Mass Spectrometric Analysis of Nitric Oxide-modified Caspase-3*

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Caspases are a family of cysteine proteases activated during apoptosis. Modification of caspasas by nitric oxide and its relevance during apoptosis is currently a controversial subject. In this study we analyzed the S-nitrosated form of caspase-3 at a molecular level. By using electrospray ionization-mass spectrometry, we detected poly-S-nitrosation of caspase-3 with an average of about 2 molecules of NO bound per enzyme. Although NO treatment completely inhibited enzyme activity, S-nitrosation was not restricted to the active site cysteine. Rather, we detected multiple relative mass increases of 30 ± 1 Da in both the p12 and p17 subunits of caspase-3, corresponding to single to triple S-nitrosation. The stability of these S-nitrosations differed in physiologically relevant concentrations of 5 mM glutathione. Whereas all S-nitroso bonds in the p12 subunit were cleaved with release of NO and partial formation of protein-mixed disulfides with glutathione, a single S-nitrosation in the p17 subunit remained stable. Since this S-nitrosation was not observed in a mutant form of caspase-3 lacking the active site cysteine, we conclude that NO nitrosates the active site cysteine of caspase-3 and that this modification is notably inert to fast trans-nitrosation with glutathione. Furthermore, we provide evidence that treatment of caspase-3 with NO can lead to mixed disulfide formation with glutathione, demonstrating the oxidative character of NO.

Nitric oxide (NO)† is a signaling molecule that has gained considerable attention because of its presence in many biological processes such as neurotransmission, endothelium-dependent relaxation, and cell-mediated immune responses (1, 2). In cellular systems, NO is produced by the enzymatic oxidation of l-arginine to l-citrulline and can directly modify proteins and thereby regulate their activity. Preferred targets for NO modification in cells are transition metal ions and sulphydryl groups. Whereas the interactions of NO with heme-iron and iron-sulfur clusters are well established, the mechanisms and end products of NO-thiol modifications are still under investigation (3). Depending on the redox state of a cell, the oxygen content, and the chemical properties of the thiol group, NO may favor S-nitrosation or induce oxidative reactions resulting in the formation of mixed disulfides with glutathione, intramolecular disulfides, or higher oxidative species such as sulfenic and sulfinic acids (4). Whereas NO in low concentrations acts as a second messenger molecule in signaling pathways, high concentrations of NO may cause harmful effects (5, 6).

Regulation of enzyme activity by NO has been established for glutathione reductase (7), tissue-type plasminogen activator, glycerophosphate-3-phosphate dehydrogenase, the small GTPase p21ras, the transcription factor oxyR, and others (for review see Ref. 8).

Recently, the possibility of employing NO to attempt to modulate the activity of the caspase family of cysteine proteases attracted increasing attention (9–12). Caspasas play a crucial role in the execution of apoptosis. The processing of procaspase-3 to its active form is considered to be a point of no return in the death signaling cascade. Thus inhibition of the enzymatic activity of caspasas might provide a mechanism to abort the apoptotic program. Although it has been reported that NO can protect certain cell lines from executing the apoptotic pathway (9, 13), it is unknown whether direct modification of the active site thiol of caspasas by NO, via formation of a nitrosothiol, contributes to this process.

We have investigated S-nitrosation of caspase-3 by NO and the subsequent effect on enzyme activity. By using electrospray ionization-mass spectrometry (14), we showed that caspase-3 is, in contrast to previous studies (11), not selectively nitrosated at a single cysteine residue. Rather, we observed multiple nitrosation of the cysteine groups in caspase-3 with an average of 2 NO molecules per enzyme. A significant difference in nitrosothiol stability is seen after treatment of the nitrosated protein with physiological equivalent concentrations of GSH (5 mM). Whereas one NO remained covalently bound to the enzyme, all other nitrosothiol bonds were cleaved with release of NO and partial formation of protein-mixed disulfides with GSH. Mutation of the active site cysteine of caspase-3 followed by nitrosation and GSH treatment of the protein led to glutathionylation with the notion that no NO remained bound to the mutated p17 subunit.

In summary, our studies show that NO promotes poly-S-nitrosation, oxidation, and glutathionylation of caspase-3.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of Recombinant Caspase-3—The full-length cDNA encoding human caspase-3 in a pET23b vector was kindly provided by Dr. G. S. Salvesen, Burnham Institute, La Jolla, CA (15). Expression was performed in Escherichia coli strain
BL21(DE3)pLyS8. The His₆-tagged protein was purified by affinity chromatography on nickel-nitrotolitric acid-agarose (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, bacterial lysates were prepared in a phosphate buffer (100 mM NaH₂PO₄, 100 mM NaCl, pH 7.5) and incubated with nickel-nitrotolitric acid-agarose resin. The column was eluted by column chromatography with an imidazole step gradient up to 150 mM. Fractions containing caspase-3 (150 mM imidazole) were pooled, concentrated by Millipore centricon, and diluted with caspase assay buffer A (100 mM Hepes, 10% sucrose, 0.1% Chaps, 1 mM EDTA, pH 7.5). Since caspase-3 spontaneously oxidized during purification, the enzyme was reduced with 30 mM DTT for 1 h at room temperature. Excess DTT was removed by anion exchange chromatography on a HiTrap Q column (Amersham Pharmacia Biotech, Freiburg, Germany), equilibrated with argon-saturated buffer A. Elution was performed with a NaCl gradient up to 120 mM. To avoid further oxidation, chromatography was carried out under an argon atmosphere, and fractions containing reduced caspase-3 were stored in septum-sealed vials at 4 °C for further use or frozen at −20 °C. The overall yield of purified caspase-3 from 1.5-liter E. coli cultures was 3–4 mg.

**Mutation of Caspase-3**—Mutation of the active site cysteine 285 of caspase-3 to alanine was performed by site-directed mutagenesis using the Quick change site-directed mutagenesis kit from Stratagene (Heidelberg, Germany) employing the following primers: ACCCAATTTT-CATCTTCGACGCCCGCGCTGCTAGACGACGCTCGCAT and AATGGCCACGTCAGCTTGTACCAACTGAAAGCTGGTTG. The C285A mutation was verified by sequencing and subsequently transferred into E. coli strain BL21(DE3)pLyS8 for expression. Expression and affinity chromatography were performed as described for wild-type caspase-3. In order to allow formation of the quaternary structure of the mutant in parallel to wild-type caspase-3, we cleaved the proform of caspase-3 by incubating caspase-3 for 2 h at 30 °C with active caspase-3 (ratio 15:1, caspase-3: caspase-3). Subsequent protein reduction and anion exchange chromatography were performed in analogy to caspase-3. Processing of caspase-3 by SNAP was checked by gel electrophoresis and mass spectrometry.

**S-Nitrosation of Caspase-3 by SNAP**—Reduced caspase-3 was diluted to a final concentration of 15 μM with buffer A containing 50 μM DTT. This concentration of DTT was necessary to avoid spontaneous oxidation during the experiments. SNAP (Alexis, Grünberg, Germany) was added at a concentration of 2 μM at room temperature, for the times indicated. Under our experimental conditions SNAP released 100–150 μM NO per h, detected by decreasing absorbance at 335 nm due to the cleavage of the S–NO bond. Reaction mixtures were stopped by buffer exchange through a Sephadex G-25 PD10 column (Amersham Pharmacia Biotech) equilibrated with buffer A for caspase activity determinations or with 50 mM Hepes buffer, pH 7.5, for UV-visible and mass spectrometry. Buffer exchange for caspase-3 by SNAP was done with 3-kDa size centrifuged fractions that contained nitrosated caspase-3 revealed an absorption at 355 nm arising from the S–NO bond.

**Quantification of S-Nitrosation**—Nitrosothiol formation was quantified by the Saville reaction (16). Briefly, the S–NO bond was cleaved by HgCl₂ in a sulfanilamide solution, acidified with hydrochloric acid. The release of NO was detected by diazodye formation with naphthylethylenediamine by UV-visible spectroscopy. Calculations are based on an extinction coefficient of 50,000 m⁻¹ cm⁻¹ at 540 nm. Free NO in solution was determined in analogy, without HgCl₂ addition. The amount of nitrosated protein was calculated as the difference between bound and free NO.

**Protein Concentration**—Protein concentrations were determined either by Bradford or by UV absorbance at 280 nm based on an extinction coefficient of 26,000 m⁻¹ cm⁻¹ (17). The latter method was used to calculate the stoichiometry of NO-modified caspase-3.

**Caspase-3 Activity Determination**—Oxidized or nitrosated caspase-3 (100 ng) was incubated in buffer A with the indicated concentrations of GSH and DTT at 30 °C for 30 min in the case of oxidized caspase-3 or for 90 min with the nitrosated enzyme. Enzyme activity was followed by the release of AMC from the fluorescent substrate N-acetyl-DEVD-AMC, 25 μM (Biomol, Hamburg, Germany), at 30 °C with a Perkin-Elmer LS50B spectrofluorimeter at 380 nm, emission wavelength 460 nm. Reactions with GSH at concentrations higher than 5 mM were performed in buffer A containing 250 mM Heps to avoid pH shifts. Caspase-3 activity is optimal at pH 7.5 (17).

**RESULTS**

**Inhibition of Caspase-3 by S-Nitrosation**—The processed cysteine protease caspase-3 consists of 2 subunits, a 12-kDa subunit that contains 3 cysteines and a 17-kDa subunit with 5 cysteines. To obtain enzymatic activity, caspase-3 dimerizes to a heterotetramer consisting of two p12 and two p17 subunits. The histidine-activated Cys-285 in the active site of the p17 subunit is conserved in the caspase superfamily and is required for enzyme activity (19–21).

The active site cysteine in caspase-3 is highly sensitive to oxidation. In accord with other results (9, 17), we found a fast inactivation of caspase-3 when reducing agents such as DTT were removed during purification. In order to obtain maximal caspase-3 activity, we performed anion exchange chromatography with reduced caspase-3 under an inert gas atmosphere. The resulting enzyme still became inactivated during activity measurements in non-reducing buffer, but addition of 50 μM DTT fully restored caspase-3 activity and kept the protein sufficiently stable for the experiments described.

In order to determine caspase-3 modification by NO, we incubated the protein with the NO-releasing compound SNAP. SNAP decreased caspase-3 activity in a time-dependent manner, with maximal inhibition at 50–60 min (Fig. 1). By assuming protein thiol nitrosation to be the underlying mechanism, we determined the rate of S-nitrosation in caspase-3 parallel to activity measurements.

Surprisingly, NO was added to the enzyme twice as fast as caspase-3 activity decreased, resulting in a stoichiometry of approximately 2 mol of NO bound per mol of caspase-3 (p12 + p17 subunit). Since the UV-visible spectra of NO-modified caspase-3 showed no significant absorbance except for that due to the protein (280 nm) and the nitrosothiol (335 nm), our results indicate that more than one cysteine in caspase-3 undergoes nitrosation in the presence of SNAP.

**ESI-MS of Nitrosated Caspase-3**—To analyze NO modification of caspase-3 further, we used nanospray ESI-MS (14). Nano-spray MS allows the detection of protein molecular masses at a molecular level with an accuracy of 1 Da in 10 kDa, which is sufficient to detect the addition of NO (29 Da) to the 12- and 17-kDa subunits of caspase-3. Noncovalent bonds such as protein subunit interactions are not retained under denaturing conditions in ESI-MS, whereas covalent modifications...
The occurrence of single to triple S-nitrosation of each subunit suggests covalent binding of one to three molecules NO to GSH (Table I). IC₅₀ values for reactivation of the oxidized and nearendnoxous and NO-enforced inactivated enzymes toward DTT and GSH (Fig. 3) (100 ng of protein) was measured by the release of AMC from the fluorescent substrate Ac-DEVD-AMC. Data were fitted to Equation 1

\[
\text{activity} = \frac{kK[\text{DTT}]}{1 + K[\text{DTT}]}
\]

Based on Scheme 1.

\[
K = \frac{k}{[\text{caspase}_{\text{inactive}} + \text{DTT} = \text{adduct} \rightarrow \text{caspase}_{\text{active}}]}
\]

**SCHEME 1**

Results are representations of three independent experiments.

By using GSH we determined an IC₅₀ value of 1.8 mM for reactivation of the oxidized protein and thus in the same concentration range as observed for DTT. In contrast, 20 mM GSH was necessary to restore 50% activity of the nitrosated enzyme. Furthermore, reactivation of the oxidized enzyme by GSH occurred approximately 5 times faster compared with the S-nitrosated enzyme.

**ESI-MS of GSH-treated S-Nitrosated Caspase-3**—Since caspase-3 activity most likely reflects the status of the active site thiol, we checked the stability of the nitrosothiol bonds toward GSH by ESI-MS (Fig. 4). In the p12 subunit, we observe...


**TABLE I**

**Reactivation of oxidized and S-nitrosated caspase-3 by DTT and GSH**

Spontaneously oxidized caspase-3 (Cas-Ox) and nitrosated caspase-3 (Cas-NO) (100 ng) were incubated with 5 mM GSH or 5 mM DTT. The time constant for the reactivation of caspase-3 was determined by measuring caspase-3 activity as a function of time by the fluorometric assay as described under “Experimental Procedures.” K values were calculated by fitting the data to a first-order kinetic function. To determine the IC50 values, caspase-3 activity was measured after incubating the inhibited enzyme species for 30 (oxidized enzyme) or 90 min (nitrosated enzyme) with increasing concentrations of GSH or DTT. IC50 values were calculated from Equation 1 shown in Fig. 3. Data represent mean ± S.D. of three independent experiments.

|          | DTT | GSH |
|----------|-----|-----|
| k (min⁻¹) | 0.085 ± 0.010 0.245 ± 0.067 0.053 ± 0.013 0.287 ± 0.070 |
| IC50 (mM) | 0.9 0.6 23.8 1.8 |

**FIG. 4.** ESI-MS of nitrosated caspase-3 following its reactivation with GSH. Nitrosated caspase-3 (3 μM) was incubated with 5 mM GSH for 90 min. ESI-MS measurements were performed as described under “Experimental Procedures.” The peak marked with + is an artifact caused by the deconvolution algorithm.

The removal of NO from all cysteine residues after treatment of nitrosated caspase-3 with 5 mM GSH. Instead we find a peak at a distance of 305 Da from that for the unmodified enzyme that accounts for incorporation of GSH. This indicates mixed disulfide formation of the cysteine residues of caspase-3 with GSH with release of NO.

In the p17 subunit that contains the active site thiol, we detected unmodified protein, a peak with a mass increase of 32 Da, and a 305-Da shift of both peaks. This indicates that in the p17 subunit one NO remains bound to the protein and that GSH is incorporated. We conclude that only one cysteine—NO bond in the p17 subunit is stable in the presence of reducing agents, whereas others are cleaved by GSH, in part at the expense of protein-mixed disulfide formation with GSH.

**An Active Site Mutant of Caspase-3 and the Reversibly Inhibited Enzyme Are Not Stably Nitrosated**—Our mass spectrometric data show that the p17 subunit of caspase-3 remains partially nitrosated in the presence of 5 mM GSH. Furthermore, we found that the enzymatic activity in these samples reached only 20–30% of unmodified caspase-3 activity. We therefore questioned whether the stable S—NO bond directly affects the active site of caspase-3.

In order to test this hypothesis, we mutated the active site of caspase-3 from cysteine to alanine. The mutation revealed an enzymatically inactive protein with the mass of about 32 kDa which does not undergo autocatalytic cleavage to the p12 and p17 subunits. Since a comparison between the active caspase-3 and caspase-3C285A demands the processed tetrameric structure of the enzyme, we cleaved caspase-3C285A using active caspase-3 in 15 times lower concentrations. As a result, we obtained two subunits of caspase-3C285A that were used for subsequent experiments. ESI-MS analysis of caspase-3C285A revealed a peak with the mass of 12,961 Da corresponding to the unchanged p12 subunit and the p17 peak with the mass of 16,583 Da, which is reduced by 32 Da (mutation of Cys (–SH) to Ala (–H)) compared with the p17 peak in wild-type caspase-3 (Table II).

We then nitrosated caspase-3C285A and analyzed the protein by mass spectrometry (Table II). In both subunits we found unmodified enzyme and intensive peaks for the addition of one NO (+29 Da). Additional nitrosation is weakly observed in the p12 subunit (+57 Da) but hardly detectable in the mutated p17 subunit. For further comparisons, we treated the nitrosated protein with 5 mM GSH. The ESI-MS spectra of those samples showed in the p12 subunit, in analogy to wild-type caspase-3, the peak for unmodified caspase-3C285A at 12,961 Da, a peak with relative mass increases of 306 Da, and a small adduct of 616 Da, respectively, which accounts for mixed disulfide formation of caspase-3C285A with one or two molecules of GSH. In the p17 subunit we observed the peak for unmodified caspase-3C285A at 16,584 Da but no additional peak at a distance of 30 Da which would indicate the addition of NO. A small peak at 16,889 Da and an additional peak at 17,198 Da represent mixed disulfides of the p17 subunit with glutathione.

The experiments with caspase-3C285A show that the protein is nitrosated but there is no stable S—NO bond in the presence of 5 mM GSH in the p17 subunit, although glutathionylation is observed in analogy to wild-type caspase-3.

To gain further insight into the binding of NO to wild-type caspase-3, the active site cysteine was protected by the reversible caspase-3 inhibitor DEVD-CHO. The inhibitor should block S-nitrosation of the active site, and following inhibitor withdrawal an active enzyme should be generated. Caspase-3 was blocked by a 50-fold excess of DEVD-CHO over protein that completely attenuated enzyme activity. The DEVD-CHO inhibited enzyme was then nitrosated by exposure to 2.5 mM SNAP as described under “Experimental Procedures.” Therefore, free DEVD-CHO and excess NO were removed, followed by activity determination with an excess of substrate (200 μM DEVD-AMC). In the presence of 10 mM DTT 35–40% of the initial enzyme activity was recovered. Since it is known that 10 mM DTT completely restores enzyme activity following oxidation or nitrosation, it is assumed that not all inhibitor had been removed from the active site tissue, thus allowing the recovery of only a maximum of 1/3 of the starting activity under these conditions. We then assayed enzyme activity in the presence of 5 mM GSH, which is known to leave the stable S—NO bond in the p17 subunit intact but to allow circumventing the oxidation of caspase-3. Again, 37 ± 1% (mean ± S.D., n = 2) of the initial activity (enzyme without inhibitor) was measured in the presence of GSH and 200 μM substrate. When caspase-3 was treated with inhibitor and subsequently nitrosated, followed by NO removal, 39 ± 1% (mean ± S.D., n = 2) of the starting activity was recovered. Obviously NO cannot directly attenuate caspase-3 activity when the active site is blocked by an reversible inhibitor, which further supports the notion that one inhibitory site affected by NO is indeed cysteine 285.

**Analysis of the Tetrameric Structure of Caspase-3**—To analyze whether the subunits of caspase-3C285A are able to form the tetrameric complex that is necessary for catalytic activity...
and probably affects the access of NO to the active site of the protein, we performed cross-linking experiments with the homobifunctional cross-linker disuccinimidyl suberate (DSS) (22). DSS covalently connects primary amines at a distance of 11 Å. Therefore, the structure of proteins in solution can be fixed for further analysis by SDS-PAGE. The processed caspase-3C285A showed two bands on silver-stained SDS gels, representing the individual subunits of the protein with the masses of 12 and 17 kDa (Fig. 5 lane 3). These bands disappear after treatment with 1.5 mM DSS. Instead a band at approximately 58 kDa is observed which accounts for the (p12/p17)2 tetramer of caspase-3C285A (lane 6).

Since we have shown that the mutation of one amino acid in caspase-3 does not lead to conformational rearrangements in the tetrameric protein complex, we investigated the influence of NO on the quaternary structure of caspase-3. Performing cross-linking experiments with the nitrosated protein, we intended to show whether NO dissociates the caspase-3 tetramer and thus inhibits enzyme activity. The comparison between caspase-3 and the nitrosated enzyme before and after cross-linking with DSS revealed no significant differences (Fig. 5, lanes 4 and 5). For the wild-type and the S-nitrosated species we observed, in analogy to caspase-3C285A, a band corresponding to the tetramer at 58 kDa. The weak band at approximately 40 kDa is caused by insufficient cross-linking and may represent a trimeric species.

In summary, neither mutation nor nitrosation of caspase-3 prevents formation of the tetrameric structure of the protein. This implies that enzyme inhibition by NO resulted from post-translational modifications rather than from overall structural changes of caspase-3.

DISCUSSION

In this study we used ESI-MS to detect poly-S-nitrosation of caspase-3 which is associated with enzyme inhibition. Exposure of the nitrosated protein to GSH led to S-glutathionylation of particular cysteine residues, but one cysteine residue in the p17 subunit remained in its S-nitrosated form. Since an active site mutant of caspase-3 does not show the stable S—NO bond, we suggest that NO modifies the active site thiol of caspase-3 by trans-nitrosation and that this S-nitrosation is rather inert to trans-nitrosation in relevant cellular concentrations of GSH.

Besides nitrosative inhibition of caspase-3, we observed a fast oxidation of the enzyme in the absence of reducing agents. Since oxidation is reversed by DTT and GSH, we suggest that disulfide formation accounts for enzyme inhibition. Moreover, the oxidation to a disulfide is not associated with significant changes in the molecular mass of caspase-3 and thus explains the presence of the unmodified protein detected by ESI-MS in enzymatically inactive protein samples.

Exposing spontaneously oxidized and nitrosated caspase-3 to physiological concentrations of GSH, we investigated a possible relevance of nitrosation and oxidation to enzyme activity under reducing conditions. Since the activity of oxidized caspase-3 is restored, intramolecular oxidation is unlikely to account for enzyme inhibition under cellular conditions in the absence of oxidative stress. In contrast, the NO modified form of one cysteine residue remains stable in the presence of GSH. Assuming that this stable nitrosothiol bond is located in the active site of caspase-3, the high stability might be explained by the higher nucleophilicity of the active site cysteine compared with the other cysteine residues. X-ray analysis showed that Cys-285 is activated by His-237 (21). This might stabilize the S—NO bond and protects the active site from the attack of nucleophilic GSH. However, at high concentrations of GSH the equilibrium between nitrosation of caspase-3 and GSH may shift to formation of S-nitrosogluthathione.

**TABLE II**

| Protein species | Detected molecular mass (Da) | Peak identification (subunit ± relative mass shift (Da)) | Interpretation of relative mass shift |
|-----------------|-----------------------------|--------------------------------------------------------|------------------------------------|
| Caspase-3C285A  | 12,962                      | p12                                                   |                                   |
|                 | 16,583                      | p17                                                   |                                   |
| Caspase-3C285A + NO | 12,962                      | p12                                                   |                                   |
|                 | 12,991                      | p12 + 29                                              | + NO                               |
|                 | 13,019                      | p12 + 57                                              | + 2 NO                             |
|                 | 16,584                      | p17                                                   |                                   |
|                 | 16,613                      | p17 + 29                                              | + NO                               |
| Caspase-3C285A + NO + GSH | 12,961                    | p12                                                   |                                   |
|                 | 12,267                      | p12 + 306                                             | + GSH                              |
|                 | 13,577                      | p12 + 616                                             | + 2 GSH                            |
|                 | 16,584                      | p17                                                   |                                   |
|                 | 16,889                      | p17 + 305                                             | + GSH                              |
|                 | 17,198                      | p17 + 614                                             | + 2 GSH                            |

**Fig. 5.** Chemical cross-linking of caspase-3 subunits. 15 µg of caspase-3 (C-3), nitrosated caspase-3 (C-3-NO), and caspase-3C285A (C-3C285A) were subjected to SDS-PAGE using 15% gels and visualized by silver staining. The same amount of protein was incubated for 30 min at 22 °C with the cross-linking reagent DSS (1.5 mM), quenched with 50 mM Tris, and analyzed by SDS-PAGE. The left lane shows molecular weight markers with the protein masses indicated. Results are representations of 3 independent experiments.
In order to explain the partial reactivation of caspase-3 by GSH, we propose that inhibition of caspase-3 is caused not only by nitrosation but also by low levels of oxidation that causes intramolecular disulfide formation. By addition of GSH, the disulfide is reduced, and caspase-3 activity is restored. Furthermore, the nitrosothiol may be cleaved by trans-nitrosation with GSH. With respect to enzyme activity, we propose a low trans-nitrosation rate at physiological GSH levels that increases with higher GSH concentrations and thus explains the recovery of 60% caspase-3 activity in the presence of 30 mM GSH.

The observed glutathionylation of nitrosated caspase-3 by GSH might be interpreted as an NO-induced oxidation. Possibly, the lower nucleophilicity of the non-activated cysteine residues in caspase-3 and the resulting weak S-nitroso bonds facilitate mixed disulfide formation with GSH. Since both nitrosation and oxidation may be induced by NO in the same molecule, this implies that the chemical property of a cysteine residue is an important factor that influences the balance between nitrosative and oxidative pathways. By using ESI-MS we detected posttranslational modifications in caspase-3 and could show the distribution of NO on the 2 subunits of the protein. To identify the modified amino acids we used an indirect approach. Comparing mutated caspase-3 with wild-type protein after treatment with NO and GSH, we found that the ESI-MS spectrum of caspase-3C285A does not obtain the peak for the stable S-NO bond, detectable in wild-type caspase-3. However, we could still detect the mass shifts of approximately 305 Da in the ESI-MS spectra of caspase-3C285A and the wild-type protein, resulting from glutathionylation. The occurrence of these peaks imply that the other cysteine residues in both protein species have similar chemical properties.

Additionally, we investigated the binding of NO to wild-type caspase-3 by reversibly blocking the active site cysteine with the inhibitor DEVD-CHO. Again, NO could not modify the enzyme in the absence of the active site, shown by the recovery of enzyme activity upon inhibitor withdrawal.

Another factor that might influence the interaction of caspase-3 with NO is structural rearrangements in the protein. Thus, for correct interpretation of ESI-MS data of caspase-3C285A in comparison to wild-type caspase-3, we analyzed the quaternary structure of both proteins. The most important and required structural characteristic for caspase-3 activity is the formation of a (p12/p17)2 tetramer (17, 21). To detect this species, we performed cross-linking experiments with caspase-3C285A and nitrosated caspase-3. Both the modified enzymes as well as wild-type caspase-3 form the tetramer in solution. Although we cannot determine minor structural changes of caspase-3 by cross-linking experiments, we provide evidence that neither NO nor the mutation of cysteine to alanine in the active site causes enzyme inhibition by dissociation of the caspase-3 tetramer.

Since our data fit to the model of active site nitrosation of caspase-3 as a result of NO-induced enzyme inhibition, one may speculate about the relevance of this process under cellular conditions. It is reported that NO prevents cell death in certain cell lines, e.g. hepatocytes at least in part by modulation of caspase-3 activity (9). If we propose caspase-3 as a target for NO in apoptosis, other members of the caspase family that are highly homologous may also be considered (10, 19). In addition, whether and how the inactive proforms of caspases are modified by NO and whether caspase inhibition by nitrosation is only possible once the enzyme is activated by proteolysis need to be investigated. Thus, the time course of caspase activation in correlation with the cellular NO concentration seems to be important. Assuming that pro-caspases are not targeted by NO, one could explain the apparent “paradox” that NO not only inhibits but also induces apoptosis in certain cell lines (6, 11, 13, 23). One possible explanation could be that in these cell lines NO initiates the apoptotic signaling cascade without affecting pro-caspases. Once caspases are processed into their active form the NO concentration is decreased to a level that is not sufficient to modulate caspase activity by S-nitrosation. Effects similar to those of NO are observed for oxidative agents (24). Oxidative stress is able to induce and inhibit apoptosis. Thus, one might also speculate whether under cellular conditions effects induced by NO are channeled into oxidative reaction pathways.

Here we have used ESI-MS to characterize NO-induced posttranslational modifications of caspase-3, and we have detected nitrosothiol formation at similar rates as published by others (9, 11). In addition, we demonstrated the distribution of NO at a molecular level, and we observed an unselective poly-S-nitrosation of the protein that is accompanied by oxidation in the absence of reducing agents. In the presence of GSH, one NO-modified cysteine, which is not detectable in caspase-3C285A, remained in its nitrosated form, whereas the other nitrosothiols were cleaved at the expense of protein-mixed disulfide formation with GSH.

Whether a similar mechanism might occur under cellular conditions and how a model of caspase-3 inhibition by S-nitrosation fits to the observation that NO can induce apoptosis with activation of caspases needs to be investigated.

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