Nitric Oxide Inhibits Caspase-3 by S-Nitrosation in Vivo*

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In cultured human endothelial cells, physiological levels of NO prevent apoptosis and interfere with the activation of the caspase cascade. In vitro data have demonstrated that NO inhibits the activity of caspase-3 by S-nitrosation of the enzyme. Here we present evidence for the in vivo occurrence and functional relevance of this novel antia apoptotic mechanism. To demonstrate that the cysteine residue Cys-163 of caspase-3 is S-nitrosated, cells were transfected with the Myc-tagged p17 subunit of caspase-3. After incubation of the transfected cells with different NO donors, Myc-tagged p17 was immunoprecipitated with anti-Myc antibody. S-Nitrosothiol was detected in the immunoprecipitate by electron spin resonance spectroscopy after liberation and spin trapping of NO by N-methyl-D-glucamine-dithiocarbamate-iron complex. Transfection of cells with a p17 mutant, where the essential Cys-163 was mutated into alanine, completely prevented S-nitrosation of the enzyme. As a functional correlate, in human umbilical vein endothelial cells the NO donors sodium nitroprusside or PAPA NONOate (50 μM) significantly reduced the increase in caspase-3-like activity induced by overexpression of caspase-3 by 75 and 70%, respectively. When human umbilical vein endothelial cells were cotransfected with β-galactosidase, morphological analysis of stained cells revealed that cell death induction by overexpression of caspase-3 was completely suppressed in the presence of sodium nitroprusside, PAPA NONOate, or S-nitroso-cysteine (50 μM). Thus, NO supplied by exogenous NO donors serves in vivo as an antiapoptotic regulator of caspase activity via S-nitrosation of the Cys-163 residue of caspase-3.

Apoptosis is central to the regulation of tissue homeostasis but also contributes to cancer, neurodegenerative diseases, and inflammation. Morphologically, the programmed form of cell death is characterized by cytoplasmic membrane blebbing, chromatin condensation, and fragmentation into apoptotic bodies. The apoptotic process is under control of a highly conserved program of gene expression, with at least three families of genes being involved termed ced-3 (C. elegans death gene 3), ced-4, and ced-9 (1). The mammalian homologues of ced-3 encode for the family of cysteine proteases, caspases, that constitute an enzyme cascade culminating in activation of caspase-3 (2). Thus, caspase-3 represents the execution enzyme of the caspase cascade that cleaves the DNase inhibitor ICAD (inhibitor of caspase-activated deoxyribonuclease) to activate DNA-degrading DNases (3). Within the molecular structure of caspase-3, the catalytic cysteine group that accounts for the proteolytic activity of the enzyme is located at position 163 of the p17 subunit (4).

NO has been implicated to be involved in regulating apoptosis in a variety of tissues (5, 6). In addition to the well-established proapoptotic effects of NO (6–8), a growing body of evidence indicates that low levels of NO function as an important inhibitor of apoptosis by interference with signal transduction pathways that control apoptotic cell death (9–12). Several studies suggested that NO may inhibit apoptosis via interacting with the caspase cysteine proteases (13, 14). As a general feature of its biochemical properties, NO is known to be capable of modifying proteins that contain cysteine residues by S-nitrosation of the thiol group of the respective cysteine (15–17). Indeed, the decrease in caspase-3-like enzyme activity by in vitro incubation of purified enzyme with NO donors could be specifically assigned to S-nitrosation of the essential cysteine residue at the active site of caspase-3 (11, 18). This could represent a potential molecular mechanism underlying the functional relationship between NO and inhibition of apoptosis signaling observed in cell culture. However, taking into account that NO could also interfere with upstream cell death signals that result in caspase activation (19–21), it still has to be proven whether caspases are directly targeted by NO in intact cells and whether an S-nitrosation-mediated inhibitory effect of NO on caspase-3-driven apoptosis does occur in vivo. Thus, in the present study we sought to demonstrate that intracellular caspase-3 is subjected to S-nitrosation of cysteine residue 163 following incubation of cells with NO donors, and we determined the functional impact of exogenous NO on caspase-3-like activity and caspase-3-induced apoptotic cell death in vivo.

EXPERIMENTAL PROCEDURES

Materials—SNP was bought from Sigma (Munich, Germany), PAPA NONOate was from Alexis (Laufeling, Switzerland), and S-nitrosopenicillamine was from Biomol (Hamburg, Germany).

Cell Culture—Human umbilical vein endothelial cells (HUVEC; Cell Systems/Clonetics, Solingen, Germany) were cultured in endothelial basal medium (Cell Systems/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., Berlin, Germany) until the third passage. After detachment with trypsin, cells were grown in culture dishes for 18 h before experiments were performed. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with glutamine (40 mM), penicillin-streptomycin, and 10% fetal calf serum.

Plasmids and Transfection—Caspase-3 was amplified by polymerase chain reaction with oligonucleotides, which were synthesized to contain BamHI and EcoRV restriction sites and subsequently cloned into the respective sites of the pcDNA3.1-MyeHis vector (Invitrogen, Leek, The Netherlands).

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1 The abbreviations used are: HUVEC, human umbilical vein endothelial cells; BCS, batocurapine-dsulfonic acid; DTTA, diethylenetriamine-pentaacetic acid; MGD, N-methyl-D-glucamine-dithiocarbamate; ESR, electron spin resonance; AMC, 7-amino-4 coumarin; SNP, sodium nitroprusside; Cys-NO, S-nitroso-cysteine.

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**NO Spin Trap and ESR Analysis**—The washed beads were resuspended in 400 μl of modified washing buffer lacking metal chelators (BCS and DTPA) and N-ethylmaleimide. A freshly prepared aqueous solution (20 μl) of FeSO₄/sodium citrate (final concentration, 0.1 mM/0.4 mM) was added to the sample 1 min later by an aqueous solution (20 μl) of N-methyl-b-glucamine-dithiocarbamate (MGD; final concentration, 24 mM). MGD served to release NO from S-nitrosothiol (23) and to generate the water-soluble NO trapping agent Fe(MGD)₃, which avidly binds NO to form a paramagnetic mononitros-iron complex (NOFe(MGD)₃) (24). After 5 min, the samples were centrifuged (900 × g for 5 min), and Na₂S₃O₇ was added to the supernatant (final concentration, 200 mM) to prevent oxidation of the spin adduct (25, 26) and to convert all nitrite eventually formed during decomposition of S-nitrosothiol into NO. The solution was then filled into teflon tubes (5-mm inner diameter; 40-mm length) and shock frozen in liquid nitrogen. The concentration of the NOFe(MGD)₃ complex was assessed by cryogenic X-band ESR spectroscopy. In frozen state, this paramagnetic complex exhibits an anisotropic triplet signal with axial symmetry at g ′ = 2.035, g = 2.02 (24). The mononitros-iron complex with diethyldithiocarbamate (NOFe(DETC)₂) (12 μM; dissolved in Me₂SO) served as a standard. ESR spectra were recorded at a microwave frequency of 9.47 GHz, a modulation frequency of 100 KHz, a modulation amplitude of 0.5 millitesla, microwave power of 20 mW, and a time constant of 655 ms. Each spectrum was collected over 160 s with 1024 points resolution.

**Caspase-3-like Activity**—HUVEC transfected with pcDNA3.1 plasmid were lysed in 200 μl of buffer (1% Triton X-100, 0.32 mM 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 for 10 min), caspase-3-like activity of the supernatant was detected by measuring the proteolytic cleavage of the fluorogenic substrate 7-aminocoumarin (AMC)-DEVD and AMC as a standard using excitation and emission wavelengths of 380 and 460 nm, respectively. Protein content was analyzed, and enzyme activity was calculated as mol AMC released × mg protein⁻¹ × s⁻¹ (11).

**Caspase-3-induced Cell Death**—To assess cell death induction, HUVEC were cotransfected as described previously (22) and incubated for 6–18 h. The transfected cells were identified by β-galactosidase staining. Viable versus dead stained cells were counted by two blinded investigators, and results were expressed as dead/viable cells × 10⁶. In addition, potential differences in cell death rate due to necrosis were excluded by measuring LDH release to indicate that death induction was apoptotic in nature.

**Statistical Analysis**—Data are expressed as the means ± S.E. from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance (variance: LSD test).

**RESULTS AND DISCUSSION**

**S-Nitrosation of Caspase-3 by Exogenous NO Donors in Vivo**—To demonstrate the in vivo occurrence of caspase-3-S-nitrosation by exogenous NO donors, a vector encoding the Myc-tagged p17 subunit of caspase-3 (pcDNA3.1-p17wt-Myc) was transiently transfected into COS-7 cells. Cells were incubated for 36 h to allow for protein expression and subsequently treated with Cys-NO (1 mM) for 10 min. Then p17wt-Myc was isolated by immunoprecipitation using an anti-Myc antibody and protein A/G-agarose beads. To remove contaminating S-nitrosothiols and other low molecular mass NO donors, the p17-Myc-anti-Myc immunocomplexes attached to the beads were extensively washed. NO bound to the free thiol group (Cys-163) available on p17 was then detected by a newly developed procedure that breaks use of the catalytic decomposition of S-nitrosothiols by dithiocarbamate and subsequent trapping of released NO by water-soluble dithiocarbamate-iron complex (Fe(MGD)₃) (cf. “Experimental Procedures”). The beads were removed by centrifugation, and the supernatant containing spin trapped NO was assessed by cryogenic ESR spectroscopy (27). The efficiency of this procedure was verified using bovine S-nitroso-albumin as a standard. The recovery of NO from 100 to 1000 nM S-nitroso-albumin kept
overnight in immunoprecipitation buffer amounted to 50 ± 5% (n = 3), and the lower limit for ESR detection (signal to noise = 2:1) was 30 nM NOFe(MGD)2 complex (data not shown). As shown in Fig. 1A, ESR spectra typical for NOFe(MGD)2 were clearly visible in samples prepared from immunoprecipitates of Cys-NO exposed p17-Myc-transfected COS cells (middle trace). For comparison, the ESR signal of a standard is shown in Fig. 1A (bottom trace). This signal was absent in samples prepared from immunoprecipitates of control cells not exposed to Cys-NO (Fig. 1A, top trace). The concentration of spin trapped NO present in the ESR probe of Cys-NO-exposed cells was 150 ± 20 nM. Considering the recovery of NO from S-nitrosothiol after the washing and trapping procedure, it can be estimated that 300 nM NO would account for the total amount of NO initially released from the immunoprecipitates, equivalent to 150 pmol NO in 0.5 ml of sample volume. As a control, one fraction of the immunoprecipitates was analyzed by Western blot with an antibody against Myc to demonstrate equal expression of Myc-tagged wild type p17 (p17 wt-Myc, data not shown).

To test whether S-nitrosation still occurs under exposure to low concentrations of NO donors, which have been shown to efficiently inhibit apoptotic cell death, COS-7 cells expressing the Myc-tagged p17 subunit were incubated for 1 h with 50 μM SNP. As shown in Fig. 1B, a significant NO release could be detected by ESR in immunoprecipitated Myc-tagged p17. Calculated NO in the immunoprecipitate derived from SNP-treated COS-7 cells expressing p17wt-Myc was 70 nM. Moreover, the S-nitrosation of caspase-3 was detectable for at least 12 h after stimulation with 50 μM SNP (data not shown).

**Caspase-3 Is S-Nitrosated at the Essential Cys-163—The essential cysteine residue of caspase-3 is located in the active center of the p17 subunit of caspase-3 at position Cys-163. To specifically assign the S-nitrosation of caspase-3 to the thiol group of Cys-163, Cys-163 was mutated into alanine by site-directed mutagenesis (p17mt-Myc). COS-7 cells expressing p17mt-Myc underwent the same incubation procedure with SNP as control cells transfected with p17 wild type (50 μM SNP, 1 h). As shown in Fig. 1B, mutation of Cys-163 completely prevented S-nitrosation of the p17 subunit, thus excluding that other cysteine residues within the molecular structure of p17 may serve as acceptor amino acids for the S-nitrosation. Again, Western blot analysis confirmed that similar amounts of p17 protein were immunoprecipitated (Fig. 1B, inset).

**Caspase-3-like Activity in Caspase-3-overexpressing HUVEC Is Inhibited by NO Donors in Vivo—To explore the functional impact of caspase-3-S-nitrosation by exogenous NO in vivo, HUVEC were transiently transfected with a plasmid containing full-length caspase-3 (pcDNA3.1Casp-3) or with the control vector (pcDNA3.1). During protein expression, cells were treated with exogenous NO donated by SNP (50 μM) or PAPA NONOate (50 μM) or were kept in the absence of an NO donor.
as a control. As shown in Fig. 2, the increase in caspase-3-like activity induced by caspase-3 overexpression is significantly reduced by 75 and 70% in the presence of SNP and PAPA NONOate, respectively (vector, 100%; *p < 0.001). Because transfection efficiency in HUVEC was 30% on average, the increase in caspase-3-like activity is likely to be even higher in those cells that were actually subjected to transfection by caspase-3 compared with the mean value of all cells.

Apoptosis Induction in HUVEC by Caspase-3 Overexpression Is Suppressed by Exogenous NO—To test whether NO also affects apoptotic cell death induced by caspase-3 overexpression, HUVEC were cotransfected with pcDNA3.1-lacZ and pcDNA3.1-caspase-3, and cell death was detected at different time points (Fig. 3, A–C). A maximal increase in cell death rate of positively stained cells was observed after 12 h (Fig. 3A). Dead cells were characterized by membrane blebs and/or a general cell shrinkage (Fig. 3C). No significant differences in LDH release as an indicator for cell necrosis were observed, when measured at time points in analogy with the determination of cell death (Fig. 3B). This indicates that cell death observed following transfection of HUVEC with full-length caspase-3 is likely due to apoptosis rather than cell necrosis. To assess the sensitivity to exogenous NO of the key apoptosis execution step driven by caspase-3, HUVEC overexpressing caspase-3 were incubated in the presence of the NO donor SNP (50 μM). As displayed in Fig. 3A, the induction of cell death by caspase-3 was abolished by NO treatment (*p < 0.001). Similar results were obtained when PAPA NONOate or S-nitroso-penicillamine were used as NO donors (Fig. 3D). In contrast, LDH activity was not affected by coinubcation with NO donors (Fig. 3B), suggesting that exogenous NO at concentrations used here has no impact on necrotic cell death.

Taken together the present results demonstrate that exogenous NO donors induce S-nitrosation of caspase-3 in intact cells. S-Nitrosation was still detected to a considerable extent, even when cells were exposed to low concentrations of NO donors, which resemble native endothelial NO production (11). In addition to the short term effect of exogenous NO on cellular caspase-3, when the NO donor was applied 1 h before protein isolation, S-nitrosation of caspase-3 was also detectable after 12 h of incubation of cells in the presence of an NO donor (data not shown). These data support the observed long lasting inhibition of caspases following the addition of exogenous NO (11).

The observed modification of cellular caspase-3 by NO leading to the inhibition of the key signal to regulate apoptosis might be of major importance in endothelial physiology where NO has been described as a major protective factor (28, 29). Disturbances in endothelial cell integrity are known to be involved in the early pathophysiological changes leading to atherosclerosis, and increased endothelial apoptosis was suggested to contribute to the initial stages of atherogenesis and plaque erosion (30, 31) that eventually can cause heart attack and stroke. One may hypothesize that the vasoprotective properties of NO could at least partially be based on its apoptosis-suppressive effects in the vascular endothelium.

In a more general point of view, the in vitro inhibition of caspase-3 by NO reported here could represent a universal mechanism by which cell death rates of a whole variety of tissues are fine tuned by NO via controlling the execution step of the caspase cascade, where the main apoptotic signal machinery converges. In such a model, the susceptibility of the cell to death signals transmitted by caspases is prone to an additional regulatory influence dependent on the cellular and exogenous NO levels. In view of the ambivalent capabilities of NO to act either in a proapoptotic or in an antiapoptotic fashion depending on cell type and NO dosage (5), a complex spectrum of NO-mediated control of apoptosis is conceivable. Thus, corresponding to the activation status of the cellular NO syntheses and to the cytosolic redox balance of the individual cell type in a certain physiological scenario, NO may either function as an apoptosis inhibitor to stabilize tissue integrity by the above mechanism or, at higher concentrations, may exert toxic effects by direct degradation of DNA (32, 33).

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