Metallothionein-3 Is a Component of a Multiprotein Complex in the Mouse Brain

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Metallothionein (MT)-3, originally called growth inhibitory factor (GIF), was initially identified through its ability to inhibit the growth of neuronal cells in the presence of brain extract. MT-3 is the brain specific isoform of the MT family whose specific biological activity associates it with neurological disorders. Indeed, studies report that MT-3 is decreased by ~30% in brains of patients with Alzheimer disease (AD). Furthermore, many lines of evidence suggest that MT-3 engages in specific protein interactions. To address this, we conducted immunoaffinity chromatography experiments using an immobilized anti-mouse MT-3 antibody. We identified five associated proteins from the pool of sixteen recovered using mass spectrometry and tandem mass spectrometry after in-gel trypsin digestion of bands from the affinity chromatography. The proteins identified were: heat shock protein 84 (HSP84), heat shock protein 70 (HSP70), dihydropyrimidinase-like protein-2 (DRP-2), creatine kinase (CK) and β-actin. Coimmunoprecipitation experiments, also conducted on whole mouse brain extract using the anti-mouse MT-3 antibody along with commercially available antibodies against HSP84 and CK, confirmed that these three proteins were in a single protein complex. Immunohistochemical experiments were then conducted on the perfused mouse brain that confirmed the *in situ* colocalization of CK and MT-3 in the hippocampus region. These data provide new insights into the involvement of MT-3 in a multiprotein complex, which will be used to understand the biological activity of MT-3 and its role in neurological disease. Exp Biol Med 231:1500–1506, 2006

Key words: metallothionein-3; Alzheimer disease; immunoaffinity chromatography; multiprotein complex

Introduction

Metallothioneins (MTs) are a class of small (6–7 kDa) cysteine-rich proteins that bind both essential (Cu^{+} and Zn^{2+}) and nonessential (Cd^{2+}, Ag^{+}, and Hg^{2+}) metal ions with the highest per mole metal content after ferritins (1). MTs bind metals with a high thermodynamic but low kinetic stability. Therefore, metal binding is very tight; however, there is facile metal exchange with other proteins (2). Based on the Fowler classification, MTs were grouped into three classes (I, II, and III), with class I being MTs in which the locations of cysteine residues closely resemble equine renal MT (3). The purification of more than 300 sequences of MTs in different species (Swiss Databank; http://us.expasy.org/) has allowed the determination of a new classification based on phylogenetic and evolutionary considerations (4). According to this classification the MTs are divided into families, subfamilies, subgroups, and isoforms. Ninety-nine families were identified, with family 1 representing vertebrate forms and family 99 the phytochelatins (4).

In mammalian MTs, there are currently four known isoforms, which are designated MT-1, 2, 3, and 4 (5). MT-1 and 2 are expressed in most organs, especially in the kidney and liver, and are similar with respect to their metal-binding characteristics. Binding of divalent Zn^{2+} and Cd^{2+} metal ions occurs in two separate domains of MT-1 and MT-2: the N-terminal β-domain binds three metals in Me_3S_9 coordination, whereas the C-terminal α-domain binds four metals in Me_4S_{11} coordination (6–8). The metal cluster arrangement is inverted in sea urchin MTA, in which four metals are bound in the N-terminal domain and three in the C-terminal cluster (9). Only six metals are bound in two 3-metal clusters in the crustacean MTI from the mud crab *Scylla serrata* (10) and the blue crab *Callinectes sapidus* (11).

MT-1 and MT-2 are thought to function in the homeostasis of zinc and copper as well as in the detoxification of heavy metals (5, 12). The kinetically labile metal ions in the β-domain can be released by oxidized glutathione, reactive oxygen species, and nitric oxide (13–15).

MT-3 was discovered in brain tissue as a protein that inhibits the growth of neurons in the presence of brain extracts from Alzheimer disease (AD) patients (16–18). Its growth inhibitory activity was established to reside in the N-terminal β-domain, and mutations of proline residues at positions 7 and 9 were found to abolish this activity (19, 20). It should be mentioned that MT-3 has also been found

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in numerous cancers, including that of the human kidney (21, 22).

To date, the native metal form of MT-3, $Cu_4^+Zn_4^{2+}$ has not been structurally characterized; however, spectroscopic studies, nuclear magnetic resonance (NMR) studies on (Cd_7MT-3), and biological activity studies have provided some fundamentally important information about this protein. Of primary importance is the finding that its biological activity is associated with the β-domain (20), which binds 4 moles of Cu^+ that are very stable against oxidation (23). NMR studies on Cd_7MT-3 revealed a dynamically disordered β–domain and a structured Cd_4^{2+} α-domain, which was superimposable with the α-domains of MT-1 and 2 except for an unstructured hexapeptide insert (24). Intrigued by the oxidatively stable Cu^+ metal ions in the biologically active β-domain, which has thus far resisted structural characterization, a literature search was conducted that revealed the crystal structure of a polynuclear Cu^+ thiolate complex containing four coppers and nine thiols (25). In yet to be published work from our laboratory,[1] the structure of the $Cu_4^+S_9$ core from the model complex, which consists of three tetrahedrally-coordinated Cu^+ and one trigonally-coordinated Cu^+, has been modeled into the homology-modeled β-domain of Cd_7MT-3, followed by energy minimization and dynamic refinement. The result reveals a very compact β-domain structure, with no Cu^+ ions accessible to water in a Connolly surface representation of the protein, consistent with the oxidative stability of Cu^+ in MT-3.

Unlike MT-1 and MT-2, MT-3 is not induced by heavy metals, indicating that this isoform is regulated differently (26). The MT-3 and MT-1 isoforms respond differently to zinc deprivation of cells in culture, as only MT-1 is downregulated under these conditions (27). Transcriptional regulation of MT-3 also differs from MT-1 and MT-2, suggesting that MT-3 is not involved in metal ion homeostasis as MT-1 and MT-2 seem to be (28–31). Overexpressed MT-3 colocalizes with Zn^{2+} in mouse neurons (32, 33). ZnMT-3, but not ZnMT-1, is able to inhibit the growth of neuronal cells in the presence of AD brain extract; however in the absence of added AD brain extract, both ZnMT-1 and ZnMT-3 stimulate neuronal growth (20). These studies support a function of MT-3, distinct from the toxic effect of Cd(II) or Cu(I) complexes of MT-3 on neurons (20), that appears to be associated with its specific structural features. The unique biological functions of MT-3 indicate its involvement in different signaling pathways and lend support to the hypothesis that there are specific interacting proteins that mediate its functionality.

AD is among the processes linked to MT-3: some studies of AD tissue have shown that MT-3 mRNA is decreased (34, 35), although other reports did not observe any decrease (36, 37). Likewise, zinc and copper have been implicated in AD (38). Copper binds with attomolar affinity to β-amyloid (39), and the neurotropic and neurotoxic effects of β-amyloid are supported by low and high levels of zinc, respectively (40, 41). MT-3, but not MT-1 or MT-2, has also been reported to modulate both the neurotropic and neurotoxic effects of β-amyloid (42). These data implicate overlapping functions of MT-3 and metal ions in normal neuronal function, and specifically in AD, although a direct connection has yet to be established.

MT-3 in Mouse Brains

Antiserum selective for mouse brain MT-3 was prepared (43) using recombinant mouse Cd_7MT-3 (24). The antiserum was found to be essentially specific for MT-3 with 100-fold greater sensitivity for MT-3 than MT-1 or MT-2. The antiserum was used in subsequent experiments essentially as a MT-3 specific antiserum. This anti–MT-3 antibody preparation was found to immunoprecipitate purified, recombinant MT-3 and MT-3 from a cellular extract. Immunoprecipitation of pure, recombinant mouse brain MT-3 showed that as little as 0.30 μg could be immunoprecipitated and detected by subsequent Western blotting (43).

MT-3 Partner Proteins

Proteins associated with MT-3 in Swiss Webster mouse brains were isolated using immunoaffinity chromatography over immobilized anti-mouse brain MT-3 antibodies (44). Proteins were identified by separation on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), digestion of bands with trypsin, and comparison of peptides obtained by mass spectrometry to the Mascot database (NCBI; http://matrixscience.com). Multiple protein bands were detected and we have identified five thus far: heat shock protein 84 (HSP84; mouse variant of heat shock protein [HSP]90), heat shock protein 70 (HSP70), dihydropyrimidinase-like protein-2 (DRP-2), creatine kinase (CK), and β-actin (Fig. 1). Additional protein bands were also analyzed, but insufficient sequence information was recovered for their identification by comparison to the Mascot database.[2] Noteworthy in this regard is the report by Kang *et al.* (45), who detected 22 signals for potential MT-3–interacting proteins using the yeast two-hybrid system. Of these signals, only the protein responsible for the strongest signal was identified—Rab3A, which was subsequently characterized using affinity precipitation and surface plasma resonance analysis (46). Because Rab3A is involved in synaptic vesicle function and neurotransmitter release, this interaction associates MT-3 with the possible regulation of neuronal signaling. Indeed, only a combination of MT-3 and Rab3A could block Zn-induced neuronal death; neither MT-3 nor Rab3A alone were able to block the neurotoxic effects of zinc (47). This finding is consistent with the hypothesis that the physiological function of MT-3 requires interactions with other proteins.

[1] J.D. Hoekman and I.M. Armitage, manuscript in preparation.

[2] I. El Ghazi, B.L. Martin and I.M. Armitage, manuscript in preparation.

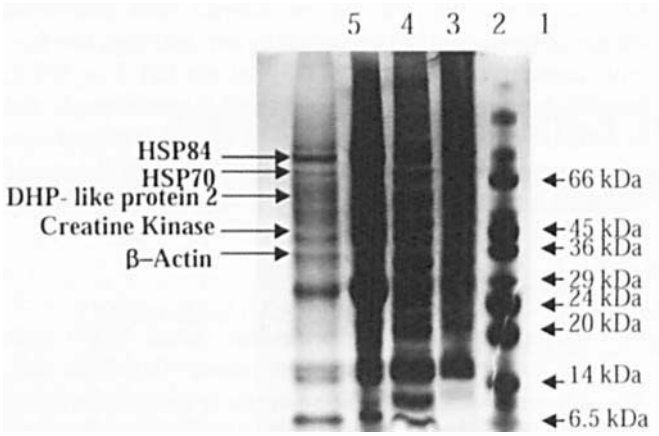

Figure 1. 4%–12% SDS-PAGE of the fractions collected from immunoaffinity chromatography of the normal mouse brain homogenate. Lane 1, molecular weight markers. Lane 2, whole supernatant. Lane 3, fractions eluted with 10m*M* sodium chloride (NaCl), 10m*M* 4-morpholinepropanesulfonic acid (MOPS), pH 7.6. Lane 4, fractions collected with 150 m*M* NaCl, 10 mM MOPS, pH 7.6. Lane 5, fractions collected with 3M sodium thiocyanate (NaSCN), 10 mM MOPS, pH 7.6.

Immunohistochemical Localization of MT-3 and Partner Proteins in Mouse Brain

Immunochemical assays have provided supporting evidence for the *in situ* association of MT-3 with partner proteins. Immunohistochemistry was performed using cryosections of mouse brain tissue obtained from mice that were deeply anesthetized with sodium pentobarbital (150 mg/kg ip) and after being perfused via the ascending aorta with saline and 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. Initial localization studies were done using normal mouse brain tissue. After protocols were established using normal mice, localization studies of MT-3–associated proteins were performed with brain tissue from a 24-month old Tg2576 mouse model of AD (48). It is noteworthy that amyloid deposition is pronounced in the hippocampus in Tg2576 mouse brains (49) and, therefore, attention was focused on the hippocampus in these preliminary experiments.

In the hippocampus, MT-3 was localized predominantly in CA1 and CA3 cells, as shown in Figure 2. Double labeling experiments revealed colocalization of MT-3 with NeuN (neuron nuclei, a marker protein for neuronal cells), confirming that MT-3 is found in neurons. Similar double labeling experiments showed that CK is also colocalized with NeuN in CA1 and CA3 neurons. From this, we conclude that MT-3 and CK are present in the same hippocampal neurons, a first step toward establishing the physiological significance of the *in vitro* biochemically-defined multiprotein complex. These results are consistent with other reports on the hippocampal localization of MT-3 and CK (50–52). Furthermore, MT-3 has been localized in CA1-CA3 pyramidal and dentate gyrus granule neurons and

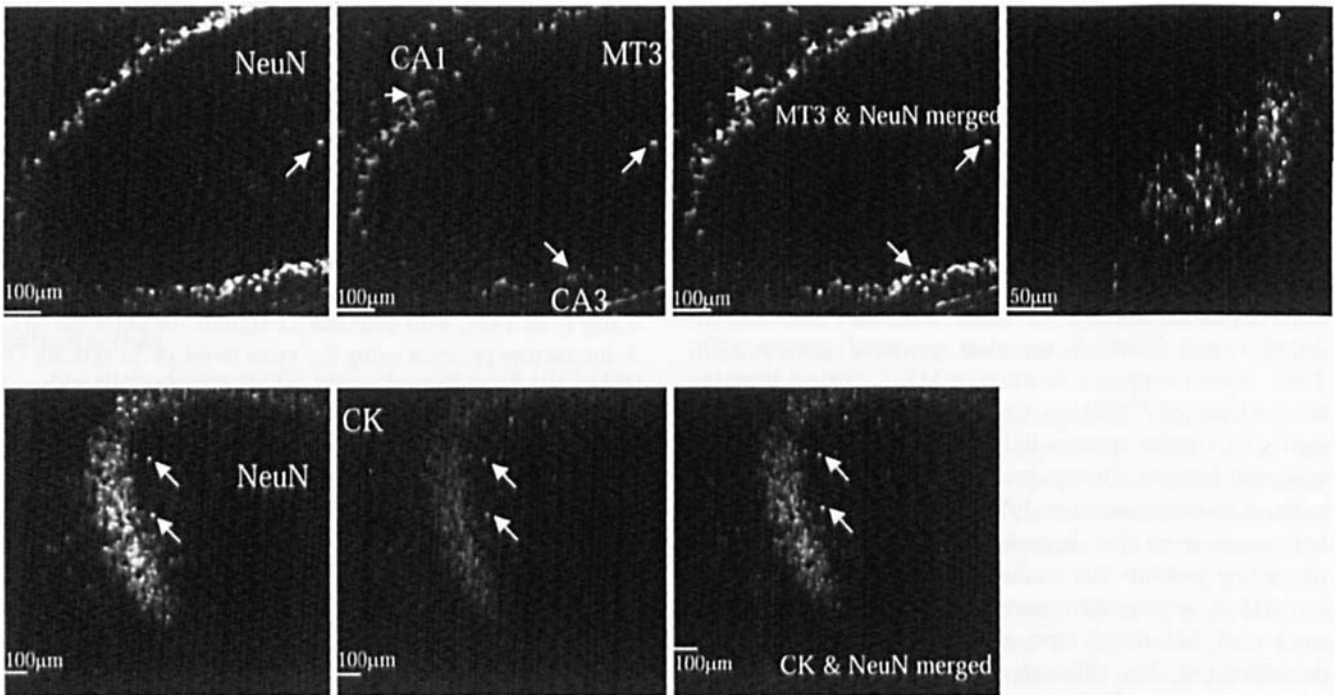

Figure 2. Colocalization of MT-3, CK, and NeuN in the same region of the hippocampus of the Tg2576 mouse brain. MT-3 and CK (Sigma Chemical, St. Louis, MO) were stained using Cy3 goat anti-rabbit secondary antibodies (red fluorescence; Jackson Lab, Bar Harbor, ME). Neuron nuclei (NeuN; Chemicon, Temecula, CA) were stained using Cy2 Donkey anti-mouse (green fluorescence; Jackson Lab). The merged panels show the appearance of a new color resulting from merging green and red. Color version available on-line.

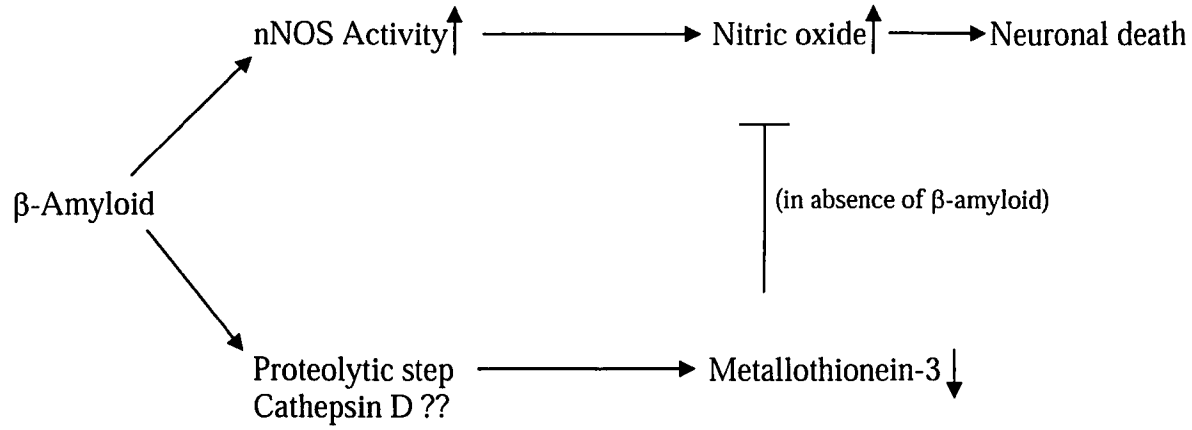

Figure 3. Model of the opposing functions of MT-3 and NOS/NO. In the absence of amyloid, MT-3 protects against the toxic effects of NO. T, block by MT-3 of the effects of NO. After the expression of amyloid, MT-3 protein levels decrease along with loss of protection against NO damage. A proteolytic event is hypothesized as the signal that leads to the reduction of MT-3 protein.

is implicated in regulating the zinc release that influences the viability of hippocampal cells (53).

MT-3 in Brains from a Mouse Model of AD

Using MT-3–specific antiserum, the amount of MT-3 protein in whole brain homogenates from the Tg2576 transgenic positive mouse model of AD was determined (48, 54, 55). Twenty-two–month old transgenic positive mice exhibited a 27% decrease in MT-3 (normalized to total protein) compared with same age, transgenic negative control mice (55). The decrease in MT-3 is attributed to its degradation in response to the pathological changes that develop in this mouse model of AD.

These data are consistent with the decrease in MT-3 found in human AD brains by other investigators (34, 35). Western blot analysis of proteins in brain samples from AD patients revealed that MT-3 was decreased by 29% and 55%, respectively, in the frontal and temporal cortexes (35). In contrast, using an anti-rat MT-3 antibody, Carrasco *et al.* (56) reported an increase of the amount of human MT-3 in brain samples from AD patients, possibly because of species differences in the antibodies used.

Consequences of Altered MT-3 Concentration

Because MT-3 has been shown to inhibit neuronal growth in culture, a plausible explanation for the decrease in MT-3 could be a compensatory mechanism used by neurons to maintain viability. An alternative explanation is that MT-3 serves a neuroprotective function and prevents the development of AD. Decreasing MT-3 induced by amyloid plaque formation may be a necessary step for the progression of AD. MT-3 has been reported to prevent the neurotoxicity of nitric oxide (NO), and injection of mice with inhibitors of nitric oxide synthase (NOS) is associated with the subsequent increase of MT-3 and MT-1 in both brain and liver (57, 58). These data are evidence of an inverse relationship between NOS activity and MT levels, and prompted us to conduct experiments to probe for the levels and activity of NOS in the mouse brain. We found a decrease in the level of neuronal (n)NOS, but the nNOS protein present had increased NOS specific activity. There was a statistically significant decrease (33%) of nNOS in AD transgenic positive mice compared with transgenic negative mice with no significant change in total protein. NOS specific activity was found to be increased by 58% in transgenic positive mice compared with transgenic negative mice (55). These changes in NOS activity are consistent with the altered viability of NOS-positive neurons (59, 60) and with observations from AD brains (61, 62). Amyloid production results in increased NO, which acts as a neurotoxic agent (63). Neuronal NOS–positive neurons kill neighboring neurons, while nNOS-positive neurons are protected by the action of Mn-superoxide dismutase (64). Our observed increase in the specific activity of NOS in the AD mouse model is consistent with the reported neurotoxic function of NO and indicates an inverse relationship between NOS activity and MT levels. This trend suggests that NOS and MT function in opposition. Thus, signaling events that result in increased amyloid production are expected to yield increased NO and decreased MT-3. A simple working model is shown in Figure 3.

Model of MT-3 Interactions

In summary, these observations indicate that the proteins thus far identified in our studies are associated with one another and targeted to respond similarly to changes in the cellular environment. A transitional model (Fig. 4) of these interactions has been proposed based on these data and information published in other reports. This model adequately incorporates the interaction of MT-3 and its partners with the conflicting interactions of NO and NOS. HSP84 is also critical to understanding the biological functions of NOS because its human variant, HSP90, has

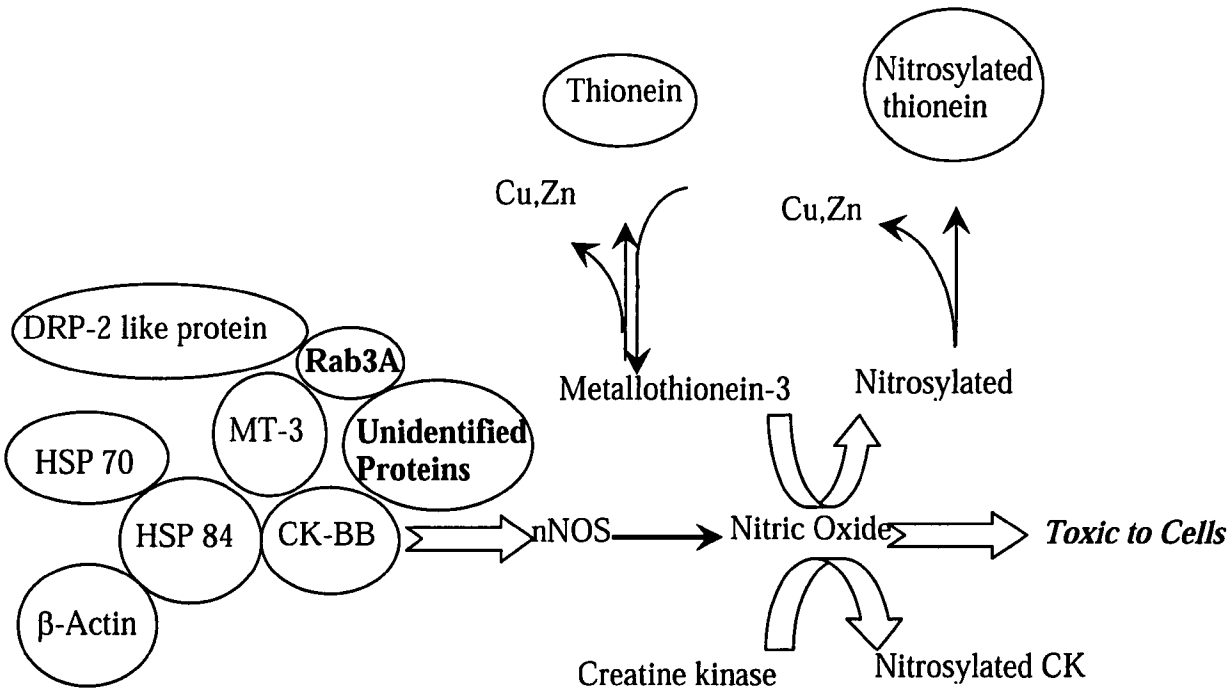

Figure 4. Hypothetical model of interactions between MT-3 and other brain proteins (44), including Rab3A (45). Rab3A is shown because it interacts with MT-3 (45), but it is not clear whether all these proteins are in the same complex.

been shown to activate human brain nNOS. The presence, however, of CK in the complex is intriguing inasmuch as this protein is reduced in AD brains (65).

Specific anti-HSP84 and anti-CK antisera both immunoprecipitate each MT-3, HSP84, and CK (44). HSP70 and HSP 84 coprecipitate (66) and HSP90, the human variant of HSP84, can activate nNOS (67) and leads to the toxic production of NO in neurons. MT-3 is neuroprotective against the toxic effects of NO (57). Furthermore, CK and DRP-2 are each nitrosylated by NO in the brain (68), and HSP84, CK, and DRP-2 are targets of oxidation (69). MT-3 and HSP70 (as well as nNOS) are induced in neural injury. Data such as these indicate the importance of these proteins in the health of neurons under changing oxygen environments. The action of NO on MT-3 causes the release of zinc from Zn_7MT-3 and may yield its apo-protein, thionein (70). The release of zinc may provide a feedback mechanism for regulating NOS activity as Zn^{2+} has been reported to inhibit nNOS (71, 72).

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