 cell extracts following MKP-3 protein expression (91% of control extracts, Fig. 2*B*). Thus, NO does not appear to modify MKP-3 phosphatase activity on a posttranslational level. Furthermore, we determined the effect of NO on the major downstream signal event ignited by MKP-3, the release of cytochrome *c* from mitochondria. In MKP-3-transfected cells, the release of cytochrome *c* into the cytosol induced by MKP-3 overexpression was not influenced by exogenous NO (Fig. 2*C*).

Down-regulation of MKP-3 mRNA and Protein Levels by Nitric Oxide—Having demonstrated that NO does not interfere with MKP-3 activity, we investigated a possible regulatory effect of NO on MKP-3 expression. Therefore, MKP-3 mRNA levels were determined following incubation of HUVEC with the NO donors SNP or SNAP for 2, 4, and 6 h. As shown in Fig. 3, *A* and *B*, MKP-3 mRNA is markedly down-regulated in the presence of the NO donor SNP ($p < 0.05$). Similar results were obtained using the NO donor SNAP (data not shown). The down-regulation of MKP-3 was confirmed on the protein level, as demonstrated by Western blot analysis (Fig. 3*C*). To assess the effect of endogenously derived NO, endothelial cells were exposed to shear stress, which activates the endothelial NO synthase (29–31). Exposure of human endothelial cells to constant laminar flow induced a time-dependent decrease in MKP-3 mRNA levels, as shown in Fig. 3*D*. Again, this effect was paralleled by a reduction in MKP-3 protein levels following the exposure to constant laminar shear stress, which was entirely prevented by the NO synthase inhibitor NG-monomethyl-L-arginine monoacetate (data not shown). To identify the mechanism by which NO decreases MKP-3 expression, MKP-3 mRNA transcription was analyzed by nuclear run-on experiments. As shown in Fig. 4*A*, the nuclear transcription rate of MKP-3 mRNA was not altered by the NO donor SNP. Therefore, MKP-3 mRNA stability was additionally analyzed by incubation of HUVEC with actinomycin D. SNP significantly reduced the stability of MKP-3 mRNA (Fig. 4*B*, $p < 0.01$), indicating that NO destabilizes rather than transcriptionally down-regulates MKP-3 mRNA. To assess a functional role of MKP-3 for TNFα-induced ERK1/2 dephosphorylation, MKP-3 mRNA levels were determined following

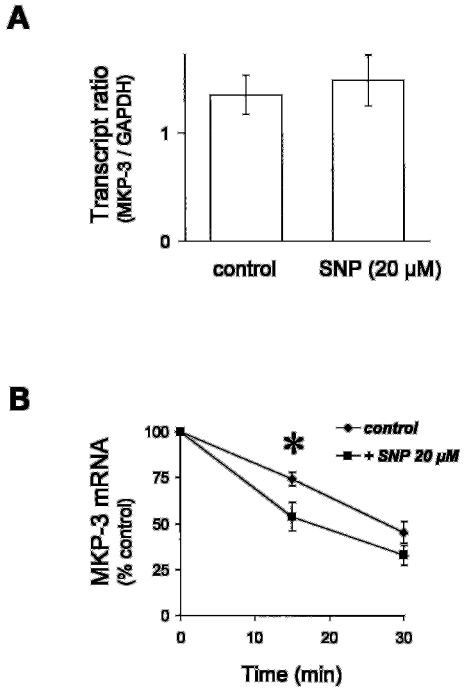

FIG. 4. **Effect of NO donor treatment on MKP-3 mRNA transcription rate and stability of MKP-3 transcripts.** *A*, MKP-3 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA transcription rates from isolated nuclei following incubation of HUVEC in the absence or presence of SNP for 2 h were determined by nuclear run-on experiments. Bar plots give the relative amount of MKP-3 mRNA in comparison with glyceraldehyde-3-phosphate dehydrogenase mRNA ($n = 4$, not shown). *B*, MKP-3 mRNA stability in the absence (♦) or presence (■) of SNP (20 μM) was detected by Northern blot analysis following the addition of actinomycin D (7.5 μg/ml). Mean values ± S.E. out of seven individual experiments are shown. *, $p < 0.01$ (ANOVA).

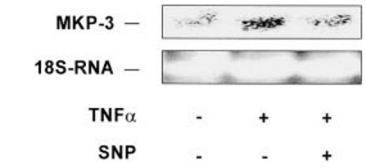

FIG. 5. **Inhibition of TNFα-induced up-regulation of MKP-3 mRNA by exogenous NO.** MKP-3 mRNA levels following stimulation of HUVEC with TNFα (50 ng/ml) for 12 h in the absence and presence of SNP (10 μM), as compared with 18 S RNA (*lower panel*, $n = 3$).

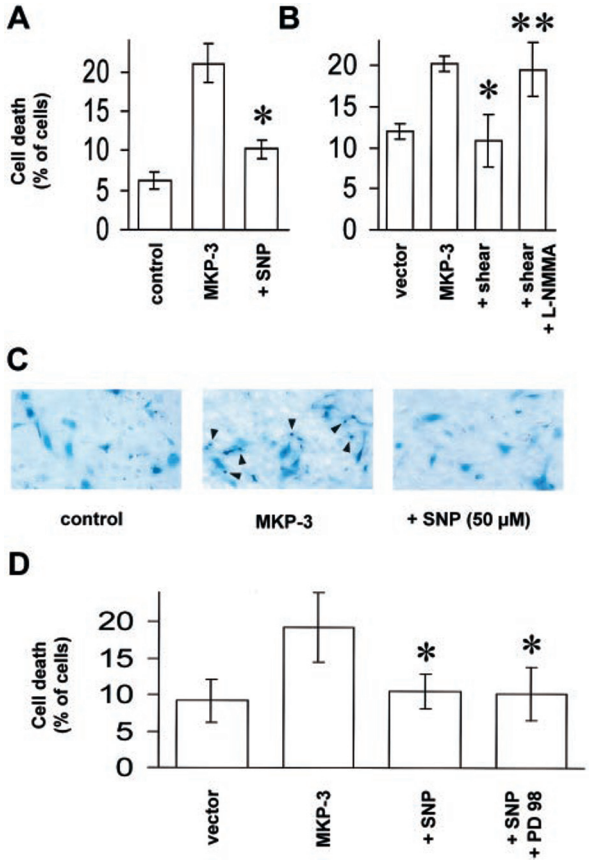

FIG. 6. **Antiapoptotic capacity of NO in MKP-3-overexpressing HUVEC.** *A–C*, HUVEC were cotransfected with mock (*left bar*, control) or a plasmid vector containing the MKP-3 insert and with the lacZ plasmid. Following an incubation period of 18 h in the absence or presence of SNP (50 μM), cells were stained by the addition of X-gal, and cell death was determined by morphological analysis of transfected cells (*A*, mean ± S.D., $n = 3$, *, $p < 0.05$). Photographs illustrate dead cell characteristics by membrane blebbing and cell shrinkage (*C*, *arrows*). *B*, cotransfected cells were exposed to constant laminar flow applied by a cone and plate apparatus (see "Experimental Procedures") in the absence or presence of the NO synthase inhibitor NG-monomethyl-L-arginine monoacetate for 18 h, and subsequently cell death induction was analyzed as given above (mean ± S.D., $n = 3$, *, $p < 0.01$ *versus* no shear stress, **, $p < 0.01$ *versus* shear stress). *D*, cells were cotransfected with lacZ and either vector or MKP-3 plasmid, in the absence or presence of the NO donor SNP (50 μM) or 2′-amino-3′-methoxyflavone (*PD 98*) (10 μM) for 18 h as indicated. Cells were stained by X-gal and the amount of cell death was determined by morphological analysis of blue cells (mean ± S.D., $n = 3$, $p < 0.001$).

stimulation of endothelial cell with TNFα. As shown in Fig. 5, TNFα induced a prolonged increase in MKP-3 mRNA levels, which was largely suppressed by NO. Taken together, NO derived from exogenous as well as from endogenous sources down-regulates MKP-3 mRNA levels in endothelial cells.

Effect of NO on MKP-3-induced Cell Death—MKP-3 was demonstrated to induce cell death in HUVEC by inactivating ERK1/2 (8, 9). Therefore, we characterized the influence of NO on cell death induction by MKP-3. Death rate of HUVEC was significantly increased by overexpression of MKP-3 compared with control cells that were transfected with the pcDNA3.1 vector with no insert ($p < 0.001$, Fig. 6, *A* and *C*). When cells were exposed to low levels of NO provided by the NO donor SNP, cell death induction by MKP-3 overexpression was almost completely abolished ($p < 0.001$, Fig. 6, *A* and *C*). Furthermore, shear stress exposure to augment endogenous NO release also reduced MKP-3-induced cell death even below control levels ($p < 0.005$, Fig. 6*B*). This effect was abolished by the addition of the competitive antagonist of NO synthase, NG-monomethyl-L-arginine monoacetate (Fig. 6*B*). Thus, death signaling in HUVEC induced by MKP-3, under conditions that do not allow for the modulation of MKP-3 expression, is sensitive to exogenous as well as to endogenous NO. Finally, to exclude a potential influence of NO on the ERK1/2-phosphorylating kinase MEK (MAP kinase kinase), we investigated the effects of NO on cell death induction by MKP-3 during pharmacological inhibition of MEK with 2′-amino-3′-methoxyflavone. As shown in Fig. 6*D*, NO was still capable of suppressing cell death induction by MKP-3 overexpression even in the presence of the MEK inhibitor 2′-amino-3′-methoxyflavone.

DISCUSSION

NO represents a key regulator of endothelial cell survival (16, 32). Various interactions of NO with intracellular signal transduction have been described to explain the prosurvival effects of low levels of NO as produced by the endothelial NO synthase. In this study, we addressed the role of the MAP kinase phosphatase MKP-3 as a potential target of the protective effect of NO in endothelial cells. We demonstrate that NO destabilized MKP-3 mRNA and, thereby, interferes with the

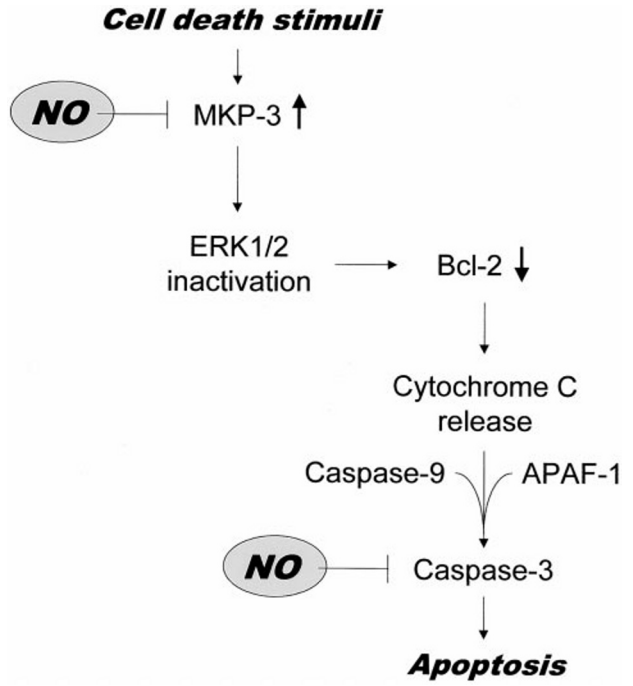

FIG. 7. **Possible interference of NO with apoptotic signaling cascades in endothelial cells at various checkpoints.** Cell death stimuli lead to the up-regulation of MKP-3, an effect which is inhibited in the presence of low levels of NO by a destabilizing influence on MKP-3 mRNA. Further downstream the apoptotic signal pathway, distal from Bcl-2 degradation and cytochrome *c* release, NO is established to interfere with the execution of apoptosis by the inhibition of caspase-3 via *S*-nitrosation (see "Discussion" for details). *APAF-1*, apoptotic protease activating factor-1.

TNFα-induced dephosphorylation of the MAP kinase p44/42 (ERK1/2). Subsequently, NO prevents Bcl-2 degradation and the release of cytochrome *c* from mitochondria, which results in the protection of endothelial cells from apoptosis.

ERK1/2 is an established player in the antiapoptotic defense network (7). The Raf/MEK/ERK pathway has previously been reported to confer protection against apoptosis induced by growth factor withdrawal (33, 34). Activation of the Ras/MEK/ERK pathway was shown to phosphorylate the Bcl-2 family member Bad, resulting in the dissociation of Bad from Bcl-x_L, which allows the protection of cells from apoptosis (35). In endothelial cells, ERK1/2-dependent phosphorylation was demonstrated to stabilize Bcl-2 and prevent its proteasome-dependent degradation (8, 9). In accordance with the prosurvival influence of phosphorylated ERK1/2, its inactivation by MAP kinase phosphatase signaling in response to cellular stress was shown to induce cytotoxicity (8, 9, 36). Thus, the maintenance of ERK1/2 activation via the regulation of MAP kinase phosphatase gene expression might represent an additional checkpoint in cellular signaling.

NO is capable of interacting with apoptosis signaling in multiple ways. The variety of interactions of NO with proapoptotic signaling events include the maintenance of sustained Bcl-2 expression in B lymphocytes (37) and the inhibition of caspase activity by *S*-nitrosation of the catalytic cysteine residue (13, 15, 38) as well as by a cGMP-dependent anti-apoptotic pathway (39). Here, we report the down-regulation of MKP-3 mRNA levels and, thus, the maintenance of ERK1/2 phosphorylation by NO as a novel mechanism that contributes to the protective effects of NO in endothelial cells. Our data demonstrate that NO exerts an inhibitory effect upstream of the ignition of the proapoptotic signaling cascade by inhibiting up-regulation of MKP-3. These findings extend the redundancy of NO to modulate apoptosis signaling to a rather proximal checkpoint of interference as compared with the integrative antiapoptotic blockade achieved by the inhibition of the downstream effector caspase-3 (Fig. 7). Moreover, by stabilizing ERK1/2 phosphorylation via down-regulation of MKP-3, NO may modulate proliferative signals that involve activation of the transcription factors Elk-1 and ATF-2 by ERK1/2 in addition to the prosurvival maintenance of Bcl-2 levels.

Remarkably, the modulation of MKP-3 protein levels by NO is accomplished by regulation of MKP-3 gene expression as opposed to the posttranslational inhibition of caspase protease activity. The down-regulation of MKP-3 mRNA by NO was independent of the transcription rate as shown by nuclear run-on experiments but is caused by a destabilization of MKP-3 mRNA. NO-induced modulation of mRNA stability has previously been reported. The inhibition of NO production in endothelial cells was shown to mimic the induction of thrombospondin-1 mRNA by hypoxia, which is known to rely on the posttranscriptional stabilization of thrombospondin-1 mRNA (40). Moreover, soluble guanylate cyclase mRNA was demonstrated to be destabilized by NO-dependent up-regulation of a destabilizing protein (41). Although the molecular mechanism underlying the regulation of MKP-3 mRNA stability by NO remains elusive, the induction of a destabilizing protein is rather unlikely given the rapid onset of the NO effect.

In conclusion, we demonstrate that following stimulation with TNFα, low levels of NO maintain ERK1/2 phosphorylation via down-regulation of MKP-3 mRNA levels, thereby providing constant phosphorylation of the ERK1/2 target Bcl-2, which prevents the degradation of Bcl-2 and, subsequently, the release of cytochrome *c* from mitochondria. Thus, the antiapoptotic multiplicity of the effects of NO extends from checkpoints up- and downstream of cytochrome *c* release and includes posttranslational modifications of protein function as well as the regulation of gene expression to control apoptotic signaling events.

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