S-Nitrosothiols React Preferentially with Zinc Thiolate Clusters of Metallothionein III through Transnitrosation†

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ABSTRACT: Metallothionein (MT) is a two-domain protein with zinc thiolate clusters that bind and release zinc depending on the redox states of the sulfur ligands. Since S-nitrosylation of cysteine is considered a prototypic cellular redox signaling mechanism, we here investigate the reactions of *S*-nitrosothiols with different isoforms of MT. MT-III is significantly more reactive than MT-I/II toward *S*-nitrosothiols, whereas the reactivity of all three isoforms toward reactive oxygen species is comparable. A cellular system, in which all three MTs are similarly effective in protecting rat embryonic cortical neurons in primary culture against hydrogen peroxide but where MT-III has a much more pronounced effect of protecting against *S*-nitrosothiols, confirms this finding. MT-III is the only isoform with consensus acid−base sequence motifs for S-nitrosylation in both domains. Studies with synthetic and zinc-reconstituted domain peptides demonstrate that *S*-nitrosothiols indeed release zinc from both the α- and the *β*-domain of MT-III. S-Nitrosylation occurs via transnitrosation, a mechanism that differs fundamentally from that of previous studies of reactions of MT with NO•. Our data demonstrate that zinc thiolate bonds are targets of *S*-nitrosothiol signaling and further indicate that MT-III is biologically specific in converting NO signals to zinc signals. This could bear importantly on the physiological action of MT-III, whose biological activity as a neuronal growth inhibitory factor is unique, and for brain diseases that have been related to oxidative or nitrosative stress.

Metallothioneins (MTs)[1] are a family of proteins that participate in cellular zinc metabolism (*1*). Among the different isoforms, MT-III is unique in having a distinct biological activity. It was first isolated and identified as a growth inhibitory factor (GIF) on the basis of its inhibiting the growth and survival-promoting effect of Alzheimer's disease brain extracts on rat embryonic cortical neurons (*2*). The expression of MT-III is predominant in the central nervous system (*3*, *4*). Like other members of the mammalian MT family, MT-III contains 20 strictly conserved cysteine residues and binds seven metal atoms. Studies of recombinant human Zn_7-MT-III have demonstrated that these are bound in two metal thiolate clusters with similar affinity as in MT-II (*5*). However, neither MT-I nor MT-II exhibits growth inhibitory activity. The GIF activity of the MT-III isoform is determined mainly by its N-terminal *β*-domain, which has a unique conserved C-P-C-P motif and a highly dynamic structure (*6*). The molecular basis for the functions of MT-III, which also include antagonizing the neurotoxic and neurotrophic effects of amyloid *β* protein (A*β*) (*7*), is not clear.

The metal atoms of MT are kinetically labile despite the fact that they are buried deep within the protein, tightly bound, and not accessible for ligand binding (*8*). The modification of surface-accessible cysteine ligands is thought to be the key that unlocks the metal atoms from the protein (*9*). Among all of the reagents that react with the sulfur atoms of MT and release zinc (*9*−*12*), two classes of biomolecules have recently become the focus of our attention: selenium compounds and nitric oxide. Selenium compounds that form catalytic selenols couple the oxidation and reduction of MT to the glutathione redox state (*12*). Nitric oxide (NO•) reacts with zinc thiolate clusters of MT with concomitant release of Zn^{2+} (*13*) and formation of either *S*-nitrosothiols in the absence of oxygen or disulfides in the presence of oxygen (*13*−*15*). Since NO• also reacts with MT in vivo, it has been suggested that NO• signaling might affect zinc homeostasis (*16*, *17*). Such an action would greatly enlarge the repertoire of targets of NO•, a diffusible messenger that regulates many physiological processes at low concentrations, such as control of blood flow, platelet adhesion, and neurotransmission. High concentrations of NO• are a defense mechanism in inflammation (*18*) but can also be cytotoxic if maintained at pathological levels for extended periods of time, a phenomenon referred to as "nitrosative stress" and implicated in the pathogenesis of age-related neurodegenerative disorders (*19*−*24*).

Owing to the high levels of cellular thiols, under physiological conditions the half-life of NO• is short, and the formation of *S*-nitrosothiols (RSNO) has been suggested to

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[1] Abbreviations: MT, metallothionein; GIF, growth inhibitory factor; ZINCON, 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene; PAR, 4-(2-pyridylazo)resorcinol; GSNO, *S*-nitrosoglutathione; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNOC, *S*-nitroso-L-cysteine.

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be a means of storage and transport of NO• (*25*, *26*). In fact, *S*-nitrosothiols are now thought to be the major signaling molecules involved in the actions of nitric oxide, including apoptosis, oxidative stress, and immune responses (*27*, *28*).

The aim of the present work is to gain further understanding of the function of MT-III by comparing its chemical reactivity and neuroprotective potential with those of MT-I/II in reactions with *S*-nitrosothiols and reactive oxygen species. We find that, among the three isoforms, MT-III is the most reactive and neuroprotective against *S*-nitrosothiols, while its reactivity and neuroprotection against H_2O_2, however, are similar to those of MT-I/II. The preferential reactivity toward *S*-nitrosothiols may stem from the presence of consensus sequence motifs for nitrosylation in MT-III and suggests that MT-III could have a function in nitric oxide and zinc metabolism of the brain.

MATERIALS AND METHODS

Materials. Rabbit liver MT-I and -II were purchased from Sigma (St. Louis, MO). Human MT-III and the synthetic β-(residues 1−31) and α-domains (residues 31−62 for MT-II; residues 31−68 for MT-III) of MT-II and -III were obtained from Prof. Milan Vašák (Institute of Biochemistry, University of Zurich) (*6*, *29*, *30*). All MT samples were reconstituted with Zn^{2+} to Zn_7-MT, Zn_4-α-MT, and Zn_3-β-MT according to the reported procedure (*31*). The metal analyses of these species were 7.0 ± 0.3, 3.0 ± 0.1, and 3.9 ± 0.1 mol of zinc. Cysteine hydrochloride, 5,5′-dithiobis-(2-nitrobenzoic acid), sodium nitrite, xanthine oxidase, xanthine, catalase, ethylenediaminetetraacetic acid (EDTA), bathocuproinedisulfonic acid disodium salt, and 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (ZINCON) were obtained from Sigma; sodium hypochlorite was from Aldrich; 4-(2-pyridylazo)resorcinol (PAR) was from Fluka; sodium peroxynitrite, *S*-nitrosoglutathione (GSNO), and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were from Cayman Chemicals (Ann Arbor, MI). *S*-Nitrosocysteine (SNOC) was prepared freshly and kept on ice before use (*32*). Superoxide ($•O_2^-$) was generated by xanthine oxidase using xanthine as substrate. Catalase (20 units) was added to decompose any H_2O_2 formed by dismutation of $•O_2^-$. HOCl was prepared just prior to the experiments by adding sodium hypochlorite to buffer. Its concentration was determined spectrophotometrically (ϵ_{290} = 350 M^{-1} cm^{-1}) (*33*). Deionized water (resistivity ≥15 MΩ cm) was used throughout.

Zinc Release Assay. Reactions of MT with nitric oxide donors and hydrogen peroxide were monitored with a CARY 1 spectrophotometer at 37 °C, employing buffers that had been purged with prepurified nitrogen gas (≤5 ppm of oxygen). Release of zinc from MT was determined spectrophotometrically by monitoring the formation of a zinc−ZINCON complex (ϵ_{620} = 175000 M^{-1} cm^{-1}) at a ZINCON concentration of 100 μM in 20 mM Hepes-Na^+ at pH 7.4. Kinetic rates were calculated by fitting progress curves with CARY (Varian) software.

Primary Cultures of Rat Cortical Neurons. Pregnant Sprague−Dawley rats (day 19; Harlan, Cambridge, MA) were deeply anesthetized with ether, and embryos were removed under sterile conditions. Cerebral cortices of the embryos were removed, coarsely minced, and digested with trypsin−versene (Biowhittaker, Walkersville, MD) for 30 min. Cells were then dissociated by passing the digested tissues gently and repeatedly through a Pasteur pipet and finally through a 62 μm nylon mesh. The neuronal cells were seeded on BIOCOAT poly(D-lysine)-coated 96-well microculture plates (Becton Dickinson, Bedford, MA) at a density of 20000 cells/well in medium A: minimum essential medium (MEM) containing 10% (v/v) fetal calf serum, 0.1 mg/mL streptomycin, and 100 units/mL penicillin G. Neurons were maintained for 2 days at 37 °C under water-saturated air/CO_2 (95/5) and were used to study the neurotoxicity of *S*-nitrosothiols and H_2O_2. Medium and all supplements were purchased from Life Technologies (Grand Island, NY).

Neurotoxicity and Neuroprotection Assays. Two days after seeding, culture medium was removed and replaced with medium B (serum-free MEM with glutamine) containing one of the MTs or MT domain peptides (dissolved in phosphate-buffered saline as stock solutions) at different concentrations. After 15 min, SNAP (1 mM) or H_2O_2 (0.5 mM) (freshly dissolved in phosphate-buffered saline) was added to the culture, which was incubated for 4 h. After these treatments, the culture medium was removed, and neurons were washed twice with medium B and incubated in medium A for 20 h. Neurons were then fixed in 90% methanol/5% acetic acid for 10 min at −20 °C and stained with Cresyl Violet, and the number of surviving neurons was counted manually under a microscope in three 0.4 mm^2 fields per well (the center and the two adjacent fields). All values are means ± SD of nine data points obtained from three separate experiments. Neuroprotection (%) was calculated as 100[($a - b$)/($c - b$)], where a is the number of MT- (or MT fragment-) treated cells, b is the number of SNAP/H_2O_2-treated cells, and c is the number of control cells without SNAP/H_2O_2 treatment; i.e., control = 100% and no MT treatment = 0%.

RESULTS

Reactivity of MT-I, -II, and -III with S-Nitrosothiols. Metal ions bound to MT are kinetically labile, and various agents can react with the zinc thiolate clusters to release zinc (*8*, *10*, *34*). Here, we investigate the reactivities of different MT isoforms toward *S*-nitrosothiols. SNOC was chosen for the kinetic studies because it is generally more reactive than GSNO and SNAP (*32*). Hence, kinetics mainly reflect the reactivity of the MT species.

Zinc release from the zinc thiolate clusters of MT can be measured spectrophotometrically by following the formation of a zinc complex with a chromophoric chelating agent such as ZINCON (log K_{zincon} = 4.9) (*35*). MT-I/II/III all react with SNOC and release Zn^{2+}. However, the reaction of SNOC with MT-III is substantially faster than that with MT-I or -II (Figure 1A). Over 72% of the zinc was released from MT-III within 1 h, whereas in the same time period only ca. 35% and 25% of the zinc were released from MT-I and -II, respectively. Zinc release from MT-I/II/III appears to proceed through a single kinetic process. With an excess of SNOC (200 μM), the rate constant for zinc release from MT-III [(3.70 ± 0.03) × 10^{-2} s^{-1}] is about three times higher than those for MT-II [(1.03 ± 0.03) × 10^{-2} s^{-1}] and MT-I [(1.45 ± 0.03) × 10^{-2} s^{-1}]. Reactions of MT-I, -II, and -III with SNAP and GSNO, two other *S*-nitrosothiols, were slower compared with the reactions with SNOC. In all cases, MT-III is the most reactive one among the three isoforms.

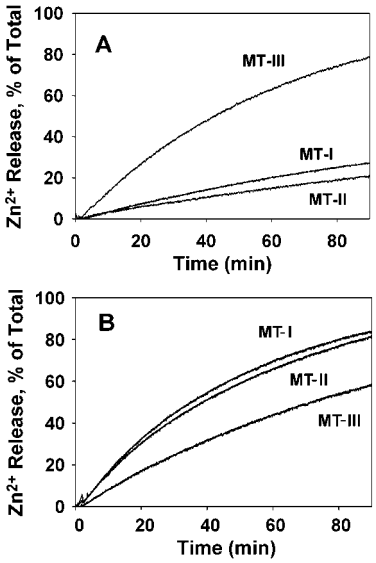


FIGURE 1: Reactions of MT-I, -II, and -III with SNOC and H_2O_2. Experiments were performed at 37 °C in 20 mM Hepes-Na^+, pH 7.4, with 0.5 μM MT. Zinc release was measured spectrophotometrically with ZINCON. Total zinc was calculated on the basis of the MT concentration and 7Zn/MT. (A) Reactions with SNOC (100 μM); (B) reactions with H_2O_2 (50 μM).

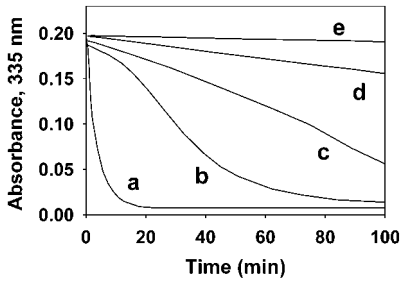


FIGURE 2: Kinetics of *S*-nitrosothiol decomposition monitored at 335 nm. Freshly prepared SNOC (final concentration 200 μM) was added to 20 mM Hepes-Na^+ buffer, pH 7.4, (a) alone or to this buffer in the presence of 0.5 μM (b) MT-III, (c) MT-I, or (d) MT-II or (e) 100 μM ZINCON. The decrease of absorbance in the presence of both MT-II and -III is similar to that shown in (c). The reactions were followed at 37 °C. Because the concentration of SNOC is much higher than that of MT, the contribution of SNOC decomposition to the formation of S-nitrosylated MT can be ignored.

To determine whether MT-III is intrinsically more prone to release its bound Zn^{2+} than the other MT isoforms, Zn^{2+} release induced by different reactive oxygen species was studied. After 1 h, H_2O_2 released only about half the amount of the zinc from MT-III compared with MT-I (Figure 1B), while in the same period of time SNOC released two times more zinc from MT-III than from MT-I (Figure 1A). The reactions all proceed through a single kinetic process. In the reactions with hypochlorite (50 μM), superoxide (xanthine oxidase, 30 milliunits; xanthine, 500 μM), or peroxynitrite (100 μM), similar or slightly lower reactivities were also observed for MT-III compared with MT-I/II.

S-Nitrosothiols are NO^+ (nitrosyl), $NO^\bullet$ (nitric oxide), or NO^- (nitroxyl) donors under physiological conditions and can undergo either transnitrosation reactions (NO^+) with other thiols or release $NO^\bullet$ in the presence of cuprous (Cu^+) ions (*36*−*39*). To gain further insight into the mechanism of the reaction between MT and *S*-nitrosothiols, the time-dependent decomposition of SNOC during the reactions was followed by monitoring the decrease of characteristic absorbance of the RS−NO bond at 335 nm (Figure 2). In the absence of MT or ZINCON, the absorbance decreased very rapidly and reached an end point after 20 min (Figure 2a). When MT was added to the buffer, the rate of decomposition of SNOC was reduced dramatically (Figure 2b−d). MT-III (Figure 2b) was much less effective than MT-II (Figure 2d) in preventing SNOC decomposition. The rate of decomposition of SNOC in the presence of MT-I (Figure 2c) was intermediate to those of either MT-II or MT-III. When 100 μM bathocuproine, a Cu^+-selective chelating agent (*40*), ZINCON, or EDTA was added, decomposition of SNOC was quenched almost completely (Figure 2e). This result indicates that trace amounts of transition metal ions, likely cuprous ions, in the buffer catalyzed the formation of $NO^\bullet$ from SNOC (Figure 2a). Because MT has a much higher affinity for Cu^+ than for Zn^{2+} (*31*), Zn-MT will sequester any free Cu^{2+}/Cu^+ in the buffer solution before MT reacts with SNOC. The fast reaction between MT-III and SNOC then releases again trace amounts of Cu^+, which then effectively catalyze the decomposition of SNOC. The reaction between MT-II and SNOC is much slower, releasing Cu^+ at a slower rate and decomposing SNOC more slowly. This conclusion is further supported by the observation that, in the presence of both MT-II and -III, MT-II binds Cu^+ when it is released from MT-III in the reaction with SNOC and thus decelerates the decomposition of SNOC. The results indicate that in our zinc release assay (Figure 1), in which ZINCON is the chelating agent and accordingly the release of $NO^\bullet$ from *S*-nitrosothiols is extremely slow, transnitrosation is the predominant pathway in the reactions of *S*-nitrosothiols with MTs.

MT contains two zinc thiolate clusters in two separate domains. To assign the specific reactivity of the intact MT molecule to its individual clusters, the synthetic zinc-reconstituted α- and β-domain peptides of MT-III were reacted with SNOC and compared with their counterparts from MT-II (Figure 3A). Significantly, one Zn^{2+} (ca. 25%) from the α-domain of MT-III is released very rapidly, within 10 min after mixing. Additional Zn^{2+} is released in a subsequent slow phase (ca. 50% in 1 h), which is similar in rate to that observed with the α-domain of MT-II. Similar to the α-domain, the reaction of the β-domain of MT-III with SNOC also proceeded through two first-order phases and released almost 80% of its zinc after 1 h. The β-domain of MT-II rapidly releases one Zn^{2+} (over 20% in 10 min) and then releases the remainder of its zinc very slowly, consistent with previous observations that one zinc in the β-domain of MT-II has high kinetic lability (*41*). The rates of the first, fast phase of zinc release from the β-domains of MT-II and -III are nearly the same. Overall, the reactivities of both the α- and β-domains of MT-III toward SNOC are much higher than those of MT-II, and the α-domain of MT-III is almost as reactive as its β-domain (Figure 3A). In contrast, the reactivities of the α- and β-domains of MT-III toward H_2O_2 are both very close to those of MT-II (Figure 3B).

Neuroprotective Effect of MT-I/II/III against S-Nitrosothiols and H_2O_2. MT-III prevents nitric oxide neurotoxicity in primary cultures of cerebellar neurons (*42*). Protection against the cytotoxic effects of nitric oxide and H_2O_2 has

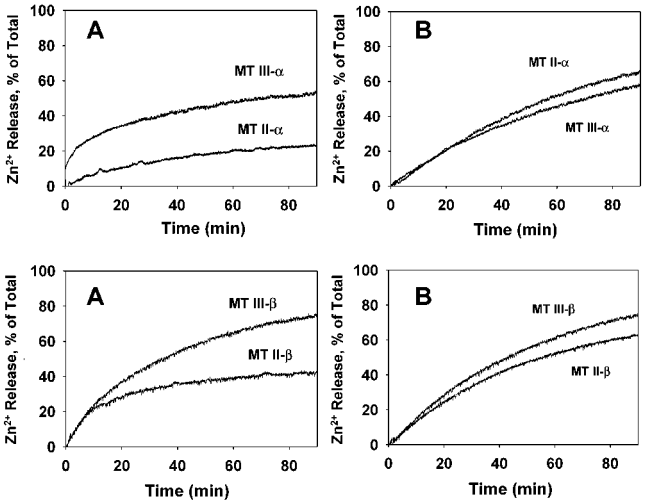


FIGURE 3: Reactions of the α- (top) and *β*-domain (bottom) peptides of MT-II and -III with SNOC and H_2O_2. Experiments were performed under the same conditions as those described in the legend of Figure 1 but with 0.5 *μ*M MT domains. Total zinc was calculated on the basis of the concentration of MT domains and 4Zn/α-MT and 3Zn/*β*-MT. (A) Reactions with SNOC (100 *μ*M); (B) reactions with H_2O_2 (50 *μ*M).

been observed with other members of the MT family (*43*, *44*). To investigate whether our in vitro results are also reflected in in vivo experiments, we compared the neuroprotective effects of MT-I/II/III isoforms against an *S*-nitrosothiol with those of H_2O_2 in primary cultures of rat embryonic cortical neurons. In these experiments, *S*-nitroso-*N*-acetylpenicillamine (SNAP) was chosen because it is less neurotoxic compared with SNOC and has been used in previous studies of MT interactions with nitric oxide in cell cultures (*42*, *43*). Different concentrations of SNAP and incubation times were examined, and the final experimental conditions were chosen on the basis of the optimal number of surviving neurons and the neuroprotective effect of MTs. Rat cortical neurons were exposed to SNAP or H_2O_2 for 4 h, and the number of surviving neurons was determined after 24 h. Both SNAP and H_2O_2 induced major neuronal death, up to ca. 65% and 90% of the control, respectively. The representative appearances of cortical neurons in culture after exposure to SNAP are shown in Figure 4.

Different concentrations of MT-I/II/III (0.001−1 *μ*M) were tested for their effect on neuronal death induced by SNAP or H_2O_2. The neuroprotective effect of MT against SNAP or H_2O_2 is dose dependent, as has been observed by others (*42*). Data for the most remarkable neuroprotection at 1 *μ*M MT are presented. Compared with control cells (Figure 4A), the survival of neuronal cells was compromised severely by SNAP (Figure 4B). The number of surviving neuronal cells decreased markedly, concomitant with severe axon damage. MT-I moderately protected neuronal cells from SNAP neurotoxicity with some part of the neuronal network remaining intact (Figure 4C). The same concentration of MT-II failed to afford any observable neuroprotection (Figure 4D). The most significant neuroprotection was observed with MT-III (Figure 4E), where the appearance of the neuronal cells was nearly indistinguishable from that of the control. The calculated neuroprotection was 71% for MT-III, compared with 42% for MT-I and no significant protection (about 6%) for MT-II (Figure 5A). On the other hand, all three MT

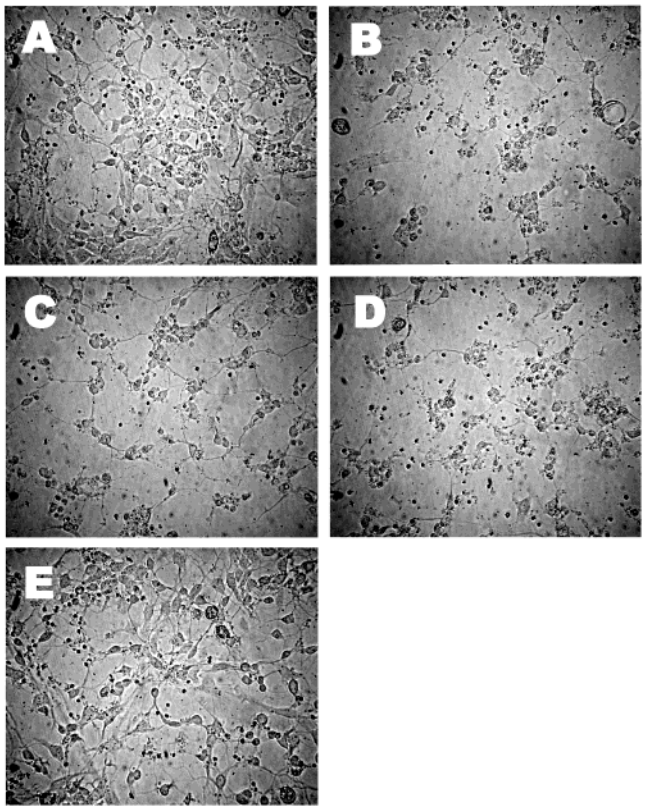


FIGURE 4: Protection of neurons against *S*-nitrosothiol-induced neurotoxicity. Representative pictures of neuronal cells in the primary culture after exposure to the NO-generating compound SNAP in the absence or presence of MTs. (A) control; (B) 1 mM SNAP alone; (C) SNAP (1 mM) + MT-I (1 *μ*M); (D) SNAP (1 mM) + MT-II (1 *μ*M); (E) SNAP (1 mM) + MT-III (1 *μ*M). See Materials and Methods for details.

isoforms showed similar neuroprotective effects against H_2O_2 (Figure 5B).

Zinc complexes of the two domain peptides of MT-II and MT-III were examined for neuroprotection also. The neuroprotective effect of the *β*-domain peptide of MT-III was close to that of the intact molecule whereas the α-domain peptide had only a modest effect (Figure 5C). Compared with MT-III, only the *β*-domain of MT-II showed a slight protection against SNAP-induced neuronal death (Figure 5C). All of these domain peptides showed similar protection against H_2O_2 (Figure 5D). These results suggest a correlation between the neuroprotective effects of the various MT species and their chemical reactivity toward *S*-nitrosothiols and H_2O_2, respectively (Figures 1 and 3). MT-III is unique in that it has the highest neuroprotection against *S*-nitrosothiols compared with the other two isoforms.

DISCUSSION

Molecular Basis for the High Reactivity of MT-III toward S-Nitrosothiols. Despite the similar three-dimensional structures of MT-I/II/III in solution (*45*, *46*), only MT-III shows GIF bioactivity. Its apparent binding constant for Zn^{2+} is close to that of MT-II, which means that the metal thiolate clusters of MT-III are thermodynamically almost as stable as those of MT-II (*5*). Therefore, it was postulated that the bioactivity of MT-III arises mainly from its different primary structure rather than from its metal binding properties (*5*, *6*). In reactions with a chelating agent (EDTA) and a thiol

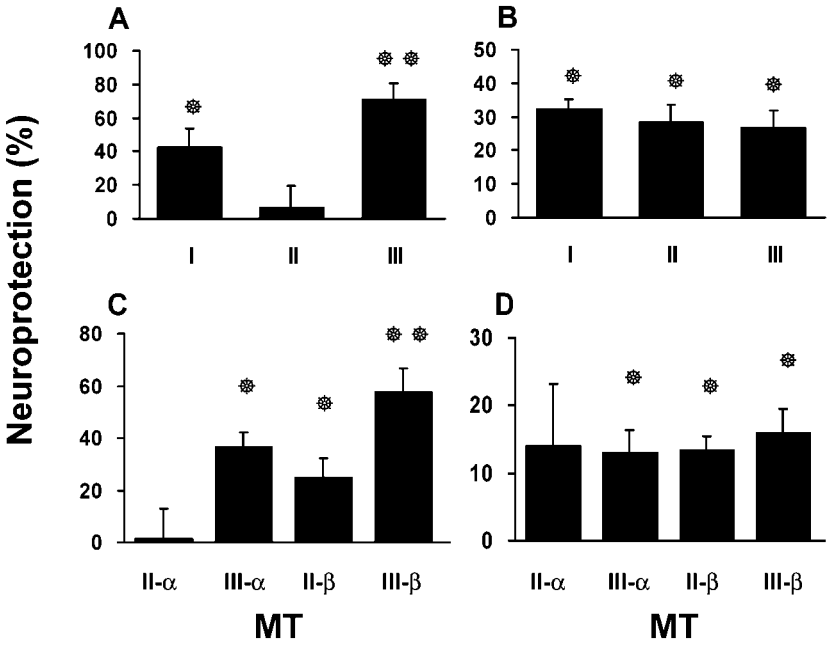
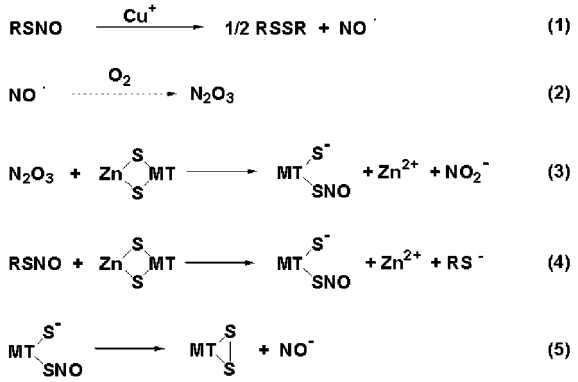


FIGURE 5: Quantification of the neuroprotective effects of MT-I/II/III and the MT domain peptides against *S*-nitrosothiol and H_2O_2. Rat cortical neurons (2 days in primary culture) were first treated with MT-I/II/III or MT domain peptides for 15 min and then exposed to SNAP (1 mM) or H_2O_2 (0.5 mM) for 4 h in the presence of MTs or domain peptides. After being washed, neurons were maintained in culture medium at 37 °C for 20 h. (A and B) Neuroprotection by MT-I/II/III (1 μM) against SNAP (A) or H_2O_2 (B) induced neurotoxicity; (C and D) neuroprotection by the α- or β-domain peptide of MT-II or MT-III (1 μM) against SNAP- (C) or H_2O_2- (D) induced neurotoxicity. Neuroprotection (%) was calculated as $100[(a - b)/(c - b)]$, where *a* is the number of MT- (or MT fragment-) treated cells, *b* is the number of SNAP/H_2O_2-treated cells, and *c* is the number of control cells without SNAP/H_2O_2 or MT treatment; i.e., control = 100% and no MT treatment = 0% [*, $P < 0.01$ by Student's *t*-test between the control and MT- (or MT fragment-) treated cells; **, $P < 0.001$ by Student's *t*-test between MT-III- and MT-I-treated cells (panel A) and between MT-IIIβ- and MT-IIIα- and between MT-IIβ- and MT-IIα-treated cells (panel B)].

reagent (Ellman's reagent), the kinetic reactivities of human Zn_7-MT-III and its α- and β-domain peptides are similar to those of the respective species of MT-I (*6*). This is also the case in the reactions of MTs with H_2O_2 and other reactive oxygen species in this study, i.e., $^{\bullet}O_2^-$, HOCl, and $ONOO^-$. The fact that MT-I/II/III and domain peptides of MT-II/III have similar kinetic reactivity toward and neuroprotective effects against H_2O_2 suggests that MT-III exhibits antioxidant ability similar to that of the other isoforms. But in the reactions of MT-I/II/III with *S*-nitrosothiols, the results were quite different. Compared with MT-I and -II, MT-III has the highest reactivity.

Nitrosation of thiols can be achieved either through oxidation by N_2O_3 formed from $NO^{\bullet}$ in the presence of O_2 (*47*) or through transnitrosation reactions involving *S*-nitrosothiols (*14*). Transnitrosation usually occurs much more rapidly than $NO^{\bullet}$ release from *S*-nitrosothiols (*36*). Moreover, the direct electrophilic attack of $NO^{\bullet}$ on thiolate groups is rather slow (*14*). *S*-Nitrosothiols are sensitive to photolytic and transition metal ion dependent breakdown to release $NO^{\bullet}$ and, consequently, are stable in the dark and in the presence of a chelating agent (*37*). Our zinc release assay contained 100 μM ZINCON, a metallochromic indicator for both Zn^{2+} and Cu^{2+} (*48*). Therefore, in this assay the free copper concentration is very low, and the rate of copper-catalyzed cleavage of SNOC to release $NO^{\bullet}$ is negligible. Thus, the reactions between *S*-nitrosothiols and MT-I/II/III followed by this assay occur mainly through transnitrosation (NO^+) (Scheme 1, reaction 4) rather than through free $NO^{\bullet}$ (Scheme 1, reactions 1−3). Under physiological conditions, the direct reaction of MT with $NO^{\bullet}$ and nitrosylation by higher oxides of nitrogen are of no practical significance (*49*). Transnitrosation is also expected to operate in vivo, based on the conclusion that there are essentially no free copper ions in the cell (*50*). The 20 cysteines of MTs are arranged spatially very close, favoring disulfide formation (*9*). The attack of a thiol in MT by a vicinal *S*-nitrosothiol in MT can lead to formation of a nitroxyl anion (NO^-) and oxidation of the thiols to a disulfide (Scheme 1, reaction 5) (*36*). Therefore, the final products of the reaction of MT-III with SNOC are most likely disulfide-bridged rather than S-nitrosylated MT species. After treatment of MT with SNOC, disulfides indeed have been detected by Raman spectroscopy (*13*).

Scheme 1



Recently, Stamler and co-workers proposed that basic and acidic amino acids in the vicinity of a target cysteine residue enhance its S-nitrosylation by acid−base catalysis (*51*, *52*). The characteristic feature of the consensus motif (K/R/H)C-(D/E) for the catalytic S-nitrosylation of proteins is an acidic amino acid following the target cysteine residue and has

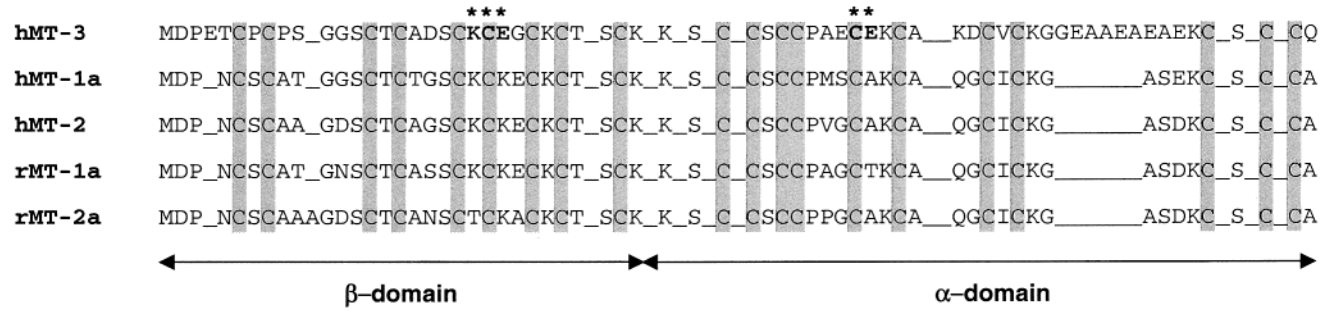


FIGURE 6: Amino acid sequences of human MT-I/II/III and rabbit MT-I/II. Consensus sequences of the acid−base motifs for nitrosylation in MT-III are highlighted with an asterisk.

proven to be predictive of S-nitrosylation in proteins (*28*). The molecular basis for this acid−base catalysis is the modulation of thiol nucleophilicity (pK_a), as thiolates are more reactive in S-nitrosylation than thiols. In MT, a continuous making and breaking of Zn−S bonds occurs (*53*). Such cluster dynamics may give rise to a fraction of free thiol(s), in particular, since thiols are ligands in zinc-finger peptides (*54*). Therefore, acid−base catalysis of S-nitrosation could apply to the sulfur coordination environment of zinc in proteins. We have compared the amino acid sequence of human MT-III (*55*) with those of rabbit and human MT-I/II (*8*) and found that only human MT-III has a unique (K)C-(E) motif in its β-domain and a (E)C(E) motif in its α-domain (Figure 6). These motifs certainly can contribute to the high reactivity of MT-III toward SNOC. Further, the high glutamate content of MT-III, i.e., 8 compared with 0−2 in MT-I/II, may also be a factor. Considering the unusually high glutamate content in MT-III and the close spatial arrangement of cysteine residues in zinc thiolate clusters, one could expect that not only the two cysteines in the two consensus motifs but also any cysteines that are juxtaposed with a glutamate in the three-dimensional structure of the protein could be targeted preferentially for S-nitrosylation (*52*, *56*).

The structure of MT-III is highly dynamic, involving folded and partially unfolded states (*5*, *57*), features that may also affect the reactivity of thiols in transnitrosation reactions. The initial S-nitrosylation and release of Zn^{2+} from MT-III could rapidly destroy the rest of the zinc thiolate clusters through a transnitrosation process (Figure 1A). For MT-I and -II, S-nitrosylation and Zn^{2+} release are much slower processes owing to their lack of acid−base motifs, low content of glutamate residues, and less dynamic zinc thiolate clusters. This notion is supported by the reactions of SNOC with the β-domains of MT-II and -III. One zinc ion in the β-domain of MT-II is particularly labile and easily released in the presence of a chelating agent or oxidants (*41*, *58*). Accordingly, one Zn^{2+} was released from the β-domain of MT-II as rapidly as from MT-III (Figure 3A). However, overall, the β-domain of MT-III is more reactive than that of MT-II (Figure 3A). This is probably due to both the (K)C-(E) motif and the C-P-C-P sequence in the β-domain of MT-III, which has been suggested to undergo a slow cis/trans isomerization leading to partial unfolding (*5*). Compared with MT-II, one Zn^{2+} in the α-domain of MT-III was released rapidly. This is likely related to the unique (E)C(E) motif and the Glu-rich six-residue insertion in the α-domain of MT-III. This insertion occurs within the only extended loop of the MT-II structure (*59*).

It has been reported recently that $NO^{\bullet}$ generated from DEA/NO, a non-*S*-nitrosothiol $NO^{\bullet}$ donor, selectively releases metals from the β-domain of MT-I (*15*). In our experiments, compared with the β-domain the α-domain of MT-II is less reactive toward *S*-nitrosothiols. However, in the α-domain of MT-III one Zn^{2+} is particularly labile in the reaction with *S*-nitrosothiols (Figure 3A). The release of Zn^{2+} from intact MT-III in the presence of SNOC is over 80% in 90 min (Figure 1A), clearly indicating that zinc release from the α-domain is significant. Neither MT-II nor its α-domain exhibited any neuroprotective effects against SNAP. On the basis of these results, we conclude that the neuroprotective effect of MT-III stems from its high reactivity toward *S*-nitrosothiols, which is mainly determined by its unique amino acid sequence and dynamic structure.

Biological Role of MT-III in NO Signaling and Nitrosative Stress. MT-III is the only MT isoform that has GIF activity. MT-III, but not MT-I/II, protects rat cortical neurons in culture from neurotoxicity induced by Aβ. This protective action of MT-III has been attributed to its ability to shift Aβ aggregation from a pathway that leads to the formation of the fibrillar, neurotoxic form of Aβ aggregates to one that favors the formation of the amorphous, nontoxic form of aggregates in vitro (*7*). Both activities clearly distinguish the roles of MT-III from those of other MTs, though the molecular basis for these activities remains unknown. The differences in tissue distribution and genetic expression between MT-III and MT-I/II also suggest that the former has distinct physiological function(s) (*60*). MT-III is expressed predominantly in the central nervous system and is particularly abundant in hippocampal glutamatergic neurons that release Zn^{2+} from synaptic terminals, while MT-I/II are expressed in virtually all tissues examined (*4*). MT-III-deficient mice have a lower concentration of Zn^{2+} in several brain regions and are more susceptible to seizures induced by kainic acid with consequent greater neuron injury in the hippocampus (*61*). Metals, glucocorticoids, and cytokines that can strongly induce expression of MT-I and MT-II have little effect on MT-III gene expression (*51*). These findings suggest that the function of MT-III in zinc metabolism is more specific.

Aside from the highly conserved cysteine residues, the amino acid sequence of MT-III differs significantly from those of other isoforms. We here show that, among the MT isoforms, only MT-III has consensus sequences for S-nitrosylation embedded in its primary structure and demonstrate that it is indeed more reactive toward *S*-nitrosothiols through transnitrosation, thus giving some rationale as to why

this protein is Glu-rich. One general effect of the reactions between MT and *S*-nitrosothiols certainly is the release of Zn^{2+}. It has been proposed that NO• affects zinc homeostasis (*16*, *17*). Our data suggest that MT-III has a function at the crossroads of nitric oxide and zinc metabolism. Zinc inhibits nitric oxide synthase (*62*), a possible mechanism of feedback inhibition for controlling NO• production. Depending on conditions, NO• can be either neurotoxic or neurotrophic (*22*). Therefore, MT-III could function as a specific NO• scavenger during stress conditions to protect neurons, or it could react with low concentrations of NO• that stimulate neuronal growth, which is perhaps the molecular basis for its action as a growth inhibitory factor (GIF).

Dramatic alterations in the expression level of MT-III have been observed during brain injury (*63*) and in the brain of Alzheimer's disease patients (*2*, *64*), although the latter is still controversial (*65*). MT-III levels are reduced in other neurodegenerative diseases, such as amyotrophic lateral sclerosis, multiple system atrophy, Parkinson's disease (*66*), and Down's syndrome (*67*). These observations suggest that MT-III, or the lack of it, may play a role during neuronal regeneration and degeneration. The neurotoxicity of Aβ is mediated at least in part by the induction of NO• and H_2O_2 production either directly or indirectly (*68*−*70*). Zinc released from MT-III can inhibit the neurotoxicity of Aβ by suppressing Cu^{2+}-dependent H_2O_2 formation (*71*), and zinc has an overall protective effect against Aβ toxicity (*72*). Further studies are needed to clarify whether MT-III has a role in the etiology of any of these conditions.

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